Results and Recommendations from the First NATO International Training Exercise on Laboratory Identification of Biological Agents

J.R. Hancock, R.E. Fulton, D.E. Bader, H.G. Thompson, G.R. Fisher, L.L. Stadnyk and B. Kournikakis
Defence Research Establishment Suffield

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Abstract

In March 1999, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the first international training exercise on the identification of biological agents. Eleven laboratories representing 10 nations: Canada, France, Germany (two laboratories), Hungary, Italy, Netherlands, Norway, Poland, United Kingdom, and the United States participated in the international training exercise. Each participating laboratory was sent five "unknown" samples, four of which contained ^{60}Co (Cobalt-60)-inactivated biological material and one blank containing phosphate buffered saline (PBS).

The United States, as the host nation, distributed PBS, *Bacillus anthracis, Coxiella burnetii,* Venezuelan Equine Encephalitis (VEE) virus, *and Yersinia pestis* as the samples for the training exercise. Canada correctly identified *Bacillus anthracis* and *Yersinia pestis* and correctly indicated that the remaining samples did not contain *Vibrio cholerae, Francisella tularensis, Brucella melitensis, Burkholderia mallei* or Vaccinia virus. As no assays were available at DRES for Yellow Fever virus, VEE or *Coxiella burnetii,* the presence of these agents could not be determined in the remaining samples.

This report summarizes the results obtained by Canada and compares the performance of DRES to the other participating international laboratories. Areas requiring improvement at DRES for the in-house identification of biological agents are discussed.

Résumé

En mars 1999, le sous-groupe du Groupe Terrestre de l'OTAN spécialisé en échantillonnage et identification des agents biologiques et chimiques (SIBCA) a conduit son premier exercice d'entraînement international d'identification d'agents biologiques. Onze laboratoires représentant dix nations : le Canada, la France, l'Allemagne (2 laboratoires), la Hongrie, l'Italie, les Pays-Bas, la Norvège, la Pologne, la Grande-Bretagne et les États-Unis ont participé à cet exercice international d'entraînement. Chaque laboratoire a reçu cinq échantillons « inconnus », quatre d'entre eux contenaient des matériaux biologiques inactivés ^{60}Co (Cobalt-60) et un blanc contenant du soluté physiologique avec tampon phosphate (PBS).

Les États-Unis, pays d'accueil, ont distribué du PBS, *Bacillus anthracis, Coxiella burnetii* , le virus de *l'encéphalite équine vénézuélienne* (VEE), *et Yersinia pestis* comme échantillons pour l'exercice d'entraînement. Le Canada a correctement identifié le *Bacillus anthracis* et la *Yersinia pestis* et a correctement indiqué que les échantillons restants ne contenaient pas de *Vibrio cholerae, Francisella tularensis, Brucella melitensis, Burkholderia mallei* ou le virus Vaccine. Les tests de vérification n'étant pas disponibles au CRDS pour le virus de la fièvre jaune, le VEE ou *Coxiella burnetii,* il n'a pas été possible de déterminer la présence de ces agents dans les échantillons restants.

Ce report analyse les résultats obtenus par le Canada et compare la performance du CRDS par rapport à celle des autres laboratoires internationaux ayant participé. Les domaines sujets à améliorations au niveau interne pour l'identification des agents biologiques au CRDS y sont aussi discutés.

Executive summary

Introduction

The Canadian Forces (CF) may be called on to perform peacekeeping or battlefield operations in regions of the world where there is a significant threat of biological warfare agent use. To operate effectively in these theatres, the CF must be able to identify the exact nature of the biological agent(s).

