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Threshold™ Immunoassays for Identification of Biological Agents: NATO SIBCA Exercise III

H.G. Thompson and R.E. Fulton
Defence R&D Canada – Suffield

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Abstract

In February 2001, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the third international training exercise on the identification of biological agents. Fourteen NATO national laboratories participated: Austria, Bulgaria, Canada, France, Germany (two laboratories), Italy, the Netherlands, Norway, Poland, Sweden, the United Kingdom and the United States (two laboratories). The designated laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). Participant laboratories were sent seven samples, four liquid (including one buffer blank) and three solid, consisting of soil. Participants were advised that samples could contain any one of the following gamma-irradiated organisms: *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis, Vibrio cholera, Burkholderia mallei,* Venezuelan equine encephalitis (VEE) virus, Vaccinia virus, *Coxiella burnetti*, or Yellow fever virus. A number of immunologically-based technologies were used at DRDC Suffield for screening of sample unknowns, one of which was the Threshold™ assay, a Light Addressable Potentiometric Sensor (LAPS) assay. Antigen capture Threshold™ assays were available for five biological agents: *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis,* and *Burkholderia mallei.* Two biological agents were identified by Threshold™ , both from the liquid samples (*B. melitensis* and *F. tularensis*). No "false positive" or "false negative" reactions were observed with SIBCA liquid samples. However, soil-extracted samples produced multiple "false positive" reactions and one "false negative" reaction, making identification of agents from this medium impossible to achieve. A comparison of the Threshold™ results with the identity of organisms in SIBCA sample unknowns, as revealed by US Dugway Proving Ground following the exercise, indicated 100% correct identification of liquid samples and 0% correct identification of soil samples.

Résumé

En février 2001, le sous-groupe VII de NATO Échantillonnage et identification des agents biologiques et chimiques (SIBCA) a conduit le troisième exercice de formation d'identification d'agents biologiques. Quatorze laboratoires des nations de l'OTAN ont participé : l'Autriche, la Bulgarie, le Canada, la France, l'Allemagne (deux laboratoires), l'Italie, les Pays-Bas, la Norvège, la Pologne, la Suède, la Grande-Bretagne et les États-Unis (deux laboratoires). Le laboratoire désigné au Canada était R & D pour la défense Canada – Suffield (RDDC Suffield). Les laboratoires participants ont reçu sept échantillons dont quatre liquides (y compris un tampon blanc) et trois solides comprenant du sol. Les participants ont été avisés que certains échantillons pouvaient contenir chacun des organismes irradiés aux rayons gamma suivants : *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis, Vibrio cholera, Burkholderia mallei,* le virus de l'encéphalite équine vénézuélienne (VEE), le virus de la vaccine, *Coxiella burnetti* ou le virus de la fièvre jaune. Un certain nombre de technologies à base d'immunologie ont été utilisées à RDDC Suffield pour cribler les échantillons inconnus dont le biotest Threshold™, un biotest avec capteur potentiométrique adressable de lumière (LAPS). Le biotest Threshold™ d'antigène de capture était disponible pour cinq agents biologiques : *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis,* et *Burkholderia mallei.* Deux agents biologiques ont été identifiés par le Threshold™, tous deux à partir des échantillons liquides (*B. melitensis* et *F. tularensis*). Aucun « faux positif » ou « faux négatif » n'a été observé avec les échantillons liquides SIBCA. Les échantillons extraits de sols ont cependant produit de multiples «faux positifs» et un «faux négatif» ce qui a rendu l'identification des agents à partir de ce médium impossible à réaliser. Une comparaison entre les résultats de Threshold™ et l'identité des organismes dans les inconnus d'échantillons SIBCA, telle que révélée par US Dugway Proving Ground à la suite de l'exercice, indique que 100% des échantillons liquides ont été identifiés correctement et que 0% des échantillons de sols ont été identifiés correctement.

Executive summary

Introduction

NATO Forces may be required to carry out military or peacekeeping 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 same to respective national laboratories, where procedures would be carried out to identify the agent unknowns and to confirm their presence in samples. In order to assess national capabilities in the NATO laboratories for identification of biological agents in samples, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) have organized several international training exercises in which participating nations have been requested to identify, within a given time period, agents in sample unknowns.

The third SIBCA training exercise for biological agents (SIBCA Exercise III) was held in February 2001. All NATO laboratories who regularly attend the SIBCA meetings were invited to participate in the SIBCA exercise. The participating laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). DRDC Suffield used a number of different immunologically-based technologies to screen the SIBCA samples, one of which was the Threshold™ assay, a Light Addressable Potentiometric Sensor (LAPS) assay. This report describes the results obtained from screening of the SIBCA samples for five different bacterial agents by Threshold™.

Results

Threshold™ assays for *Bacillus anthracis, Yersinia pestis, Francisella tularensis, Brucella melitensis,* and *Burkholderia mallei* were developed previously for use in the SIBCA I exercise and used to screen the SIBCA III liquid and liquid-extracted soil samples.

Two bacterial agent unknowns, *B. melitensis* and *F. tularensis*, were identified by Threshold™ assay from the liquid samples. No "false positive" or "false negative" reactions were observed with the SIBCA liquid samples. However soil extracted samples produced multiple "false positive" reactions and one "false negative" reaction, making identification of agents from this medium impossible to achieve. A comparison of the Threshold™ results with the identity of organisms in SIBCA sample unknowns, as revealed by US Dugway Proving Ground following the exercise, indicated 100% correct identification of liquid samples and 0% correct identification of soil samples.

Significance of Results

Results demonstrated that Threshold™ technology is a sensitive assay tool for identification of biological agents of concern to the military community. Results indicated that the ease of agent identification is influenced by the medium in which the biological agent is contained. Hence, biological agent in buffer was easily identified while problems with "false positive" and "false negative" reactions were encountered when agent was extracted from soil. Further research is required to identify useful methods for extraction of biological agents from environmental matrices such as soil. DRDC Suffield participation in NATO SIBCA exercises provides a measure of in-house capabilities for identification of biological agents in sample unknowns.

Future Goals

DRDC Suffield will continue to develop assays for the Threshold™ to complete the identification capability of this technique for agents of concern to the Canadian Forces (CF). In addition, samples in matrices other than liquid will be studied including those in various types of soil and vegetative matter. The effect of battlefield interferents will also be investigated. Improvements to the assay will include using larger volumes of more dilute sample, longer incubation times, and stabilization of the incubation temperature.

Thompson, H.G. and Fulton, R.E. 2002. Threshold™ Immunoassays for Identification of Biological Agents: NATO SIBCA Exercise III. DRDC Suffield. TR 2002-008. Defence R&D Canada – Suffield.

Sommaire

Introduction

Les Forces de l'OTAN 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 telle attaque 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 les acheminer aux laboratoires nationaux respectifs où ils seront soumis à aux procédures d'identification des agents inconnus et où la présence de ces agents dans les échantillons sera confirmée. Pour évaluer la capacité des laboratoires de l'OTAN à identifier les agents biologiques à partir d'échantillons, le sous-groupe de l'OTAN Échantillonnage et identification des agents biologiques et chimiques (SIBCA) a organisé plusieurs exercices internationaux de formation durant lesquels chaque nation participante a été requise d'identifier des agents à partir d'échantillons inconnus, durant une période de temps limitée.

