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Immunoassays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise VI

H.G. Thompson and R.E. Fulton
DRDC Suffield

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Abstract

In March 2005, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the sixth international training exercise on identification of biological agents. Fifteen biological sample unknowns, diluted in PBS, were sent to each laboratory and participants were advised that biological agents could consist of varying concentrations of any of the following three gamma-irradiated organisms: *Bacillus anthracis, Yersinia pestis,* or vaccinia virus. The object of the exercise was to assess each nation's current capabilities in assay sensitivity for these agents. An immunological-based technology, the enzyme-linked immunosorbant assay (ELISA), was used at DRDC Suffield for screening of sample unknowns. Antigen capture ELISAs for each of the three possible biological agents were used to screen the samples. In addition, a fluorescent hand-held assay, the Rapid Analyte Measurement Platform (RAMP^{TM}), manufactured by Response Biomedical Corp., was used to analyze the samples for the presence of *Bacillus anthracis* and vaccinia virus. Five biological agent unknowns were identified by ELISA in the SIBCA samples, three samples containing *B. anthracis* and two samples containing *Y. pestis*. Two biological agent unknowns were identified by RAMP^{TM} in the SIBCA samples, both containing *B. anthracis*. No agents were identified in 11 of the SIBCA sample unknowns. A comparison of the immunoassay results with the identity of organisms in SIBCA sample unknowns, as revealed by Dugway Proving Ground following the exercise, indicated confirmed identification of five agents and the sample blank, and 12 false negative identifications.

Résumé

En mars 2005, le Sous-groupe du Panel VII d'échantillonnage et identification des agents chimiques et biologiques (SIBCA) de l'OTAN a conduit son sixième exercice international de formation portant sur l'identification des agents biologiques. Quinze échantillons biologiques inconnus, dilués dans PBS, ont été envoyés à chacun des laboratoires et les participants ont été avisés que des agents biologiques pouvaient consister en des concentrations variées de n'importe lequel des trois organismes gamma irradiés suivants : *Bacillus anthracis, Yersinia pestis* ou virus de la vaccine. L'objectif de cet exercice était d'évaluer les capacités actuelles de chaque nation dans le domaine de la réactivité des biotests à ces agents. Une technologie à base immunologique, le dosage immunoenzimatique (ELISA) a été utilisé à RDDC Suffield pour cribler les échantillons inconnus. Des antigènes de capture ELISA pour chacun des trois agents biologiques possibles ont été utilisés pour cribler ces échantillons. De plus, un biotest fluorescent manuel, *the Rapid Analyte Measurement Platform* (RAMP^{TM}), manufacturé par Response Biomedical Corp., a été utilisé pour analyser les échantillons et vérifier la présence du *Bacillus anthracis* et du virus de la vaccine. Cinq agents biologiques inconnus ont été identifiés par ELISA dans les échantillons SIBCA, trois échantillons contenant du *B. anthracis* et deux échantillons contenant *Y. pestis*. Deux agents biologiques inconnus ont été identifiés par RAMP^{TM} dans les échantillons SIBCA, chacun contenant *B. anthracis*. Aucun agent n'a été identifié dans 11 des échantillons SIBCA inconnus. Une comparaison des résultats des immuoessais avec l'identité des organismes contenus dans les échantillons inconnus SIBCA, telle que révélée par Dugway Proving Ground après l'exercice, a confirmé l'identification de cinq agents et d'un échantillon blanc ainsi que 12 identifications de faux négatifs.

Executive summary

Immunoassays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise VI

H.G. Thompson, R.E. Fulton; DRDC Suffield TM 2005-223; Defence R&D Canada – Suffield; December 2005.

Background

NATO/Partner for Peace (PfP) 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/PfP 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. In order to assess national capabilities in the NATO/PfP laboratories for identification of biological agents in samples, the NATO group 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.

In March 2005, DRDC Suffield participated in the sixth international exercise on identification of biological agents (SIBCA VI) hosted by US Dugway Proving Ground (DPG), UT. Fifteen biological sample unknowns, diluted in PBS, were sent to each laboratory and participants were advised that biological agents could consist of varying concentrations of any of the following three gamma-irradiated microorganisms: *Bacillus anthracis*, *Yersinia pestis*, or vaccinia virus. The object of the exercise was to assess each nation's current capabilities in assay sensitivity. DRDC Suffield used several different technologies to screen the SIBCA samples, two of which were the enzyme-linked immunosorbent assay (ELISA) and the rapid analyte measurement platform (RAMP^{TM}), a fluorescent hand-held assay (HHA). This report describes the results obtained in screening SIBCA samples for three different biological agents, present at varying concentrations in samples, by ELISA and RAMP^{TM}.

Principal results

Antigen-capture ELISAs for *B. anthracis, Y. pestis,* and vaccinia virus were used to screen SIBCA samples for homologous agents. In addition RAMP^{TM} HHAs for *B. anthracis* and vaccinia virus were used to screen SIBCA samples. *B. anthracis* was identified in two SIBCA samples by both ELISA and RAMP^{TM} HHA, *B. anthracis* was identified in an additional sample by ELISA, and *Y. pestis* was identified by ELISA in two samples. One sample was correctly identified as a blank. A total of 10 false negative SIBCA samples (12 false negative agent identifications) by ELISA and RAMP^{TM} technologies combined, was observed.

Significance of results

Participation of DRDC Suffield in NATO/PfP SIBCA training exercises is valuable, as it provides a means for DRDC Suffield to measure and evaluate, against international standards, in-house capabilities in the identification of biological agents from sample unknowns. The results of the current exercise confirmed the utility of ELISA and RAMP™ HHAs as useful tools for immunological identification of biological agents in samples. Also highlighted was the finding that, to accurately compare the sensitivity of different identification systems used by different countries, standardized antigens for use among nations are required. Further work is necessary to establish a repository and mechanism for distribution of standard antigens for use as international standards for assay development and validation.

Future work

While all of the three agent ELISAs used in this exercise had been standardized and optimized for sensitivity, only one of these assays had been evaluated for reactivity with common battlefield materials and potential assay interferents. Further work is required to complete the screening of all standardized agent ELISAs at DRDC Suffield against a standard panel of related and unrelated agents and potential battlefield and assay interferents, and to assess ELISAs for assay reproducibility on a statistical basis. In addition, incorporation of monoclonal antibodies into developed ELISAs would result in greater long-term assay reproducibility, as well as enhanced specificity for a number of assays in which species-specific identification would be an asset e.g., *Bacillus spp.* Furthermore, each agent ELISA should be tested with live agent, to confirm assay sensitivity with live materials. In the longer term, investigation of alternative, more sensitive immunological techniques for identification of biological agents is desirable, as is the development of multiplexed immunological assay systems for the simultaneous screening of samples for multiple agents. A thorough evaluation of the RAMP™ using *B. anthracis* is planned for the fall of 2005.

Sommaire

Immunoassays for Identification of Biological Agents in Sample Unknowns: NATO SIBCA Exercise VI

H.G. Thompson, R.E. Fulton; DRDC Suffield TM 2005-223; R & D pour la défense Canada – Suffield; décembre 2005.

Contexte

Les forces du Partenariat pour la paix (PPP) de l'OTAN risquent d'être appelées à effectuer des opérations de maintien de la paix dans certains endroits du monde où il existe une menace d'attaque avec des agents biologiques ou bien dans certains endroits où l'existence d'une attaque biologique est suspectée ou confirmée. Dans de telles circonstances, les forces PPP de l'OTAN doivent prendre des échantillons des matériaux suspectés de contenir des agents biologiques et envoyer ces derniers aux laboratoires nationaux respectifs, dans lesquels les procédures d'identification des agents inconnus seront effectuées. Pour évaluer les capacités nationales des laboratoires PPP de l'OTAN concernant l'identification d'échantillons d'agents biologiques, le groupe de l'OTAN d'Échantillonnage et identification des agents biologiques et chimiques (SIBCA) a organisé des exercices internationaux de formation. Durant ces exercices, les nations participantes étaient requises d'identifier, durant une période de temps déterminée, des agents contenus dans des échantillons inconnus.