In 1999, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the first international training exercise on the identification of biological agents. Ten possible agents were selected by the United States (host nation); from this list, four agents and a blank were distributed to all the participating laboratories. Eleven laboratories representing 10 nations (Canada, France, Germany (two laboratories), Hungary, Italy, Netherlands, Norway, Poland, United Kingdom, and the United States) participated in the international training exercise. Participants were informed that the samples could contain any of the following ten ^{60}Co- inactivated agents: *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.*

Results

The United States distributed PBS, *Bacillus anthracis*, *Coxiella burnetii,* VEE virus, *and Yersinia pestis* as agent unknowns for the training exercise. Using immunological techniques, Canada correctly identified *Bacillus anthracis* and *Yersinia pestis* and correctly concluded that the samples did not contain *Vibrio cholerae*, *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei,* or Vaccinia virus. As no immunoassays were available at DRES for Yellow Fever virus, VEE or *Coxiella burnetii*, the presence of these agents could not be determined in the remaining samples. Using DNA techniques, Canada detected 16S rRNA amplicons in two of the samples, indicating the presence of bacterial or ricketessial organsims. Based on the NATO identification criteria, Canada provisionally identified two of the four agent unknowns. By comparison, eight of the participating laboratories obtained provisional identification for three of the four agent unknowns, with four laboratories able to confirm the identification for three of the four agents.

Significance of Results

This training exercise allowed Canada to assess current in-house capabilities and identify areas where problems exist in BW identification. While DRES could identify two of the four agents distributed as test samples, the lack of antibodies and specific gene probes and associated assays for additional agents was noted. Equipment requirements for the BL3 suite and problems associated with import permits need to be resolved prior to the next training exercise. Under the conditions used during this training exercise it was demonstrated that biological agents inactivated by gamma irradiation can be successfully identified in sample unknowns using either immunoassay or genetic methods.

Future Goals

At DRES, the research effort in identification of biological agents is focused on identification of bacteria, viruses, and biological toxins through application of immunoassay- and genetic probe-based technologies. DRES needs to increase the number of pathogens that can be identified using immunological and genetic methods. The development of a nucleotide sequencing capability would also be beneficial for providing definitive analysis of amplified genetic sequences.

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Sommaire

Introduction

Les Forces canadiennes (FC) risquent d'être appelées à exécuter des opérations de maintien de la paix et des opérations sur les champs de bataille dans les régions du monde où il existe une menace importante d'utilisation d'agents chimiques et biologiques. Pour opérer de manière efficace dans ces théâtres, les FC doivent être en mesure d'identifier la nature exacte des agents biologiques.

En 1999, le sous-groupe du Groupe Terrestre de l'OTAN spécialisé en échantillonnage et identification des agents biologiques et chimiques (SIBCA) a conduit son premier exercice d'entraînement international d'identification d'agents biologiques. Dix agents potentiels ont été sélectionnés par les États-Unis (le pays d'accueil) et à partir de cette liste, quatre agents et un blanc ont été distribués à tous les laboratoires participant. Onze laboratoires représentant dix nations : le Canada, la France, l'Allemagne (2 laboratoires), la Hongrie, l'Italie, les Pays-Bas, la Norvège, la Pologne, la Grande-Bretagne et les États-Unis ont participé à cet exercice international d'entraînement. Les participants ont été informés que les échantillons pouvaient contenir l'un des dix agents désactivés ^{60}Co: *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, le virus de l'encéphalite équine vénézuélienne (VEE), *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, le virus de la fièvre jaune, le virus Vaccine ou *Coxiella burnetii.*

Résultats

Les États-Unis ont distribué du PBS, *Bacillus anthracis*, *Coxiella burnetii*, le virus VEE, et la *Yersinia pestis* comme agents inconnus pour l'exercice d'entraînement. À l'aide des techniques immunologiques, le Canada a correctement identifié le *Bacillus anthracis* et la *Yersinia pestis* et a correctement conclu que les échantillons restants ne contenaient pas de *Vibrio cholerae*, *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei* ou le virus Vaccine. Les tests de vérification n'étant pas disponibles au CRDS pour le virus de la fièvre jaune, le VEE ou *Coxiella burnetii,* il n'a pas été possible de déterminer la présence de ces agents dans les échantillons restants. À l'aide de techniques ADN, le Canada a détecté des amplicons ARNr 16S dans deux des échantillons, indiquant ainsi la présence d'organismes bactériens et rickettsies. En se basant sur les critères d'identification de l'OTAN, le Canada a provisoirement identifié deux des quatre agents inconnus. En comparaison, huit des laboratoires participant ont réussi à identifier provisoirement trois agents inconnus sur quatre et quatre laboratoires ont réussi à confirmer l'identification de trois agents sur quatre.