Le troisième exercice de formation SIBCA pour les agents biologiques (les exercices SIBCA III) ont eu lieu en février 2001. Tous les laboratoires des pays de l'OTAN ayant participé aux réunions SIBCA de manière régulière ont été invités à y participer. Le laboratoire représentant le Canada était R & D pour la défense Canada – Suffield (RDDC Suffield). Ce dernier a utilisé un certain nombre de technologies à base immunologique pour cribler les échantillons SIBCA dont le biotest Threshold™, un biotest avec capteur potentiométrique adressable de lumière (LAPS). Ce rapport décrit les résultats obtenus à partir du criblage par Threshold™ d'échantillons SIBCA contenant cinq différents agents bactériens.

Résultats

Les biotests Threshold™ pour les *Bacillus anthracis, Yersinia pestis, Francisella tularensis, Brucella melitensis* et *Burkholderia mallei* avaient été mis au point auparavant pour être utilisés dans les exercices SIBCA I; ils ont été utilisés pour cribler les échantillons liquides et les échantillons liquides extraits de sols pour les exercices SIBCA III.

Deux agents bactériens inconnus, *B. melitensis* et *F. tularensis*, ont été identifiés par les biotests Threshold™ à partir des échantillons liquides. Aucun « faux négatif » ou « faux positif » n'a été observé avec les échantillons liquides SIBCA. Les échantillons extraits de sols ont cependant produit de multiples « faux positifs » et un « faux négatif » ce qui a rendu l'identification des agents par ce médium impossible à réaliser. Une comparaison entre les résultats de Threshold™ et l'identité des organismes dans les inconnus d'échantillons SIBCA, telle que révélée par US Dugway Proving Ground à la suite de l'exercice, indique que 100% des échantillons liquides ont été identifiés correctement et que 0% des échantillons de sols ont été identifiés correctement.

La portée des résultats

Les résultats indiquent que la technologie Threshold™ est un outil sensible de biotest d'identification des agents biologiques préoccupant la communauté militaire. Les résultats indiquent que la facilité d'identification réside dans le médium contenant l'agent biologique.

Ainsi, l'agent biologique contenu dans un tampon a été facilement identifié alors qu'il existe des problèmes de «faux positifs» et de «faux négatifs» quand l'agent a été extrait du sol. De plus amples recherches sont requises pour déterminer les bonnes méthodes d'extraction d'agents biologiques à partir de matrices environnementales comme celle des sols. La participation de RDDC Suffield aux exercices SIBCA de l'OTAN permet de mesurer les capacités internes du laboratoire en matière d'identification d'agents biologiques à partir d'échantillons inconnus.

Les buts futurs

RDDC Suffield continuera à mettre au point des biotests Threshold™ avec pour objectif de compléter la capacité d'identification de cette technique pour les agents dont se préoccupent les Forces canadiennes (CF). De plus, des matrices autres que celles des liquides seront étudiées y compris une variété de matières de sol et végétales Les effets des interférences sur les champs de bataille seront aussi examinés. Les améliorations apportées aux biotests comprendront des volumes plus importants d'échantillons dilués, des durées plus longues d'incubation et la stabilisation des températures d'incubation.

Thompson, H.G. and Fulton, R.E. 2002. Threshold™ Immunoassays for Identification of Biological Agents: NATO SIBCA Exercise III. DRDC Suffield. TR 2002-008. Defence R&D Canada – Suffield.

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Introduction

NATO Forces may be required to carry out military or peacekeeping 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 same to respective national laboratories, where procedures would be carried out to identify the agent unknowns and to confirm their presence in samples. In order to assess national capabilities in the NATO laboratories for identification of biological agents in samples, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) organized international training exercises 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 I, was held in March 1999. Eleven national laboratories participated in the exercise: Canada, France, Germany (two laboratories), Hungary, Italy, the Netherlands, Norway, Poland, United Kingdom, and the United States. Participant nations 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 and Development - Suffield. DRDC Suffield screened sample unknowns by two different antibody-based identification technologies, the Threshold™ device, a light addressable potentiometric sensor (LAPS), and immunochromatographic assays [1, 2]. In addition, a limited analysis by genetic techniques was also used [3].

A second SIBCA training exercise (SIBCA II), again hosted by DPG, was held in February 2000. With the exception of Hungary, all laboratories that participated in SIBCA I also participated in SIBCA II. In addition, Austria and Sweden joined this exercise, for a total of 12 participating laboratories. Six sample unknowns from the list of ten agents used in the SIBCA I exercise were sent to the participating laboratories. Two of the samples also contained common battlefield interferents, either burning vegetation or burning diesel fuel. Two technologies were employed by DRES to assess the samples, one genetic-based method [4] and one antibody-based method, enzyme-linked immunosorbent assay (ELISA) [5]. In addition, a rapid, limited survey (*B. anthracis, B. melitensis, F. tularensis, B. mallei, Y. pestis,* Vaccinia virus) of the samples was performed on the Threshold™ , which succeeded in identifying three bacteria (*B. anthracis, B. melitensis,F. tularensis)*, for which assays were available. Vaccinia virus was not successfully identified due to problems with antibody conjugates that later proved to be inactive (unpublished results).

In February 2001, Dugway Proving Ground (DPG) again hosted a SIBCA training exercise (SIBCA III) in which fourteen NATO laboratories (Canada, France, Germany (two laboratories), Italy, the Netherlands, Norway, the United States (two laboratories), the United Kingdom, Poland, Austria, Sweden, and Bulgaria took part. Seven samples, six containing agent and one blank, from the same list of ten inactivated agents as were used in the previous two SIBCA exercises, were sent to each participating laboratory. Three of the samples were

agent suspended in soil. Concentrations were to be in the order of 10^6-10^7 cfu/mL for bacteria and 10^7-10^8 pfu/mL for viruses and rickettsia. Analyses were to be completed within ten working days and results forwarded to DPG for compilation and analysis. DRDC Suffield employed three different technologies for this exercise, including one genetic-based technique and two antibody-based techniques, namely enzyme-linked immunosorbent assay (ELISA) and Threshold™ immunoassay. This report describes the results obtained on screening of the SIBCA III sample unknowns by Threshold™ assay. A discussion of the literature pertaining to Threshold™ immunoassay, as well as a complete description of the assay mechanism and procedures, have been previously reported [1].

The Threshold™ immunoassay was used in SIBCA III for confirmation of the results found by ELISA. Threshold™ assays were performed on all sample unknowns, both liquid and soil, for the following agents: *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Bacillus mallei,* and *Yersinia pestis.* Threshold™ assays for *Coxiella burnetii, Vibrio cholerae*, VEE, Vaccinia, and Yellow fever were unavailable for SIBCA III and therefore were not performed. Agents identified by Threshold™ were compared with the known agent content of samples as revealed by DPG following the exercise. Results indicated that all biological agents present in the liquid sample unknowns for which Threshold™ assays were available, were identified correctly by Threshold™. However, none of the agents present in soil samples could be identified, due to the presence of numerous false positive test results and one false negative test result.