En mars, 2005, RDDC Suffield a participé au sixième exercice international d'identification d'agents biologiques (SIBCA VI) et a eu lieu sur le polygone d'essais américain, Dugway Proving Ground (DPG), UT. Quinze échantillons biologiques inconnus, dilué dans du PBS, ont été envoyés à chaque laboratoire et les participants ont été avisés que les agents biologiques pouvaient consister en une variété de concentration de n'importe lesquels des trois microorganismes gamma irradiés suivants : *Bacillus anthracis*, *Yersinia pestis* ou le virus de la vaccine. Le but de l'exercice était d'évaluer les capacités actuelles de chaque nation dans le domaine de la réactivité des biotests à ces agents. RDDC Suffield a utilisé plusieurs technologies différentes pour cribler les échantillons ELISA. Deux d'entre elles étaient le dosage immunoenzimatique (ELISA) et *Rapid analyte measurement platform* (RAMP^{TM}), un biotest manuel à fluorescence (HHA). Ce rapport décrit les résultats obtenus avec le criblage des échantillons SIBCA, par ELISA et RAMP^{TM}, de trois agents biologiques différents qui étaient présents à des concentrations variées dans les échantillons.

Résultats principaux

Des antigènes de capture ELISA pour *B. anthracis, Y. pestis* et le virus vaccine ont été utilisés pour cribler des échantillons SIBCA et trouver les agents homologues. De plus, on a utilisé HHA RAMP^{TM} pour *B. anthracis* et le virus de la vaccine pour cribler les échantillons SIBCA. *B. anthracis* a été identifié dans deux échantillons SIBCA par les deux méthodes ELISA et HHA RAMP^{TM}. *B. anthracis* a été identifié dans un échantillon additionnel par ELISA et *Y. pestis* a été identifié par ELISA dans deux échantillons. Un échantillon a été correctement identifié comme blanc. On a observé un total de 10 échantillons SIBCA faux négatifs (sur 12 identifications de faux négatifs) en combinant les deux technologies ELISA et RAMP^{TM}.

Portée des résultats

La participation de RDDC Suffield aux exercices de formation SIBCA du PPP de NATO est précieuse ; elle permet à RDDC de mesurer et d'évaluer ses capacités internes par rapport aux normes internationales en matière d'identification d'agents biologique à partir d'échantillons inconnus. Les résultats des exercices actuels ont confirmé l'utilité d'ELISA et du HHA RAMP™ comme étant des outils utiles dans le domaine de l'identification d'agents biologiques dans des échantillons. On souligne aussi la conclusion que, pour être en mesure de comparer avec exactitude la réactivité des différents systèmes d'identification utilisés par différents pays, il faut que les antigènes soient normalisés entre les nations. Il faudra continuer les travaux pour établir une réserve et un mécanisme de distribution des antigènes normalisés qui seront utilisés comme normes internationales pour la mise au point et la validation des biotests.

Travaux futurs

Bien que tous les trois agents ELISA utilisés dans cet exercice aient été normalisés et optimisés pour leur réactivité, un seul de ces biotests a été évalué pur sa réactivité avec les matériaux communs dans les champs de bataille et dans les interférants de biotests potentiels. Il faudra continuer les travaux à RDDC Suffield pour compléter le criblage de tous les agents ELISA normalisés par rapport à un panel normalisé d'agents reliés et non reliés et d'interférents de biotests et de champs de bataille potentiels et d'évaluer ELISA pour la reproductibilité des biotests sur une base statistique. De plus, l'incorporation d'anticorps monoclonaux dans les ELISA développés résulterait en une reproductibilité à plus long terme des biotests, ainsi qu'en une spécificité améliorée pour un certain nombre de biotests dans lesquels l'identification spécifique aux espèces serait un avantage (ex : *Bacillus spp*). Plus encore, chaque agent ELISA devrait être testé avec un agent vivant pour confirmer la réactivité du biotest avec les matériaux vivants. À long terme, il est désirable d'investiguer des techniques immunologiques d'identification d'agents biologiques alternatives qui soient plus sensibles, telles que la mise au point de systèmes de biotests immunologiques multiplexés permettant le criblage simultané d'échantillons d'une multitude d'agents. Une évaluation exhaustive de RAMP™ utilisant *B. anthracis* est prévue pour l'automne 2005.

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Acknowledgements

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Introduction

NATO/Partner for Peace (PfP) 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/PfP 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. In order to assess national capabilities in the NATO/PfP laboratories for identification of biological agents in samples, the NATO group 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 hosted by Dugway Proving Ground (DPG), UT, in March 1999. Samples, consisting of phosphate buffered saline (PBS) spiked with biological agents, were number coded by DPG, then shipped to participant nations for analysis. 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 Defence Research Establishment Suffield (DRES)[1]. DRES 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. Six sample unknowns from the list of 10 agents used in the SIBCA I exercise, again suspended in PBS, were sent to the participating laboratories. Two of the samples also contained common battlefield interferents, either burnt vegetation residue or burnt diesel fuel residue. 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 February 2001, DPG again hosted a SIBCA training exercise (SIBCA III) in which seven samples, six containing agent and one blank, from the same list of 10 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, while the remainder were in a PBS matrix. DRDC Suffield employed three different technologies for this exercise, including one genetic-based technique [6] and two antibody-based techniques, namely ELISA [7] and Threshold™ immunoassay [8].

In January 2002, SIBCA conducted the fourth international training exercise on identification of biological agents. For the fourth SIBCA exercise (SIBCA IV), also hosted by DPG, six swab samples, bearing sample unknowns from the same list of 10 potential agents as previous exercises, were sent to participating countries. DRDC Suffield employed two different technologies for this exercise, one genetic-based technique [9] and an antibody-based technique, namely ELISA [10], complemented by limited Threshold™ assays and bacterial stains techniques.

[1] DRES: renamed Defence Research and Development Canada – Suffield (DRDC Suffield) in January, 2001

The fifth international training exercise on identification of biological agents (SIBCA V), again hosted by DPG, was held in March 2004. Samples received consisted of eight leaves on which agent unknowns, from the same list of 10 potential agents as described for previous exercises, had been adsorbed. DRDC Suffield employed the same two technologies as for the SIBCA V exercise, namely one genetic-based technique [11] and ELISA, an antibody-based technique [12].

In March, 2005 DRDC Suffield participated in the sixth international exercise on identification of biological agents (SIBCA VI) hosted by DPG. Fifteen biological sample unknowns, diluted in PBS, were sent to each laboratory and participants were advised that biological agents could consist of varying concentrations of any of the following three gamma-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, or vaccinia virus. The object of the exercise was to assess each nation's current capabilities in assay sensitivity.

In the SIBCA VI exercise, reported herein, antigen-capture ELISAs were used to analyze sample unknowns for the three SIBCA agents. Assays for each agent were configured in such a way that liquid sample unknowns were screened in parallel with homologous agents (positive controls). In addition, a fluorescent hand-held assay, the Rapid Analyte Measurement Platform (RAMP^{TM}), manufactured by Response Biomedical Corp., Burnaby, BC, was used to analyze the samples for the presence of *Bacillus anthracis* and vaccinia virus. Five biological agent unknowns were identified by ELISA in the SIBCA samples. Two biological agent unknowns were identified by RAMP^{TM} in the SIBCA samples. A comparison of the ELISA and RAMP^{TM} results with the identity of organisms in SIBCA sample unknowns, as revealed by US DPG following the exercise, indicated that *Bacillus anthracis* had been correctly identified in samples 296 and 328 by both ELISA and RAMP^{TM}, *Bacillus anthracis* was correctly identified in sample 364 by ELISA and *Yersinia pestis* was correctly identified by ELISA in samples 101 and 364. A total of 10 false negative SIBCA samples (12 false negative agent identifications) by ELISA and RAMP^{TM} technologies combined, was observed: vaccinia virus in samples 113, 168,183, 230, 231, and 234 (for both ELISA and RAMP^{TM}), *Yersinia pestis* in samples 231, 390, and 420 (ELISA), *Bacillus anthracis* in samples 203, 234, and 320 (for both ELISA and RAMP^{TM}), and *Bacillus anthracis* in sample 364 (RAMP^{TM} only).

Materials and Methods

SIBCA test samples

Pre-exercise information

Fifteen liquid sample unknowns, 5 mL each, numbered 101, 113, 122, 168, 183, 203, 230, 231, 234, 296, 320, 328, 364, 390, and 420, were received at DRDC Suffield from US DPG on 4 March, 2005. Participants were informed that samples would contain any one, or combination of, varying concentrations of the following killed (cobalt-irradiated) agents: *B. anthracis*, *Y. pestis*, and vaccinia virus. Ten working days, not necessarily consecutive, were allowed for completion of the analyses, after which time, results were to be sent to DPG for collation.

Sample preparation

Each 5 mL SIBCA sample was split into two aliquots, with one of each aliquot used for genetic analyses, and the other used undiluted for the two immunoassays.