Portée des résultats

Cet exercice d'entraînement a permis au Canada d'évaluer ses capacités internes actuelles et d'identifier les domaines des problèmes existants en termes d'identification d'agents de guerre biologique. Le CRDS a réussi à identifier deux des trois agents distribués comme échantillons d'essais, cependant on a noté le manque d'anticorps et d'analyseurs de gènes spécifiques ainsi que les tests de vérification y étant associés pour les agents additionnels.

L'équipement requis pour le laboratoire étanche (BL3) et les problèmes reliés aux permis d'importation devront être résolus avant le prochain exercice d'entraînement. On a démontré que, dans les conditions des exercices d'entraînement, il est possible d'identifier des échantillons inconnus d'agents biologiques désactivés par les irradiations aux rayons gamma, en utilisant les tests de vérification immunologique ou des méthodes génétiques.

Buts futurs

Au CRDS, la recherche dans le domaine d'identification des agents biologiques est concentrée sur l'identification des bactéries, des virus et des toxines biologiques à travers l'application des technologies de tests de vérification immunologique et des technologies d'analyse de gènes. Le CRDS doit augmenter le nombre d'agents pathogènes pouvant être identifiés par des méthodes immunologiques ou génétiques. Le développement de ses capacités de séquençage de nucléotides permettrait aussi de fournir des analyses définitives de séquences génétiques amplifiées.

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Introduction

NATO may be called upon to deploy military forces in support of peacekeeping or battlefield operations in regions of the world where there is a significant threat of the use of biological warfare (BW) agents. To operate effectively in these theatres, NATO forces must be able to detect and identify BW agents.

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

Each participating laboratory was sent five sample unknowns, four of which contained biological material and one blank containing phosphate buffered saline (PBS). Participants were informed that the samples could contain any of the following ten ^{60}Co-inactivated agents: *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 United States, as the host nation, distributed PBS, *Bacillus anthracis*, *Coxiella burnetii,* VEE virus, *and Yersinia pestis* as agent unknowns for the training exercise. Participating laboratories were requested to complete their analyses within 10 working days.

In Canada, the participating laboratory was the Defence Research Establishment Suffield (DRES). Three technologies, Light Addressable Potentiometric Sensor (LAPS) assay, Immunochromatographic Assay (ICA), and Polymerase Chain Reaction targeting 16S ribosomal RNA genes (16S rDNA PCR) were employed in this exercise. LAPS was considered a high priority candidate technology for laboratory identification of biological agents at DRES. For identification of biological agents in the field, an automated identification system based on ICA technology, is installed in the Canadian Integrated Biological Agent Detection System (CIBADS). Thus, for the SIBCA training exercise, LAPS was selected as the prime immunological laboratory-based technology, while ICAs were included as a means of confirmatory back-up identification. The genetic amplification technology, 16S rDNA PCR, was included in this training exercise to assess the suitability of PCR-based techniques on gamma-irradiated samples, given that gamma irradiation has been shown to damage RNA [1] and DNA [2].

Results from the SIBCA training exercise reflect the capabilities and deficiencies of the DRES laboratories for in-house identification of the specific biological agents at the time of the exercise. Antibodies and antigens used in the LAPS assay were those held in DRES inventory at the time of the exercise and no attempts were made to procure immunological reagents solely for the purpose of the exercise. Similarly, ICAs used reflect the commercial availability of ICAs for the ten agents selected for this training exercise.

This report summarizes the results obtained by Canada and compares the performance of DRES to the other participating international laboratories. Areas requiring improvement at DRES for the in-house identification of biological agents are discussed.