Materials and methods

SIBCA test samples

Samples of killed (cobalt-irradiated) biological materials, four in liquid and three in soil, were received at DRDC Suffield from DPG. The DRDC Suffield sample numbers were 211, 227, 242 and 352 for the liquids and 320, 326, and 353 for the soils. Six samples were to contain agent in the order of $10^6 - 10^7$ cfu/mL bacteria or $10^7 - 10^8$ pfu/mL virus or rickettsia, and one sample was to be a blank. Sample unknowns were to be suspended in 10 mL PBS or 10 g soil and were to contain any of the following inactivated agents: *Bacillus anthracis, Brucella melitensis, Bacillus mallei, Coxiella burnetti, Francisella tularensis, Yersinia pestis, Vibrio cholerae*, Vaccinia virus, VEE virus, or Yellow fever virus. Participant laboratories were requested to complete their analyses within ten working days.

Threshold™ assays

Materials

Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) and Triton X-100 were from BDH Chemicals (Toronto, ON). Bovine serum albumin (BSA), sodium chloride (NaCl), sodium hydroxide (NaOH), Tween-20, and urea were from Sigma Chemical Co. (St. Louis, MO). Streptavidin (Scripps Laboratories, San Diego, CA), 10 mg/mL in distilled water, was prepared previously (for SIBCA I) and stored at 4° C. 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

Cobalt-irradiated antigen stocks were gifts from DPG. *B. mallei* mallein was a gift from Animal Diseases Research Institute, Nepean, ON (Table 1).

Anti-bacterial antibodies were from the DRDC Suffield collection and were the same antibodies as used in SIBCA I [1]. Biotin and fluorescein conjugates of these antibodies were prepared for use in SIBCA I [1] and stored since that time at 4° C. These antibody conjugates were verified for activity prior to use in SIBCA III. Lyophilized anti-fluorescein urease-conjugated antibody was prepared prior to the assay by reconstitution in 30 mL of assay buffer to a stock concentration of 7.5 mg/mL and stored no longer than one week.

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°, no longer than one week.

The substrate solution was 100 mM urea in wash buffer (pH 6.5), prepared fresh daily.

For the Threshold™ assay, reagents were added together in the following order to make a cocktail mixture (total volume of 1 mL): assay buffer, streptavidin, biotin-labelled antibody, and fluorescein-labelled antibody (Table 2).

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

Liquid samples

Sample unknowns were assayed undiluted.

Soil samples

Soil (1.5 g) was suspended in PBS (3 mL) containing 0.025% Triton-X 100. The suspension was stirred for one hour, the mixture filtered under vacuum through Whatman #1 filter paper, and the filtrate used directly in the assays.

Analysis of SIBCA unknowns

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. In order to minimize the effect of stick to stick variation, assays for each bacteria were confined to a single stick. A typical configuration for a sample assay is depicted in Figure 1. A volume of 125 µL of cocktail reaction mixture was added to each of the tubes, mixed by pipet, 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.

Statistics

All unknown samples and negative controls were tested in replicates of three. Positive controls were assayed in replicates of two. Threshold™ readings were considered positive if the value of the sample mean minus one standard deviation was greater than the negative control plus three standard deviations.

Results

Results from screening individual SIBCA sample unknowns by Threshold™ assay are presented in Figures 2 to 8. A summary of the Threshold™ assay identification results compared with the agents known to be present in these samples is presented in Table 3.

Positive control concentrations (Table 1) were chosen to give an unequivocal positive signal while maintaining a mid-range response. However, *Y. pestis* positive control concentrations were chosen to minimize fouling of the reader [1]. Although lower than for the other analyses, the signal from the *Y. pestis* positive control was, in all cases, higher than the negative control. Assay reproducibility (stick to stick) was evaluated by assessing the percent coefficient of variation (%CV) of the negative (no antigen) and the positive controls, stick to stick, for each of the agents tested. CVs ranged from 11% to 67% (Table 4). Similarly, spot to spot variation within a single stick was evaluated by determining the %CV for data points within a single stick (Figures 2 – 8) and was found to range from 1-51% with a median of 13%.

Liquid samples #242 and #352 were identified by Threshold™ assay as *Brucella melitensis* and *Francisella tularensis*, respectively. Liquid samples #211 and #227 screened negative for any of the bacteria for which Threshold™ assays were available, although the F. *tularensis* assay should be considered invalid as the postive control was below the three standard deviation cut-off. This test could not be repeated due to depletion of liquid sample #211. Threshold™ results were consistent with results obtained by ELISA, where *B. melitensis* was identified in sample #242 and *F. tularensis* in sample #353 [6].

SIBCA soil sample #320 tested positive for *Y. pestis* and *B. mallei*. No reliable results were obtained for the stick used to test for *F. tularensis* because filtering had to be abandoned due to a plugged nitrocellulose membrane. This test could not repeated due to depletion of soil sample #320. SIBCA soil #326 tested positive for *B. anthracis, B. melitensis, F. tularensis* and *Y. pestis*. SIBCA soil #353 showed positive results for all five bacterial agents tested. Results for soil samples by Threshold™ assay were consistent with the findings by ELISA, in that SIBCA sample unknowns were positive for more than one agent [6].

Discussion

Threshold™ assays were initially used for verification of the presence of *Brucella melitensis* and *Francisella tularensis* in liquid samples #242 and #352, respectively, as detected by ELISA. Later, it was decided to test all seven samples by Threshold™ for agents for which antibody conjugates were on hand. In the liquid samples, two bacterial agents were identified by Threshold™ assay. However, analyses of the soil samples proved to be beyond the capabilities of the Threshold™. The soil extraction process provided a clear, slightly coloured filtrate which yielded multiple false positives and one false negative when analyzed. Although the signals obtained in false positive assays were much lower than would have been anticipated had a bacterial agent been present in the concentration expected (10^6-10^7 cfu/mL), readings were statistically significant.

The current format of the Threshold™ assay was developed at a time when the Threshold™ was being considered as an identification tool in a mobile field laboratory. Over the years, other methods of identification have taken the forefront for use in the field e.g., immunochromatographic assays, IGEN electrochemiluminescence assays. The Threshold™ assay is probably of more value as a laboratory-based tool. However, assay precision, evaluated by examining data reproducibility, stick to stick and spot to spot within a single stick, revealed %CVs ranging from 11-67% and 1-51%, respectively. On signal readings taken from individual spots, it has been observed that CVs tended to increase with the magnitude of the signal generated (unpublished observation). The signal (in mV/sec) is generated from the slope of the straight line applied to the output as the stick is being "read". Higher signals tend to be less linear near the start of the curve and, therefore, are subject to greater error when a straight line is imposed on the data points. This enhanced error can be eliminated if the instrument is adjusted to ignore the first 5-6 data points of the curve. The CV might also be improved by increasing incubation times to enhance sensitivity, and using larger, more dilute volumes of Streptavidin and anti-fluorescein urease conjugated antibody, as suggested in the Threshold™ operator's manual, to increase precision. This adjustment has, in fact, been made in assays performed post SIBCA III; preliminary results from these assays have indicated improved CVs in individual sticks, ranging from 2-14% with the median at 7%.

Reproducibility has, historically, been a problem in Threshold™ analyses performed at DRDC Suffield (unpublished observation). Factors that may account for this problem include incubation temperature, variability of membrane thickness on the sticks, and age of the sticks. Threshold™ assays are routinely perfomed in this laboratory at "room temperature". However, the temperature of the laboratory can range from a low of 15 °C to a high of 35 °C. This variation in temperature is likely to affect the rate of formation of immune complexes. In future, it would be advisable to standardize the incubation temperature of 37 °C and ascertain whether there is an improvement in reproducibility. In addition, anecdotal information suggests that the membrane may vary in thickness from stick to stick. Thus, while comparison of signals within any one stick may be valid, it may not be possible, for example, to use the background from one stick to compare with the sample signal from another stick. Finally, the sticks used in the analyses of the SIBCA III samples had an expiry date of 1997,

or earlier. Whether the capture ability of the biotinylated membrane deteriorates unevenly, or at all, over time, has never been investigated.