Post-exercise identity of sample unknowns

After each laboratory had completed their analyses and the results had been reported, participants were informed by DPG of the identities of agents present in SIBCA sample unknowns. The identities and concentrations of the agents in the fifteen sample unknowns received by Canada are presented in Table 1.

Table 1: Agents in SIBCA exercise VI samples[2]

Sample No.	Agent	Concentration
101	*Yersinia pestis*	10^7 cfu/mL
113	vaccinia virus	10^4 pfu/mL
122	PBS	NA
168	vaccinia virus	10^5 pfu/mL
183	vaccinia virus	10^6 pfu/mL
203	*Bacillus anthracis*	10^4 cfu/mL

[2] Information provided by US DPG following reporting to DPG of SIBCA VI results

Sample No.	Agent	Concentration
230	vaccinia virus	10^8 pfu/mL
231	*Yersinia pestis* vaccinia virus	10^4 cfu/mL 10^7 pfu/mL
234	vaccinia virus *Bacillus anthracis*	10^7 pfu/mL 10^5 cfu/mL
296	*Bacillus anthracis*	10^6 cfu/mL
320	*Bacillus anthracis*	10^3 cfu/mL
328	*Bacillus anthracis*	10^7 cfu/mL
364	*Bacillus anthracis* *Yersinia pestis*	10^5 cfu/mL 10^6 cfu/mL
390	*Yersinia pestis*	10^5 cfu/mL
420	*Yersinia pestis*	10^3 cfu/mL

Enzyme-linked immunosorbent assays

Antibodies

Unlabelled antibodies

Antibody stocks developed under DRES contract by SciLab Consulting Inc. (Redcliff, AB) [13] were as follows: *B. anthracis*, goat IgG, lot no. SC97Ant001, 5 mg/mL, serial no.[3] CABAC70P0090797; *B. anthracis,* rabbit IgG, lot no. SC97Ant002, 5 mg/mL, serial no. CABAC71P0090797; *Y. pestis,* goat IgG, lot no. SC97YP001, 4 mg/mL, serial no. CAYER3810/08/99; *Y. pestis,* rabbit IgG, lot no. SC97YP002, 3 mg/mL, serial no. CAYER9310/08/99; vaccinia virus, goat IgG, lot no. SC97V001, 5 mg/mL, serial no. CAVac61P0090797; and vaccinia virus, rabbit IgG, lot no. SC97002, 5 mg/mL, serial no. CaVac69P0090797.

[3] Serial no. assigned by DRDC Suffield MOU database

Labelled antibody

The following horseradish peroxidase-labelled antibody was purchased from Sigma Aldrich Canada Ltd. (Oakville, ON); goat anti-rabbit IgG, (whole molecule, lot no. 90H8990).

Antibody purification

Antibodies produced by Scilab Consulting Inc. were purified on a Bio-Gel^{R} Protein G Fast Flow Gel column (Bio-Rad Laboratories, Mississauga, ON) by a High-Performance Liquid Chromatography system (Spectral Physics, San Jose, CA) [13].

Antigens

The following ^{60}Co-irradiated antigen stocks were gifts from DPG: *Y. pestis*, India 195/P strain (F1+), 3.6 x 10^8 cfu/mL; *B. anthracis*, Vollum strain, lot no. 96092, 1.3 x 10^8 cfu/mL; and vaccinia virus, Lister strain, 1 x 10^8 pfu/mL.

Format

ELISAs for the identification of *B. anthracis, Y. pestis,* and vaccinia virus were performed in indirect (antigen capture) assay format. By this method, capture antibody (CAb) is adsorbed to the solid phase and is used to capture the target antigen or sample unknown. Agent ELISAs were used to screen the SIBCA VI samples for homologous agents.

Procedures

Agent ELISAs performed on the SIBCA samples included positive and negative (no antigen) controls, and the sample unknowns. Samples were assayed one agent per plate. All samples were tested undiluted in replicates of three wells.

ELISAs were performed in 96-well NUNC^{TM} Maxisorb microtiter plates purchased from Canadian Life Technologies, Burlington, ON. Positive control antigens and sample unknowns were detected with unlabelled detector antibody (DAb) and indicated by enzyme-labelled indicator antibody (IAb). Washes were performed using a Bio-Tek ELX-50 autostrip washer (Fisher Scientific). Wash steps consisted of five cycles of washing with a volume of 300 µL wash buffer (PBS containing 0.1% bovine serum albumin (Roche Diagnostics, Laval, QC) and 0.1% Tween-20) per wash cycle. Antigen, DAb, and IAb were diluted, as required, in ELISA buffer (PBS containing 2% BSA and 0.1% Tween-20). Unless otherwise specified, all incubation steps were at 37 °C for 1 hr.

Wells were coated with 100 µL of CAb in coating buffer (carbonate-bicarbonate buffer, 0.05M, pH 9.6) and incubated at 4° C overnight. Plates were washed, then blocked by the addition of 300 µL of blocking buffer (PBS containing 2% BSA), and incubated. Plates were washed, then 100 µL of antigen, or sample unknown, was added, and the plates incubated. Plates were again washed, 100 µL of DAb was added, plates were incubated, then washed. One hundred µL of IAb was added and the plates were incubated, then washed. Two hundred µL of substrate solution ((2.2'-azino-di- (3-ethyl-benzthiazoline sulfonate) (ABTS) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD)) was added and the plates incubated at room temperature for 30 min, after which the coloured reaction product was measured in an automated plate reader.

Immunoreagents

The identity and working concentrations of CAbs, DAbs, IAbs, and positive control antigens used in respective agent ELISAs, are shown in Table 2. All assays had been optimized previously, although for the *Y. pestis* assay in this study, the working concentrations of Cab, Dab, and IAb were not the optimized concentrations, but rather the concentrations used in previous SIBCA exercises [2, 5, 7, 10, 12].

Table 2: Working concentrations of capture, detector, and indicator antibodies, and positive control antigens used in agent ELISAs

AGENT ELISA	CAB	DAB	IAB (ABTS SUBSTRATE)	POSITIVE CONTROL ANTIGEN
B. anthracis	goat α-*B. anthracis* 10 µg/mL	rabbit α-*B. anthracis* 15 µg/mL	goat α-rabbit-HRP 1:1000	*B. anthracis*, vollum: 1.3×10^7 cfu/mL
Y. pestis	goat α-*Y. pestis* 10 µg/mL	rabbit α-*Y. pestis* 25 µg/mL	goat α-rabbit-HRP 1:1000	*Y. pestis*-India (F1+): 3.6×10^6 cfu/mL
vaccinia	goat α-vaccinia 15 µg/mL	rabbit α-vaccinia 15 µg/mL	goat α-rabbit-HRP 1:2000	vaccinia, Lister: 1.0×10^7 pfu/mL

Data acquisition, reduction, and analysis

ELISA reaction products were read at A_{405} nm in a Molecular Devices Thermomax^R automated plate reader (Fisher Scientific). Preliminary data analysis was performed using Softmax 3.0 software (Molecular Devices, Menlo Park, CA). Data was then exported to Microsoft Excel 7.0 for reduction and statistical analysis. Reduced data was further exported to Grapher 4 (Golden Software Inc., Golden, CO) for plotting of graphs.

Statistics

SIBCA samples and the positive controls were tested in replicates of three wells. Negative controls were tested in replicates of six wells. Unless otherwise indicated, data points represent the mean of three determinations for SIBCA samples and positive controls and six determinations for negative controls.

ELISA readings were considered positive if the mean absorbance reading was significantly greater than the background absorbance reading. Statistical significance was established at 20% above the background (no antigen control) absorbance. This reading represents a value of ten times the average coefficient of variation among replicates. Thus, a signal was considered positive if the value of the sample signal to background signal was 1.2 or greater.