The antibodies used in these assays were tested by ELISA for cross-reaction with a pool of heterologous agents and were found to be specific at the concentrations tested [6]. However, aqueous solutions of SIBCA soil samples (clay, loam, or sand) produced non-specific positive reactions when used to challenge agent-specific Threshold™ assays. These "false positive" reactions could have been caused by non-specific attachment of soil particles to the antibody conjugates, resulting in Ab/biotin-soil-Ab/fluorescein "sandwiches", which, in turn, reacted with the anti-fluorescein/urease and substrate. Soil particles are known to be surrounded by a layer of polyvalent cations [7] and would thus carry a net positive charge. Antibodies (IgG) are heterogeneous in charge, exhibiting a wide range of electrophoretic mobilities [8], thus it is likely that IgG molecules with a net negative charge would be electrostatically attracted to soil particles.

Prior to the SIBCA III exercise, no soil extraction methods were investigated, but it was decided to add Triton-x-100, a non-ionic detergent, to the extraction media to act as a surfactant, reducing the surface tension surrounding the soil aggregates, thus dispersing soil particles and releasing microorganisms. However, as noted above, the results obtained on the SIBCA 3 samples were inconclusive. Further discussion of the difficulties in assaying soil samples may be found elsewhere [6].

Table 1. *Working amounts of positive control antigens used in Threshold™ assays*

ORGANISM	DESCRIPTION	WORKING CONC.	AMOUNT PER TEST SITE
B. anthracis	Vollum strain, lot no. 96092, 1.3 x 10^8 cfu/mL from DPG, Co^{60}-irrad	1 ng/µL	100 ng
B. mallei	Mallein complement fixation (CF) antigen (opthalmic); serial no. 91-94; expiry date 95.12.31 from ADRI (Nepean, ON)	1 ng/µL	100 ng
B. melitensis	Type 2, 4.3 x 10^8 cfu/mL, from DPG, Co^{60}-irrad	2 ng/µL	200 ng
F. tularensis	Schu 4 strain, lot no. 95306, 7.8 x 10^7 cfu/mL, from DPG, Co^{60}-irrad	2 ng/µL	200 ng
Y. pestis	India 195/P strain (F1+), 3.6 x 10^7 cfu/mL, from DPG, Co^{60}-irrad	5 ng/µL	500 ng

Table 2. *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
Y. pestis	200	100	500

F-Ab: fluoresceinated antibody

B-Ab: biotinylated antibody

SA: streptavidin

Table 3. *Agents identified by Threshold™ assay compared to agents known present in SIBCA III samples*

SIBCA SAMPLE #	SAMPLE Matrix	*Bacillus anthracis*	*Brucella melitensis*	*Francisella tularensis*	*Burkholderia mallei*	*Yersinia pestis*	ACTUAL AGENT PRESENTt[1]
211	liquid	-ve	-ve	invalid	-ve	-ve	*Vibrio cholerae*, Inaba strain (5.7 x 10^6 cfu/mL)
227	liquid	-ve	-ve	-ve	-ve	-ve	PBS
242	liquid	-ve	+ve	-ve	-ve	-ve	*Brucella melitensis*, biovar3 (2.4 x 10^7 cfu/mL)
352	liquid	-ve	-ve	+ve	-ve	-ve	*Francisella tularensis*, strain Schu S4, (7.8 x 10^6 cfu/mL)
320	soil	-ve	-ve	abandoned[a]	+ve	+ve	*Bacillus anthracis*, strain Vollum 1B (1.4 x10^7 cfu/g)
326	soil	+ve	-ve	+ve	-ve	+ve	*Yersinia pestis*, India strain (1.1 x 10^7 cgu/g)
353	soil	+ve	+ve	+ve	+ve	+ve	*Coxiella burnetti*, 9 mile strain, phase 1 (2.3 x 10^7 ID_{50}/g)

a. filtering abandoned due to faulty stick

[1] As provided by DPG post exercise

Table 4. *Variability of Threshold™ assays from stick to stick*

ASSAY TYPE	MEAN OF NEGATIVE CONTROLS	CV OF NEGATIVE CONTROLS	MEAN OF POSITIVE CONTROLS	CV OF POSITIVE CONTROLS
B. anthracis	106 +/- 44	41%	636 +/- 225	37%
B. melitensis	134 +/- 14	11%	756 +/- 90	25%
F. tularensis	106 +/- 38	36%	431 +/- 227	53%
B. mallei	104 +/- 13	13%	799 +/- 271	34%
Y. pestis	105 +/- 22	21%	247 +/- 83	34%

Sample unknown A, replicate 1	Sample unknown A, replicate 2	Sample unknown A, replicate 3
Positive control, bacteria	not useable	Positive control, bacteria
Negative control, bacteria	Negative control, bacteria	Negative control, bacteria

Figure 1. *Typical sample assay configuration depicting reaction test site locations on a single Threshold™ stick*

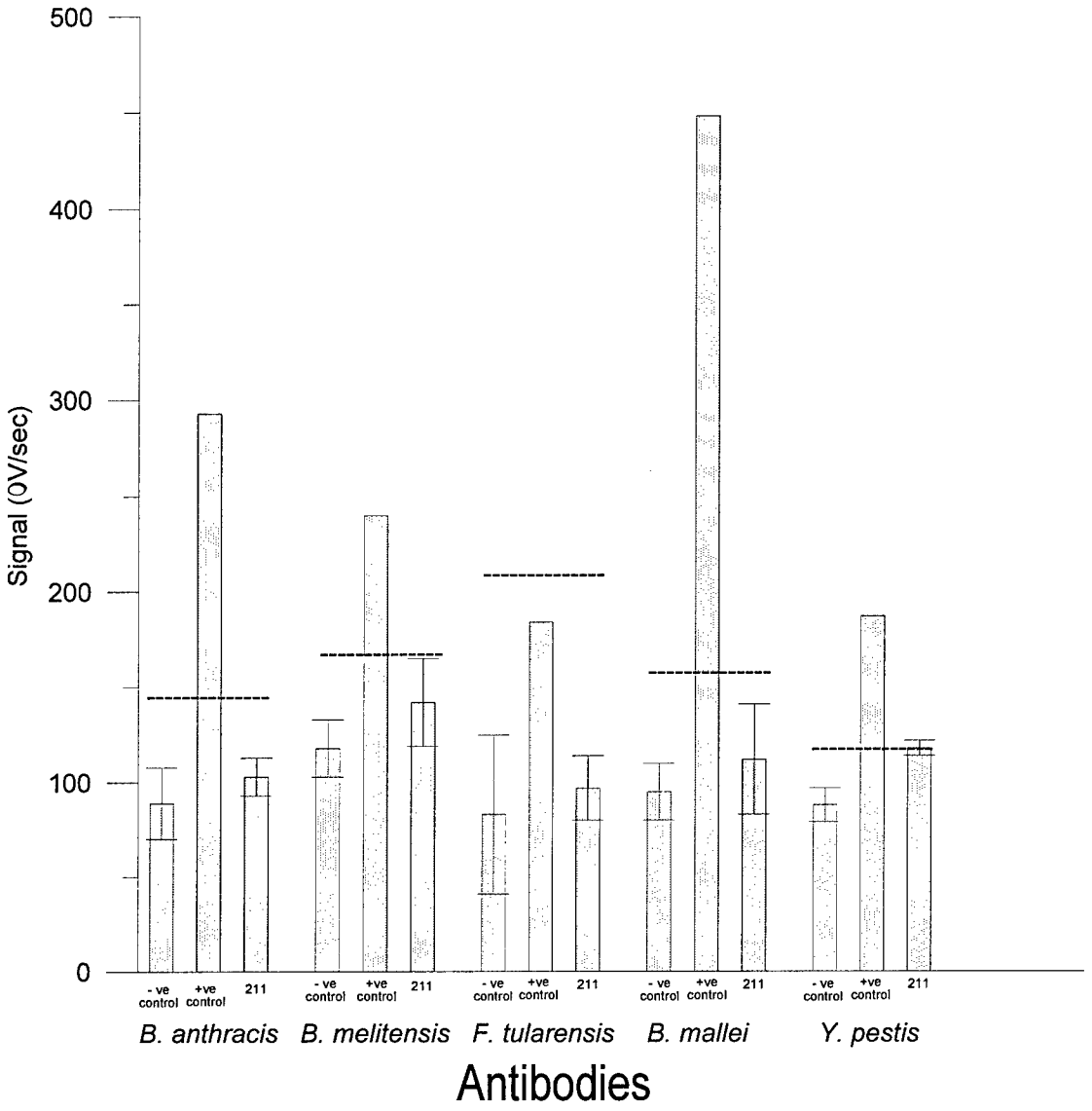

Figure 2. *Threshold™ analysis of SIBCA sample #211 (liquid). Biotin and fluorescein labelled anti-analyte antibodies were reacted by Threshold™ assay with SIBCA sample #211. Data points for negative (no antigen) controls and SIBCA sample unknowns represent the mean of three readings. Positive controls are the mean of duplicate readings. Error bars indicate one standard deviation. ---------- indicates signal equal to negative control plus three standard deviations. Results for F. tularensis are considered invalid since the positive control is below the three standard deviation cut-off.*

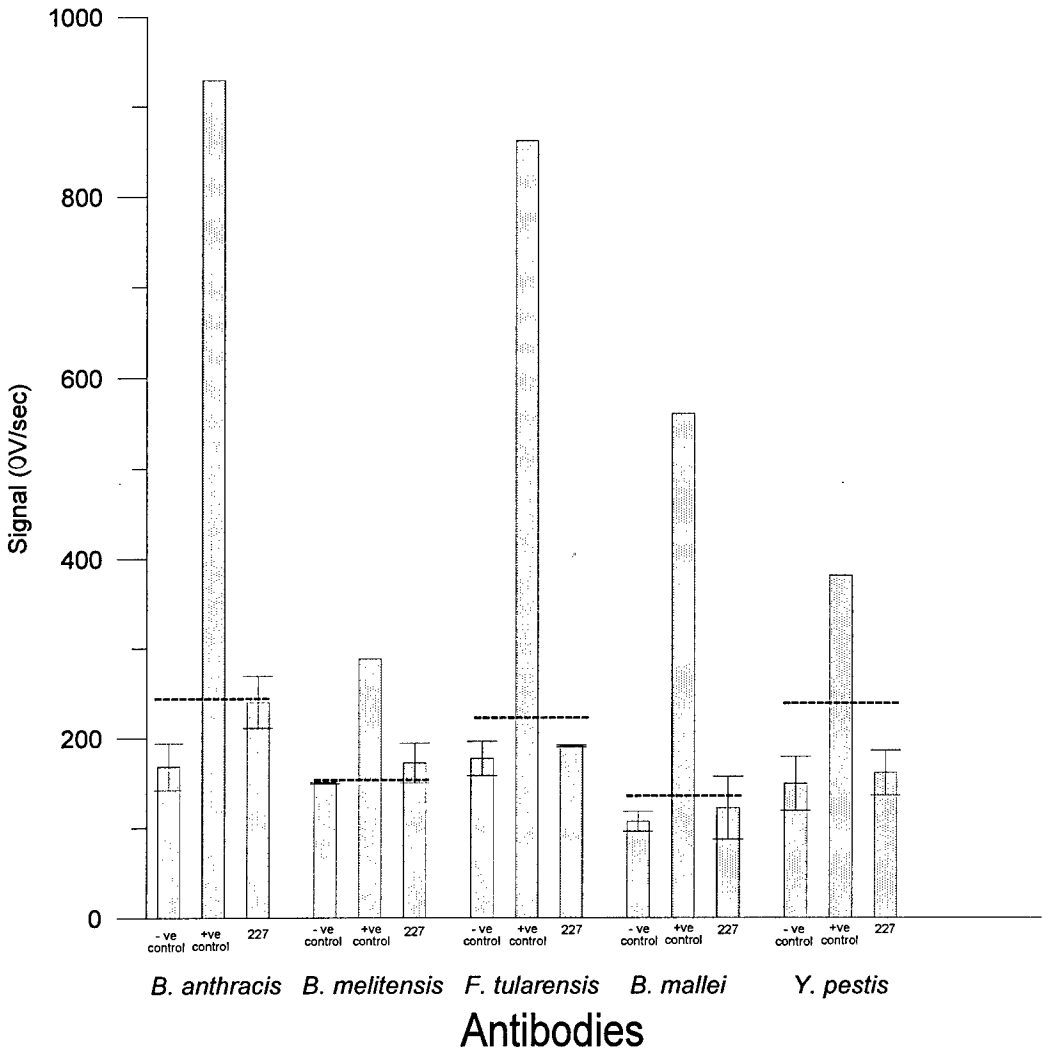

Figure 3. *Threshold™ analysis of SIBCA sample #227 (liquid). Biotin and fluorescein labelled anti-analyte antibodies were reacted by Threshold™ assay with SIBCA sample #227. Data points for negative (no antigen) controls and SIBCA sample unknowns represent the mean of three readings. Positive controls are the mean of duplicate readings. Error bars indicate one standard deviation. ---------- indicates signal equal to negative control plus three standard deviations.*