RAMP™ HHAs

Principle of operation

The RAMP™ system (Response Biomedical Corp., Burnaby, BC) is composed of a portable fluorescence reader and disposable test cartridge that encloses an analyte-specific nitrocellulose immunochromatographic strip. The membrane strip uses fluorescence-labelled latex beads that are coated with analyte-specific detector reagent (usually antibody) to generate two signals at two different "capture" locations on the assay strip, the test-specific detection zone and an internal control zone. A small volume of test sample containing analyte is mixed with RAMP™ sample buffer and a fixed volume is transferred to the cartridge sample well via a pipette tip that has been pre-infused with fluorescent latex beads coated with analyte-specific detector reagent. The sample is drawn by capillary action along the membrane. Analyte in the sample interacts and binds with the mobile analyte-specific reagent-coated fluorescent latex beads, forming an analyte-reagent-bead complex. The fluid sample, along with analyte-bound and unbound latex beads, are transported through the strip to the detection and internal control zones. A second analyte-specific reagent (usually antibody) (capture reagent) embedded at the detection zone "captures" the latex bead complex and arrests its migration in the strip. Unbound latex beads migrate past the detection zone to the internal control zone, where they are "captured" by an immobilized reagent-specific molecule e.g., anti-species antibody (control reagent). When the reaction is complete, the reader scans the test strip through an opening in the bottom of the cartridge and detects fluorescence in the detection and internal control zones. A bar code on the test cartridge containing test-specific information e.g., lot number and calibration details is read by the reader. The reader calculates the ratio between the concentration of fluorescing beads in the detection and the internal control zones and refers to an analyte-specific calibration curve to convert fluorescence signal to analyte concentration. By calculating the final assay results as a ratio between two measurements, the RAMP™ system automatically accounts for variation in sample and membrane properties. Cartridges are supplied together with specific lot cards which provide lot information, including lot number, expiration date, and standard curve (positive/negative cut-off).

A diagrammatic representation of the principle of operation of the RAMP™ cartridges is provided in Figure 1

Cartridges

Bacillus anthracis and vaccinia RAMP™ environmental assay kits containing the test cartridges (lot numbers M00087 and M00117, respectively) were purchased from Response Biomedical Corp. The *B. anthracis* and vaccinia antibodies incorporated into these cartridges were of an unknown source. Similarly, the sources of anti-species antibodies used as internal controls for the RAMP™ cartridges were unknown.

The identity and configuration of detector, capture, and control reagents in the RAMP™ cartridges are presented in Table 3.

Table 3: Detector, capture, and control reagents used in RAMP™ HHAs

Agent	Detector	Capture	Control
B. anthracis	goat anti-*B. anthracis* (Vollum) IgG	goat anti-*B. anthracis* (Vollum) IgG	anti-goat IgG
vaccinia	rabbit anti-vaccinia IgG	rabbit anti-vaccinia IgG	Anti-rabbit IgG

Equipment

Two RAMP™ environmental test kits were purchased from Response Biomedical Corp. Each kit consisted of lot cards for respective anthrax and vaccinia RAMP™ cartridges, vials each containing 150 uL sample RAMP™ buffer, and a MiniPet® pipette (70 µL fixed volume) (TriContinent Inc., Grass Valley, CA), the latter not used in this study. The two fluorescence scanners (RAMP™ reader) were purchased separately and each RAMP™ reader was connected to a desktop computer for data acquisition.

Procedures

The positive control antigens for the RAMP™ *B. anthracis* and vaccinia HHAs were from the same source as those used in the ELISAs (see above) and were used at concentrations of 1.3×10^7 cfu/mL and 1×10^8 pfu/mL, respectively. The negative antigen control for the RAMP™ HHAs was PBS.

Procedures for performance of RAMP™ assays were as previously carried out in a RAMP™ study of ricin [14] and are briefly described below. A 10 µL aliquot of each sample negative control, positive control, or sample unknown was transferred to a 500 µL vial containing 90 µL of RAMP™ sample buffer which had been removed from the original buffer vial supplied by Response Biomedical Corp. The RAMP™ cartridge pouch was opened and the enclosed cartridge and pipette tip removed. The pipette tip (containing the detector reagent-coated fluorescent beads) was fitted to a variable volume pipette set to 70 µL and the sample mixed thoroughly by triturating up and down 15 times. A 70 µL volume of the sample mixture was delivered to the sample well of the RAMP™ cartridge, and the cartridge inserted into the RAMP reader. After the reader had detected the flow as indicated by a message generated by the reader, the assay was allowed to develop automatically for 12.5 min. The reader then scanned the cartridge and the data was automatically sent to the attached computer.

Data acquisition, reduction and analysis

Samples were tested in replicates of three with the exception of the vaccinia positive control which consisted of two replicates. The raw data from the computer connected to the RAMP™ reader was reduced manually, and inserted into Microsoft Excel 7.0 for exporting to Grapher 4 (Golden Software Inc., Golden, CO) for plotting of graphs.

RAMP™ readings were considered positive if the mean RAMP™ ratio was significantly greater than the negative control. Statistical significance was established at 2 standard deviations above the background (negative antigen control).

Results

ELISAs

Results obtained by challenge of three agent ELISAs with the fifteen SIBCA sample unknowns, including results of homologous agent positive controls, are summarized in Table 4 and graphically represented in Figures 2-4.

Positive controls for all three agent ELISAs (*B. anthracis, Y. pestis,* and vaccinia virus) produced positive reactions in respective homologous assays, thus confirming the validity of each of the agent assays for identification of respective homologous agents. Five positive reactions were observed in four SIBCA samples for two of the SIBCA agents (*B. anthracis* and *Y. pestis*). Samples 296 and 328 were positive for *B. anthracis*, sample 101 was positive for *Y. pestis*, and sample 364 was positive for both *B. anthracis* and Y. *pestis*. No positive reactions were observed with any of the 15 SIBCA samples in the vaccinia ELISA.

Table 4: ELISA results: SIBCA VI samples

	Agent ELISAs		
Sample	***B. anthracis***	***Y. pestis***	**Vaccinia**
Positive Control	+	+	+
101	-	+	-
113	-	-	-
122	-	-	-
168	-	-	-
183	-	-	-
203	-	-	-
230	-	-	-
231	-	-	-
234	-	-	-
296	+	-	-
320	-	-	-

	Agent ELISAs		
Sample	***B. anthracis***	***Y. pestis***	**Vaccinia**
328	+	-	-
364	+	+	-
390	-	-	-
420	-	-	-

RAMP™ HHAs

Results obtained by challenge of two agent HHAs (*B. anthracis* and vaccinia virus) with the fifteen SIBCA sample unknowns are summarized in Table 5 and graphically represented in Figures 5 and 6.

Positive antigen controls for both agent HHAs produced positive results in respective homologous assays, thus confirming the validity of each of the agent assays for identification of the respective homologous agents. Two SIBCA samples were positive for *B. anthracis*, namely 296 and 328. None of the 15 SIBCA samples produced a positive reaction on vaccinia HHAs.

Table 5: RAMP™ HHA results: SIBCA VI samples

	Agent RAMP™ HHA	
Sample	***B. anthracis***	**Vaccinia**
Positive control	+	+
101	-	-
113	-	-
122	-	-
168	-	-
183	-	-
203	-	-
230	-	-

	Agent RAMP™ HHA	
Sample	*B. anthracis*	Vaccinia
231	-	-
234	-	-
296	+	-
320	-	-
328	+	-
364	-	-
390	-	-
420	-	-

Agents identified compared with known sample content

Table 6 is a summary of the agents identified by immunoassay (ELISA and RAMP™ HHA), compared with the agent content as revealed by US DPG following the SIBCA exercise.

In four of the fifteen samples (samples nos. 101, 296, 328, and 364), the agents confirmed present by DPG were correctly identified by ELISA. In two of the fifteen samples (sample nos. 296 and 328), the agents confirmed present by DPG were also correctly identified by RAMP™ HHA. In addition, the blank, containing PBS only, was correctly identified as negative for agent by both ELISA and RAMP™ HHA. A total of 10 false negative SIBCA samples (12 false negative agent identifications by either ELISA or RAMP™) were observed: vaccinia virus in samples 113, 168, 183, 230, 231, and 234 (both ELISA and RAMP™), *Y. pestis* in samples 231, 390, and 420 (ELISA, RAMP™ not applicable), *B. anthracis* in samples 203, 234, and 320 (both ELISA and RAMP™), and *B. anthracis* in sample 364 (RAMP™ only).

Table 6: Agents identified compared with known agent content of SIBCA VI samples.

SIBCA sample no.	Agent present[4] (concentration)	Agent identified by ELISA	Agent identified by RAMP™ HHA[5]	Agent identified by ELISA or RAMP™ HHA
101	*Y. pestis* (10^7 cfu/mL)	*Y. pestis*	NA	*Y. pestis*
113	vaccinia (10^4 pfu/mL)	-	-	-
122	PBS	-	-	-
168	vaccinia (10^5 pfu/mL)	-	-	-
183	vaccinia (10^6 pfu/mL)	-	-	-
203	*B. anthracis* (10^4 cfu/mL)	-	-	-
230	vaccinia (10^8 pfu/mL)	-	-	-
231	*Y. pestis* (10^4 cfu/mL) vaccinia (10^7 pfu/mL)	- -	NA -	- -
234	vaccinia (10^7 pfu/mL) *B. anthracis* (10^5 cfu/mL)	- -	- -	- -
296	*B. anthracis* (10^6 cfu/mL)	*B. anthracis*	*B. anthracis*	*B. anthracis*
320	*B. anthracis* (10^3 cfu/mL)	-	-	-
328	*B. anthracis* (10^7 cfu/mL)	*B. anthracis*	*B. anthracis*	*B. anthracis*
364	*B. anthracis* (10^5 cfu/mL) *Y. pestis* (10^6 cfu/mL)	*B. anthracis* *Y. pestis*	- NA	*B. anthracis* *Y. pestis*
390	*Y. pestis* (10^5 cfu/mL)	-	NA	-
420	*Y. pestis* (10^3 cfu/mL)	-	NA	-

[4] Information provided by US DPG following the SIBCA exercise
[5] No assay available for *Y. pestis*

Discussion

The agents present in SIBCA samples 296 and 328 were correctly identified by both ELISA and RAMP™ HHA as *B. anthracis*. In addition, sample 101 was correctly identified by ELISA as containing *Y. pestis*. Sample 364 was also correctly identified by ELISA as containing both *B. anthracis* and *Y. pestis*. These results were consistent with those obtained by genetic analysis (Doug Bader, DRDC Suffield, personal communication), thus allowing Canada to report identification of agents in these samples at the NATO Confirmed[6] level. SIBCA sample 122 was correctly identified by both immunoassay (ELISA and RAMP™ HHA) and genetic analysis as negative. Samples 230 and 231 were not identified by immunoassay, while genetic analysis correctly identified these samples at the NATO Provisional[6] level as containing vaccinia virus.

The LODs for ELISAs at DRDC Suffield for identification of *B. anthracis*, *Y. pestis* and vaccinia virus ELISAs were determined in previous studies to be 10^4 cfu/mL, 10^3 cfu/mL and 10^6 pfu/mL, respectively (unpublished data). Antigens for these previous studies were ^{60}Co-irradiated antigens from DPG and were the same antigens as those used as positive controls in this present SIBCA exercise. In this SIBCA exercise, false negatives in the ELISA for *B. anthracis* were observed at concentrations of 10^5 cfu/mL and below (samples 234, 203, and 320 at 10^5 cfu/mL, 10^4 cfu/mL, and 10^3 cfu/mL, respectively), for *Y. pestis* at concentrations of 10^5 cfu/mL and below (samples 390, 231, and 420 at 10^5 cfu/mL, 10^4 cfu/mL, and 10^3 cfu/mL, respectively), and for all the vaccinia samples (sample 230 at 10^8 pfu/mL, samples 231 and 234 at 10^7 pfu/mL, and samples 183, 168, and 113 at 10^6 pfu/mL, 10^5 pfu/mL, and 10^4 pfu/mL, respectively). ELISA results obtained for SIBCA samples containing varying concentrations of *B. anthracis* are consistent with those expected from the predetermined LOD of the *B. anthracis* assay, given predicted signal variations at or near the end-point. For samples containing *Y. pestis*, where the predetermined LOD was 10^3 cfu/mL, one would expect that the 10^4 and 10^5 cfu/mL concentrations would have been detected. Although the antibodies used in the *Y. pestis* assay were not at optimized values, the LODs observed using varying concentrations of capture, detector, and indicator antibodies during the optimization of the *Y. pestis* assay were all in the range of 1-9 x 10^3 cfu/mL (unpublished data). The results of the vaccinia ELISA are similarly unexpected and cannot be readily explained. Although the LOD of the vaccinia ELISA was previously determined at DRDC Suffield to be 10^6 cfu/mL, and the SIBCA positive control in this exercise was detectable at 10^7 pfu/mL, SIBCA VI samples containing 10^8 pfu/mL and below were not detectable. In previous SIBCA exercises at DRDC Suffield, absorbance signals of the SIBCA sample unknowns at given concentrations also did not correspond well with absorbances previously determined at DRDC Suffield (unpublished data). The ELISA positive controls for *B. anthracis, Y. pestis,* and vaccinia virus used in all SIBCA exercises at DRDC Suffield to date, as well as the antigens used in LOD studies of these three agents at DRDC Suffield, were all provided by US DPG. It is not known, however, whether these latter agents and those provided in the SIBCA VI samples were from the same antigen batches. If from different batches, procedural differences in batch preparation may have

[6] NATO SIBCA level of identification [15] where "Confirmed" means identification by two different methods in the presence of a positive control; "Provisional" means identification by one method in the presence of a positive control.

contributed to discrepancies in amount of immunological reactive material between batches. A good check on the antigenic stability of these three antigens stocked at DRDC would be to re-titrate them for LODs in respective ELISAs to confirm consistency with LODs previously determined.

The LODs of the RAMP™ assays according to the manufacturer are 3×10^5 cfu/mL and 7×10^6 pfu/mL for *B. anthracis* and vaccinia virus, respectively. As similarly observed with the ELISAs, false negatives for *B. anthracis* were observed in SIBCA samples at 10^5 cfu/mL and below (samples 234 and 364 at 10^5 cfu/mL, and samples 203 and 320 at 10^4 cfu/mL and 10^3 cfu/mL, respectively) and no vaccinia was observed in any SIBCA sample (sample 230 at 10^8 pfu/mL, samples 231 and 234 at 10^7 pfu/mL, and samples 183, 168, and 113 at 10^6 pfu/mL, 10^5 pfu/mL, and 10^4 pfu/mL, respectively). Results on the *B. anthracis* RAMP™ HHAs were consistent with those expected from the manufacturer's stated LODs. Results obtained with the vaccinia HHAs were not as expected since, with a LOD of 7×10^6 pfu/mL (as per the manufacturer), vaccinia in samples 231 and 234 (10^7 pfu/mL) and 230 (10^8 pfu/mL) should have been detectable. Furthermore, although the positive control produced a strong positive signal at 10^8 pfu/mL, vaccinia at 10^8 pfu/mL and lower in the SIBCA samples was not detectable.

The combined ELISA and HHA results of the SIBCA exercise indicate serious discrepancies between concentrations of agent in SIBCA VI samples, as stated by DPG post-exercise, and the positive control antigenic materials in use at DRDC Suffield. This is particularly obvious with the vaccinia ELISA, where vaccinia was readily detectable in positive controls but undetectable in any of the SIBCA samples containing what was believed to be equivalent concentrations of virus. It is possible that the SIBCA samples did not actually contain the concentrations of vaccinia as stated by DPG. This suggestion is supported by the observation that genetic methods, which theoretically can detect a single virus particle in a sample unknown, could detect vaccinia in only two of the SIBCA VI samples (10^7 and 10^8 pfu/mL) (Doug Bader, personal communication). Due to the observed discrepancies between agent concentrations in SIBCA VI samples, as provided by DPG following the exercise, and those of control antigens in use at DRDC Suffield, it was not possible to validate, with certainty, the sensitivity of the ELISAs and HHAs in use at DRDC Suffield. To overcome this problem, the use of standardized antigenic materials among the NATO nations, should be adopted.

Conclusions

Participation in NATO SIBCA VI has provided DRDC Suffield with the opportunity to evaluate several immunological technologies, namely ELISA and RAMP™ HHAs, for sensitivity in the detection and identification of several biological threat agents, namely *B. anthracis*, *Y. pestis*, and vaccinia virus. Based on agent concentrations provided by DPG following the SIBCA exercise, ELISAs at DRDC Suffield successfully detected *B. anthracis* in SIBCA samples at concentrations of 10^5 cfu/mL and greater, *Y. pestis* at concentrations of 10^6 cfu/mL and greater, and vaccinia vius not at all, even at concentrations in excess of 10^7 cfu/mL. Similarly, RAMP™ HHAs detected *B. anthracis* in SIBCA samples at concentrations exceeding 10^5 cfu/mL, and vaccinia in no samples, even in those with concentrations exceeding 10^7cfu/mL. A discrepancy between agent concentrations in SIBCA samples as provided by DPG post-exercise, and concentrations of the same agent in in-house positive control materials, was observed. In order to accurately validate and compare assay identification systems for sensitivity on an international basis, the use of standardized positive control materials among nations should be instituted. It may be concluded from the results of this work, that ELISA and RAMP™ HHAs are useful tools for the immunological identification of biological agents in samples, but that further work is required, using standardized reference antigens, to establish international standards for assay sensitivity.