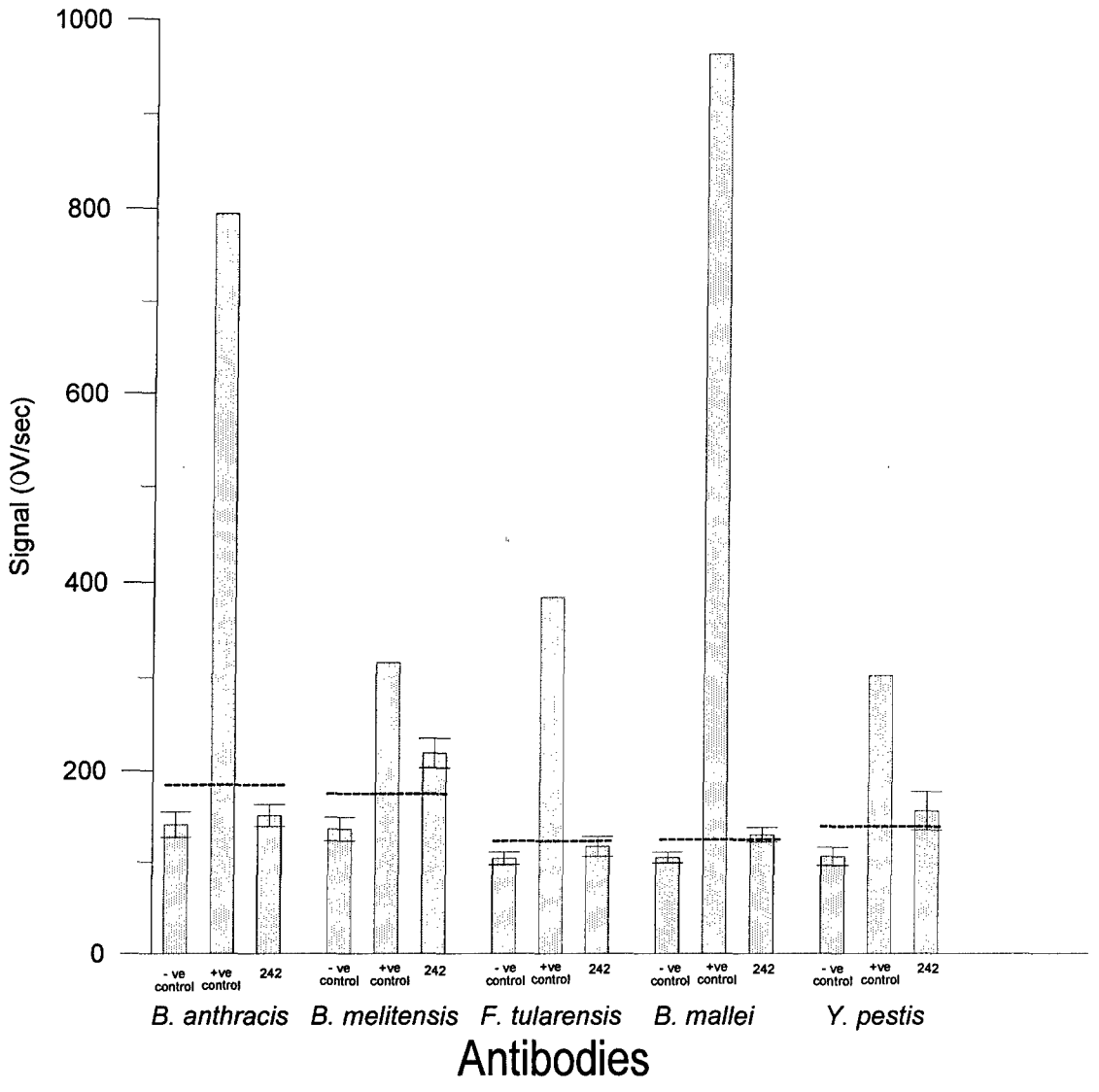

Figure 4. *Threshold™ analysis of SIBCA sample #242 (liquid). Biotin and fluorescein labelled anti-analyte antibodies were reacted by Threshold™ assay with SIBCA sample #242. Data points for negative (no antigen) controls and SIBCA sample unknowns represent the mean of three readings. Positive controls are the mean of duplicate readings. Error bars indicate one standard deviation. ---------- indicates signal equal to negative control plus three standard deviations.*

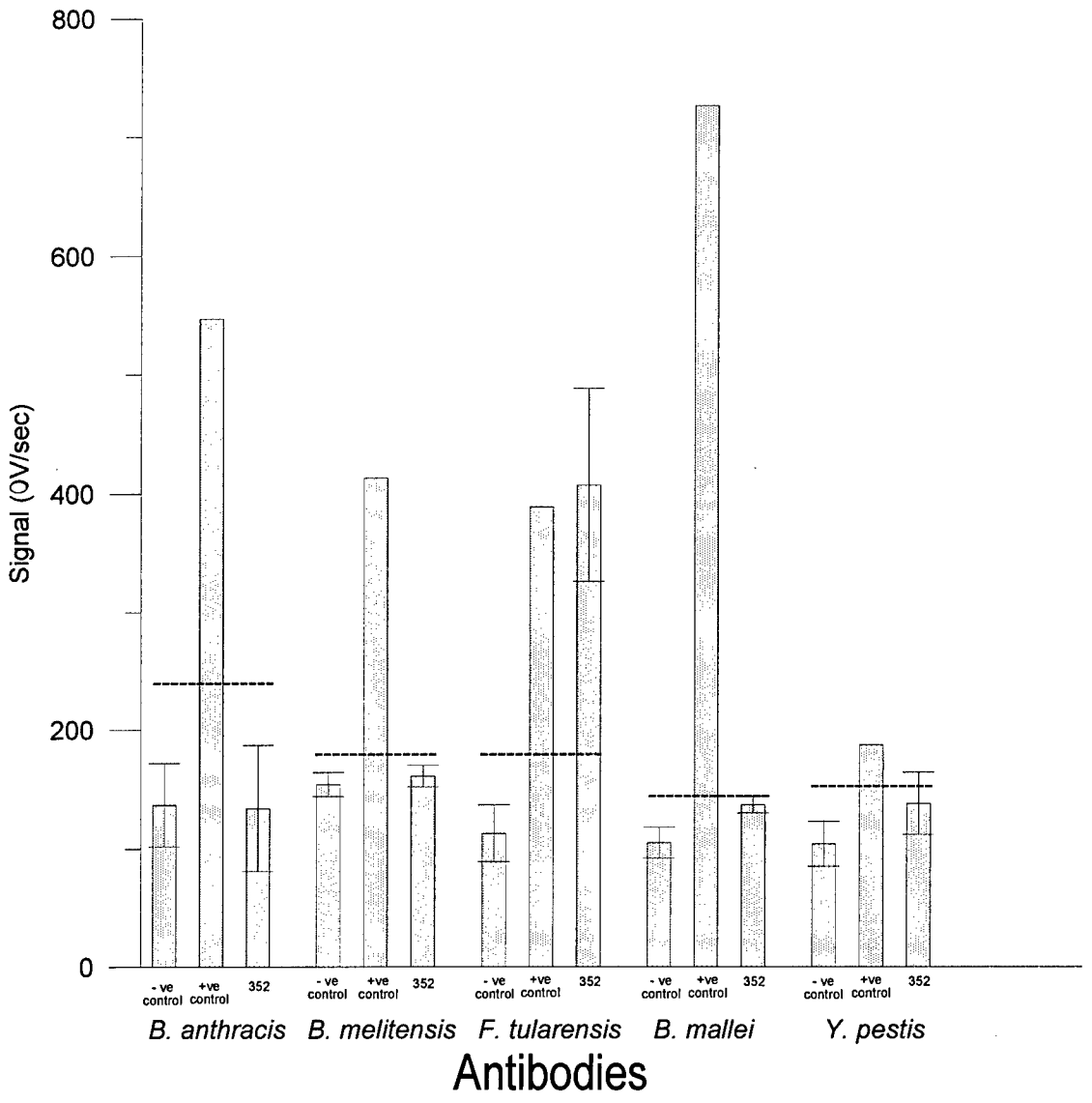

Figure 5. *Threshold™ analysis of SIBCA sample #352 (liquid). Biotin and fluorescein labelled anti-analyte antibodies were reacted by Threshold™ assay with SIBCA sample #352. Data points for negative (no antigen) controls and SIBCA sample unknowns represent the mean of three readings. Positive controls are the mean of duplicate readings. Error bars indicate one standard deviation. ---------- indicates signal equal to negative control plus three standard deviations.*