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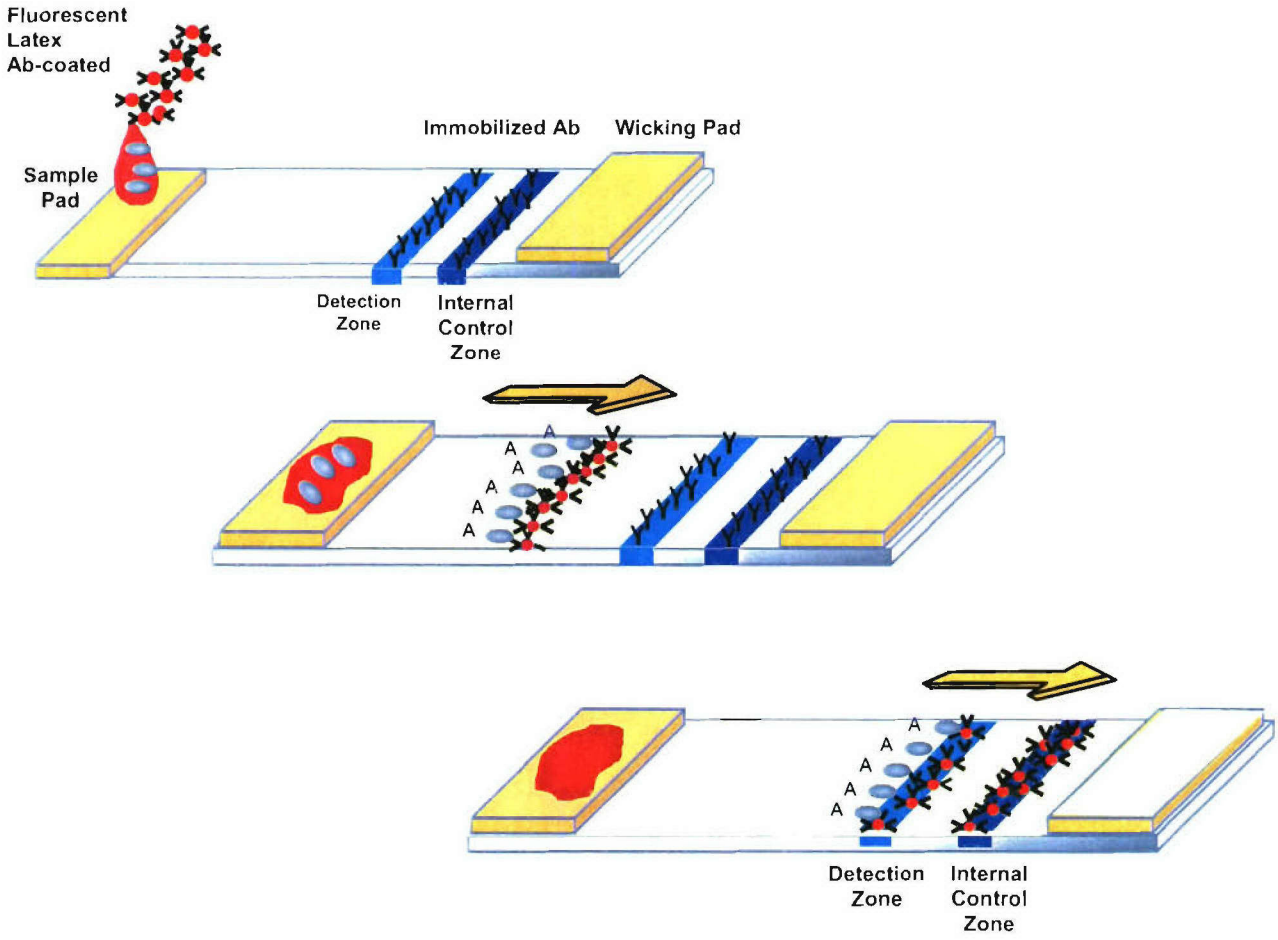

Figure 1: Diagrammatic representation of the principle of operation of the RAMP™ system (reproduced courtesy of W Radvak, (Response Biomedical Corp.)

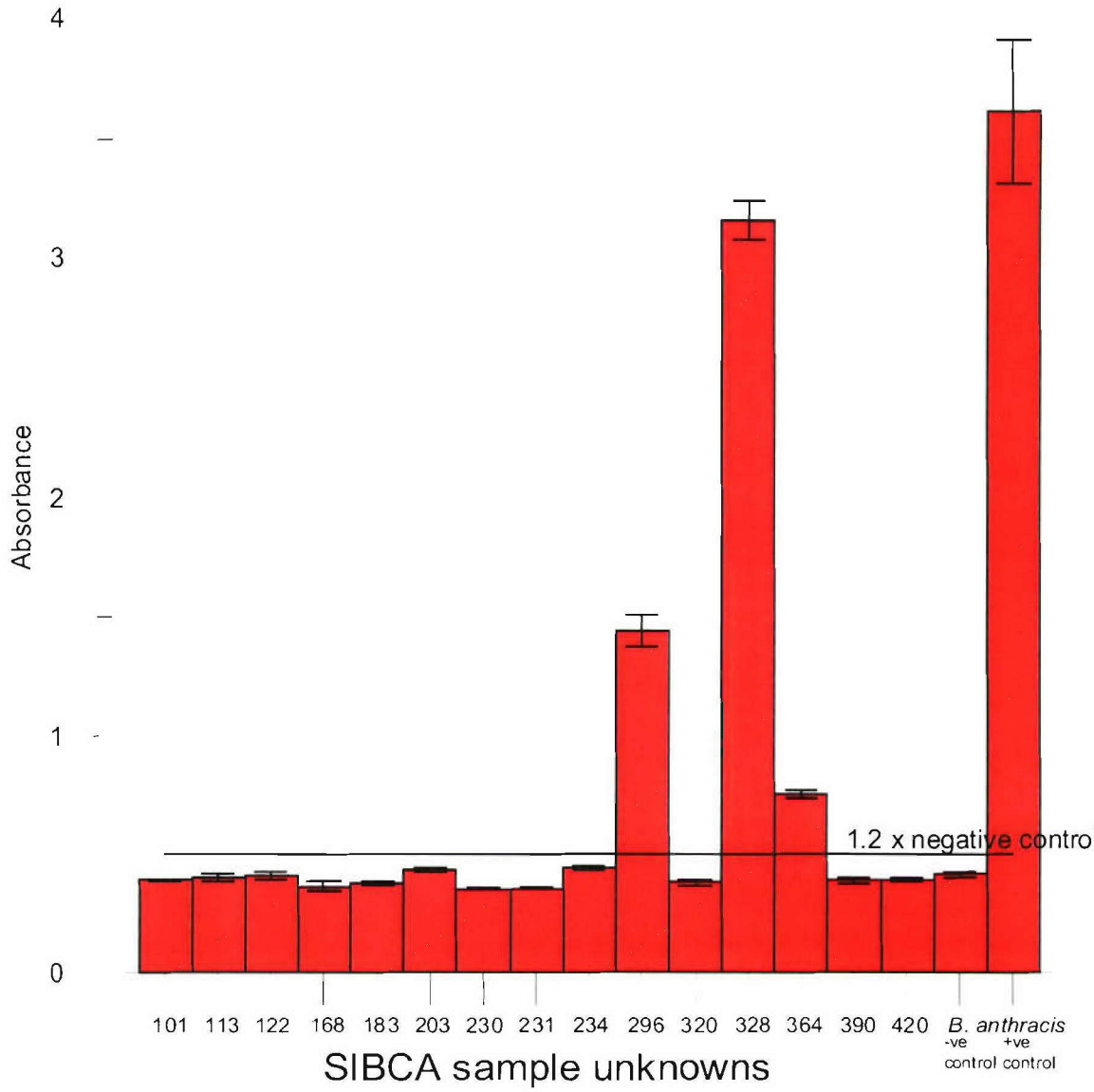

Figure 2: B. anthracis ELISA challenged with SIBCA VI sample unknowns. Positive control was $1.3x10^7$ cfu/mL dilution. Error bars represent the mean +/- one standard deviation