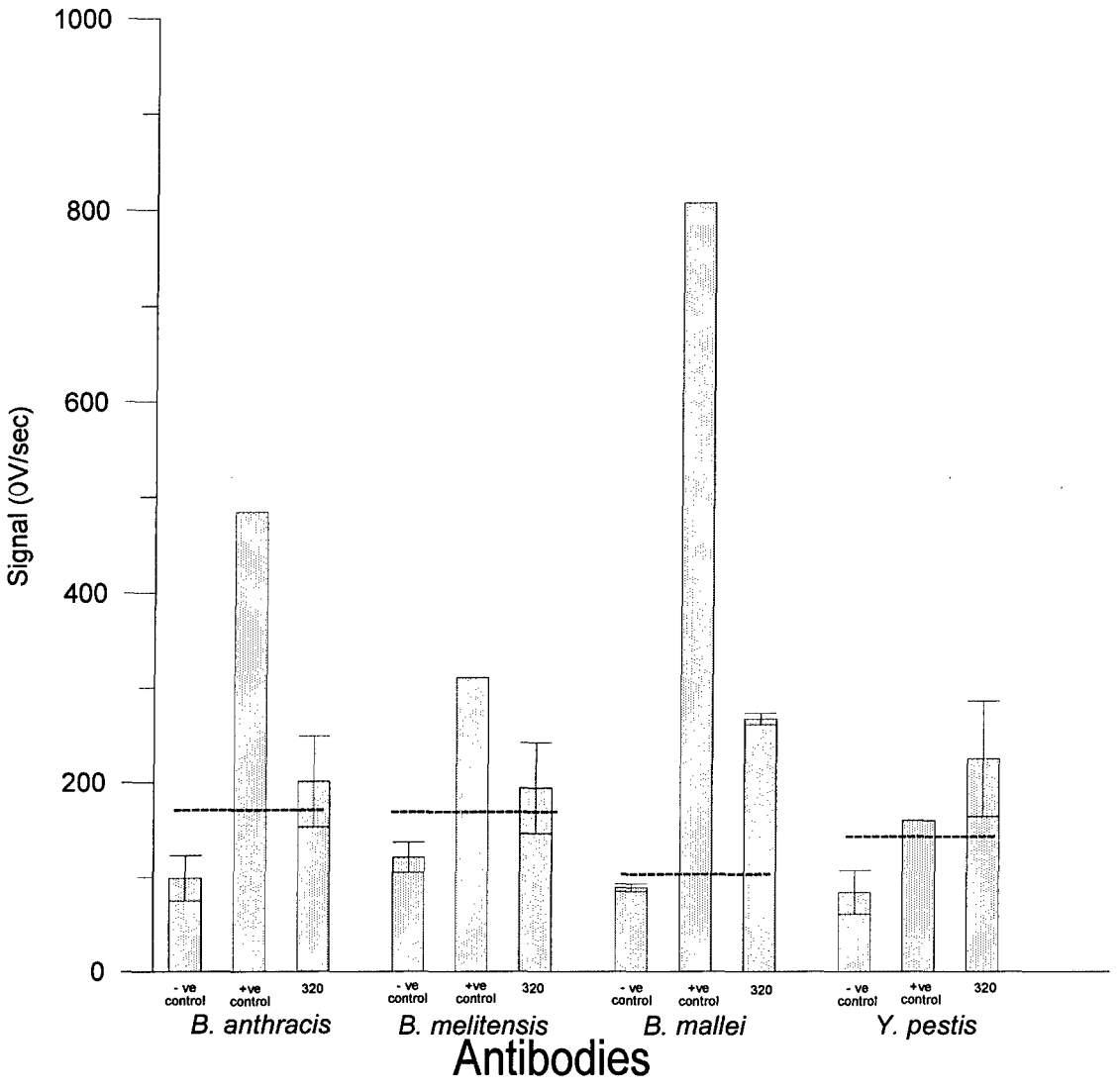

Figure 6. *Threshold™ analysis of SIBCA sample #320 (soil). Biotin and fluorescein labelled anti-analyte antibodies were reacted by Threshold™ assay with SIBCA sample #320. Data points for negative (no antigen) controls and SIBCA sample unknowns represent the mean of three readings. Positive controls are the mean of duplicate readings. Error bars indicate one standard deviation. ---------- indicates signal equal to negative control plus three standard deviations. No results were obtained for F. tularensis as filtering was discontinued due to a plugged membrane.*

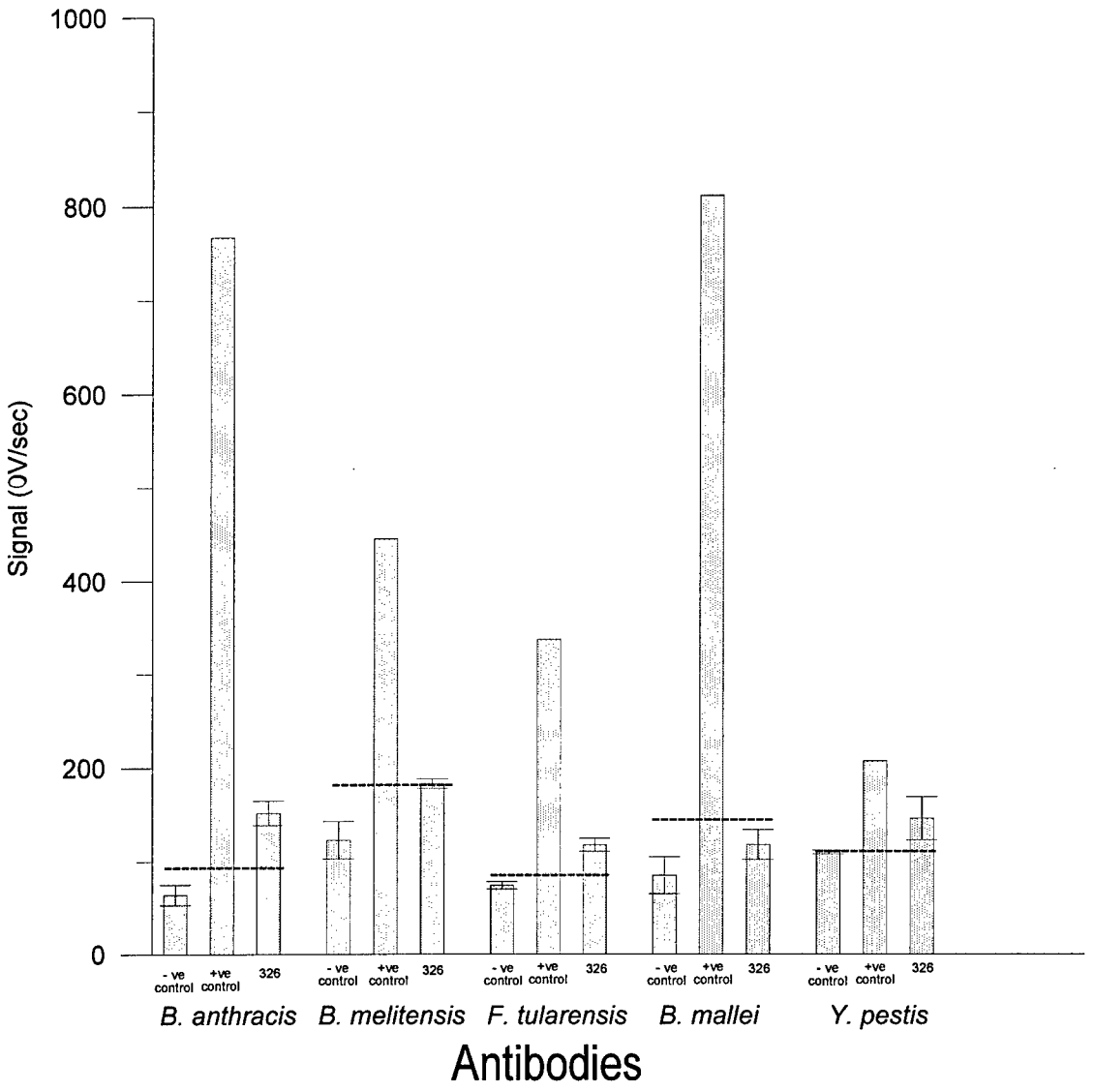

Figure 7. *Threshold™ analysis of SIBCA sample #326 (soil). Biotin and fluorescein labelled anti-analyte antibodies were reacted by Threshold™ assay with SIBCA sample #326. Data points for negative (no antigen) controls and SIBCA sample unknowns represent the mean of three readings. Positive controls are the mean of duplicate readings. Error bars indicate one standard deviation. ---------- indicates signal equal to negative control plus three standard deviations.*

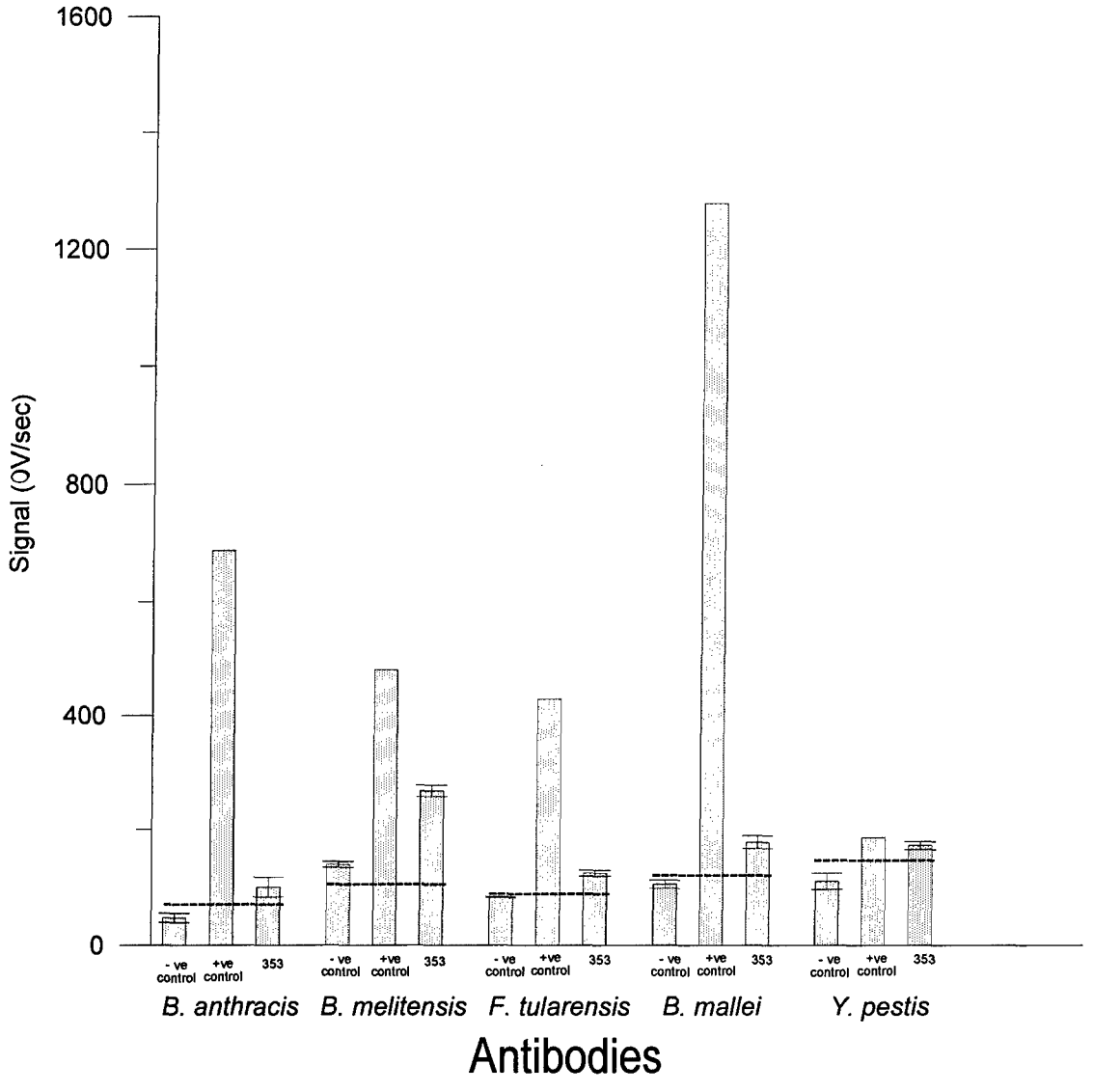

Figure 8. *Threshold™ analysis of SIBCA sample #353 (soil). Biotin and fluorescein labelled anti-analyte antibodies were reacted by Threshold™ assay with SIBCA sample #353. Data points for negative (no antigen) controls and SIBCA sample unknowns represent the mean of three readings. Positive controls are the mean of duplicate readings. Error bars indicate one standard deviation. ---------- indicates signal equal to negative control plus three standard deviations.*

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In February 2001, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the third international training exercise on the identification of biological agents. Fourteen NATO national laboratories participated: Austria, Bulgaria, Canada, France, Germany (two laboratories), Italy, the Netherlands, Norway, Poland, Sweden, the United Kingdom and the United States (two laboratories). The designated laboratory for Canada was Defence R&D Canada – Suffield (DRDC Suffield). Participant laboratories were sent seven samples, four liquid (including one buffer blank) and three solid, consisting of soil. Participants were advised that samples could contain any one of the following gamma-irradiated organisms: *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis, Vibrio cholera, Burkholderia mallei,* Venezuelan equine encephalitis (VEE) virus, Vaccinia virus, *Coxiella burnetti,* or Yellow fever virus. A number of immunologically-based technologies were used at DRDC Suffield for screening of sample unknowns, one of which was the Threshold™ assay, a Light Addressable Potentiometric Sensor (LAPS) assay. Antigen capture Threshold™ assays were available for five biological agents: *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Francisella tularensis,* and *Burkholderia mallei.* Two biological agents were identified by Threshold™, both from the liquid samples (*B. melitensis* and *F. tularensis*). No "false positive" or "false negative" reactions were observed with SIBCA liquid samples. However, soil-extracted samples produced multiple "false positive" reactions and one "false negative" reaction, making identification of agents from this medium impossible to achieve. A comparison of the Threshold™ results with the identity of organisms in SIBCA sample unknowns, as revealed by US Dugway Proving Ground following the exercise, indicated 100% correct identification of liquid samples and 0% correct identification of soil samples.

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