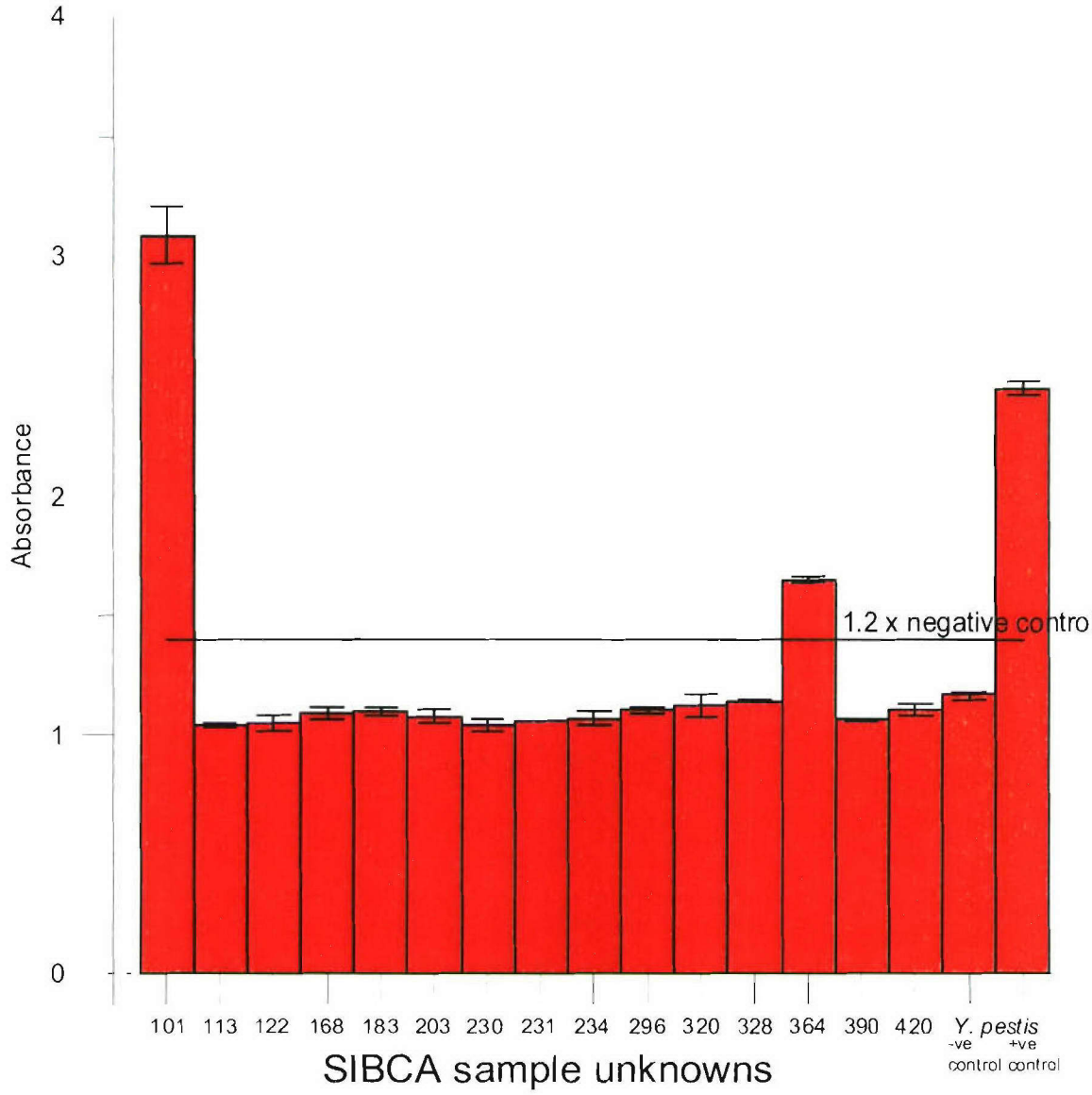

Figure 3. Y. pestis ELISA challenged with SIBCA VI sample unknowns. Positive control was $3.6x10^6$ cfu/mL dilution. Error bars represent the mean +/- one standard deviation

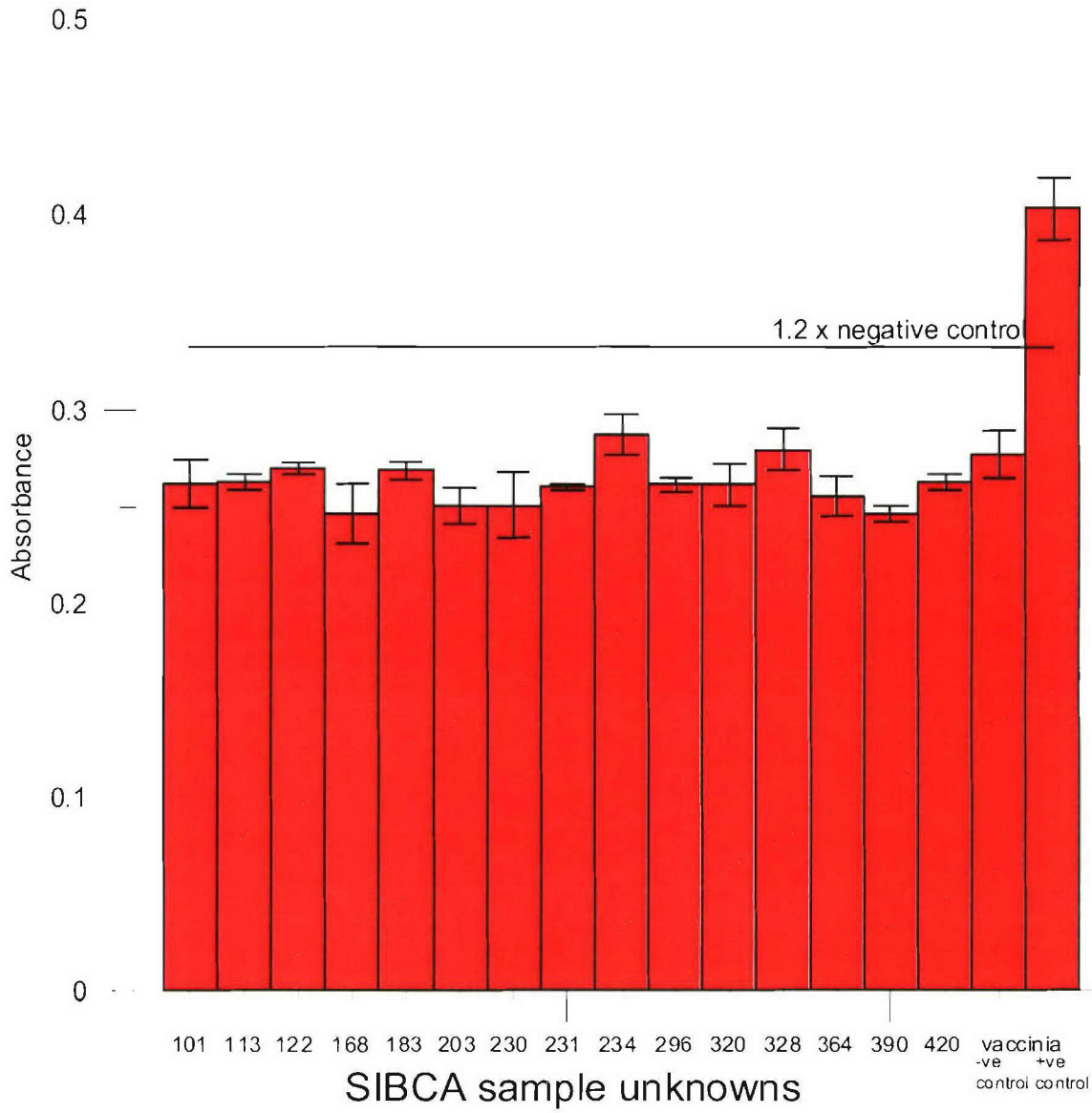

Figure 4: Vaccinia ELISA challenged with SIBCA VI sample unknowns. Positive controls was $1x10^7$ pfu/mL dilution. Error bars represent the mean +/- one standard deviation

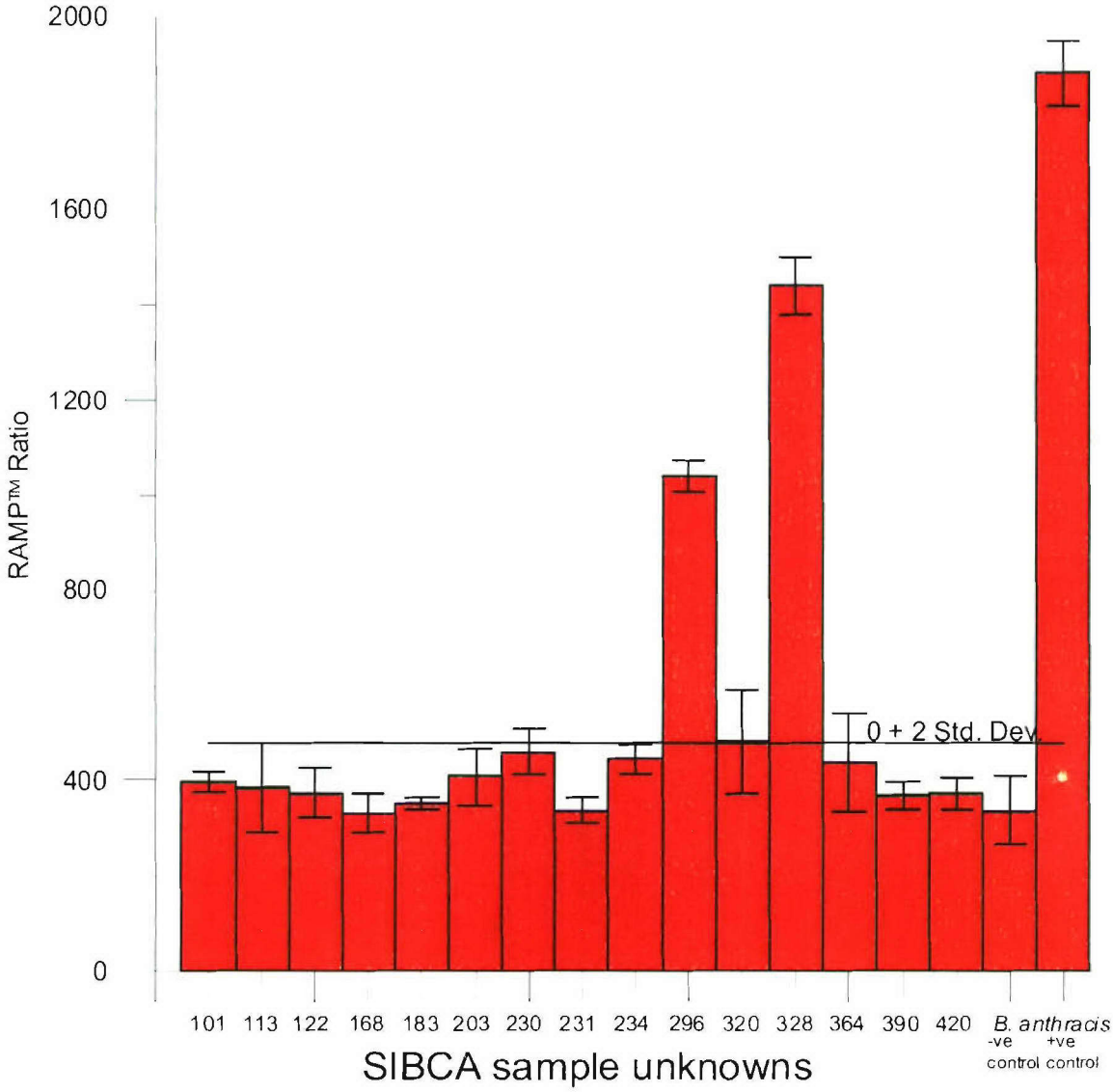

Figure 5: B. anthracis RAMP™ IIIA challenged with SIBCA VI sample unknowns. Positive control was $1.3x10^7$ cfu/mL dilution. Error bars represent the mean +/- one standard deviation

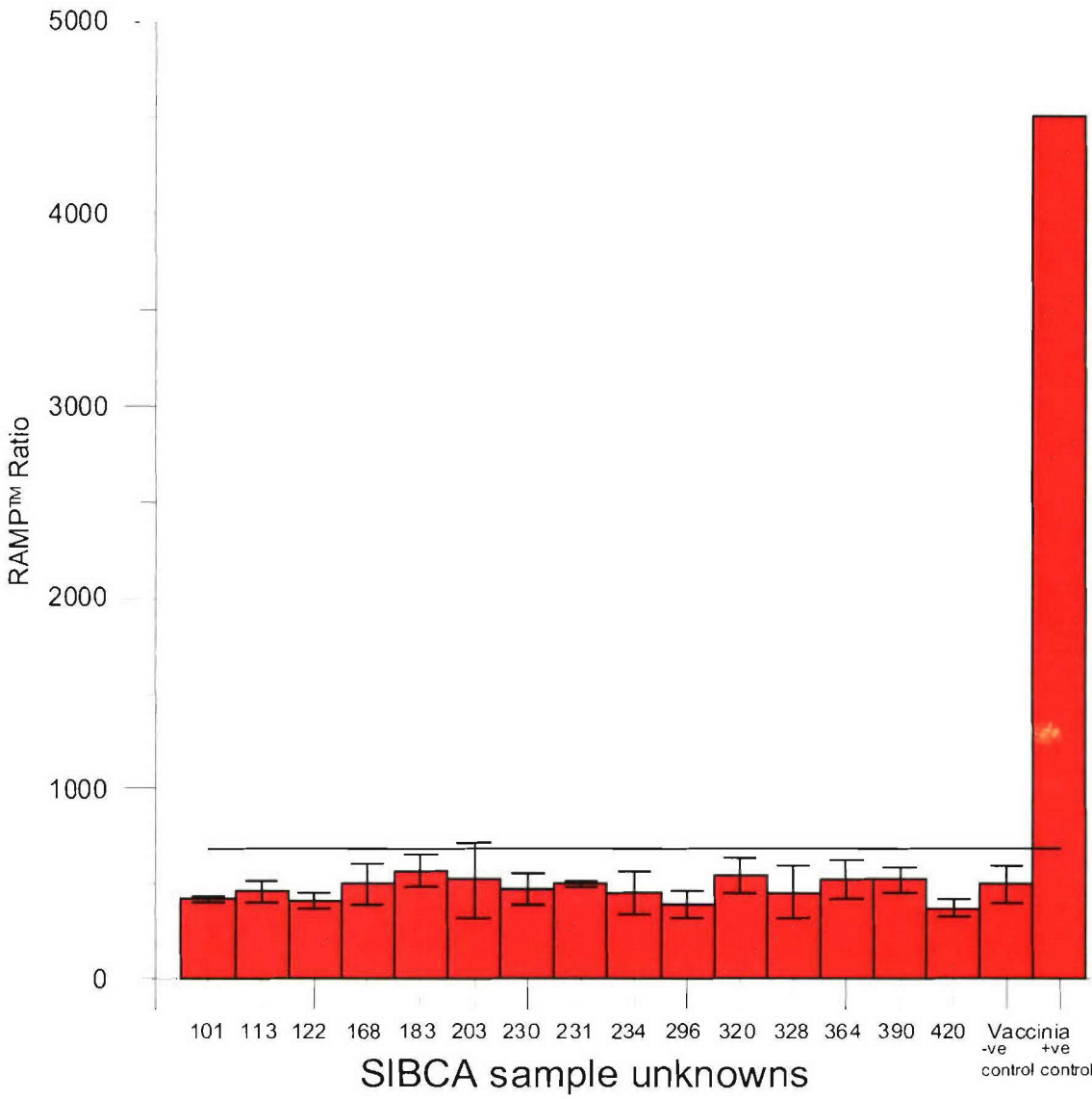

Figure 6: Vaccinia RAMP™ IIIA challenged with SIBCA VI sample unknowns. Positive control was 1x10⁸ pfu/mL dilution. Error bars represent the mean +/- one standard deviation

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In March 2005, the NATO Panel VII Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the sixth international training exercise on identification of biological agents. Fifteen biological sample unknowns, diluted in PBS were sent to each laboratory and participants were advised that biological agents could consist of varying concentrations of any of the following three gamma-irradiated organisms: *Bacillus anthracis*, *Yersinia pestis*, or vaccinia virus. The object of the exercise was to assess each nation's current capabilities in assay sensitivity for these agents. An immunological-based technology, the enzyme-linked immunosorbant assay (ELISA), was used at DRDC Suffield for screening of sample unknowns. Antigen capture ELISAs for each of the three possible biological agents were used to screen the samples. In addition, a fluorescent hand-held assay, the Rapid Analyte Measurement Platform (RAMP™), manufactured by Response Biomedical Corp., was used to analyze the samples for the presence of *Bacillus anthracis* and vaccinia virus. Five biological agent unknowns were identified by ELISA in the SIBCA samples, three samples containing *B. anthracis* and two samples containing *Y. pestis*. Two biological agent unknowns were identified by RAMP™ in the SIBCA samples, both containing *B. anthracis*. No agents were identified in 11 of the SIBCA sample unknowns. A comparison of the immunoassay results with the identity of organisms in SIBCA sample unknowns, as revealed by Dugway Proving Ground following the exercise, indicated confirmed identification of five agents and the sample blank, and 12 false negative identifications.

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Rapid Analyte Measurement Platform
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