Materials and methods

NATO/SIBCA test samples

Samples of biological agents inactivated by ^{60}Co gamma irradiation were prepared at Dugway Proving Ground (Utah, USA) and were dispatched by courier to participating NATO SIBCA laboratories during March 1999. The biological agents were suspended in PBS (10 mL) and contained either bacteria at a concentration of 10^6 - 10^7 cfu/mL or virus/rickettsia at 10^7 - 10^8 pfu/mL Five samples were received at DRES on 24 March 1999. These samples, labelled 110, 137, 143, 157, and 160 were, upon receipt, immediately subdivided into separate 5 x 2 mL sub-samples. Sub-samples for immediate use were stored at 4 °C, while the remaining sub-samples were transferred to a -70 °C freezer for longer term storage.

Assays

Detailed descriptions of the materials, methods and instrumentation used for the analysis of the SIBCA samples by LAPS, ICA and PCR have been reported elsewhere [3-5].

Results

LAPS assay

Prior to the SIBCA training exercise, antibodies for *Bacillus anthracis*, *Yersinia pestis*, VEE, Vaccinia virus, *Francisella tularensis*, *Brucella melitensis*, and *Burkholderia mallei* were conjugated with fluorescein and biotin, respectively, and each labelled antibody was tested by LAPS assay against the respective homologous antigens. All agent-specific assays were positive with respective homologous antigens (positive controls) with the exception of VEE and *Bacillus anthracis*, both of which were negative. Subsequent studies indicated that, in the case of the *Bacillus anthracis* assay, the negative result on the positive control was due to inactivity of the antigen; in the case of VEE, however, the biotinylated antibody was responsible for the negative result. In spite of the poor quality of the positive control in the *Bacillus anthracis* assay, this assay was included in the screening of the SIBCA training exercise samples. Immunoreagents for *Coxiella burnetii*, *Vibrio cholerae*, and Yellow fever virus were not available, therefore, LAPS assays for these agents could not be performed. Hence, assays for Vaccinia virus, *Yersinia pestis, Francisella tularensis, Brucella melitensis, Burkholderia mallei,* and *Bacillus anthracis*, were performed on SIBCA sample unknowns. A summary of sample identification results based on LAPS is provided in Table 1. *Bacillus anthracis* was provisionally identified in sample 137 and *Yersinia pestis* was provisionally identified in sample 143. A more detailed account of the LAPS results for SIBCA exercise I has been reported [3].

Table 1. Summary of sample identification results based on LAPS analysis

Sample No.	Vaccinia	*Yersinia pestis*	*Francisella tularensis*	*Brucella melitensis*	*Bacillus anthracis*	*Burkholderia mallei*
110	-	-	-	-	-	-
137	-	-	-	-	+	-
143	-	+	-	-	-	-
157	-	-	-	-	-	-
160	-	-	-	-	-	-

Immunochromatographic assay

Immunochromatographic assays were performed on the SIBCA training exercise samples using ICA tickets purchased from or donated by New Horizons Diagnostics Corp. (NHDC) and/or Majesco Biologicals Inc. (MBI). Tickets were validated for use in the exercise if they gave positive reactions with the homologous agents and did not react with heterologous agents, water, or buffer. Based on these criteria, tickets from NHDC were validated for identification of *Vibrio cholerae* and *Francisella tularensis*. Tickets from MBI were validated for identification of Vaccinia virus, *Yersinia pestis*, *Francisella tularensis*, *Brucella melitensis*, and *Bacillus anthracis*. ICAs were unattainable for *Burkholderia mallei*, VEE virus, Yellow fever virus, and *Coxiella burnetii*. A summary of the results obtained by ICA is presented in Table 2. *Bacillus anthracis* was provisionally identified in sample 137 and *Yersinia pestis* was provisionally identified in sample 143. A more detailed account of the ICA results for SIBCA exercise I has been previously reported [4].

Table 2. Summary of sample identification results based on immunochromatographic assay

Sample No.	Ticket					
	Vaccinia[a]	*Yersinia pestis*[a]	*Francisella tularensis*[a, b]	*Brucella*[a] *melitensis*	*Bacillus anthracis*[a]	*Vibrio cholerae*[b]
110	-	-	-	-	-	-
137	-	-	-	-	+	-
143	-	+	-	-	-	-
157	-	-	-	-	-	-
160	-	-	-	-	-	-

[a] MBI
[b] NHDC

Polymerase chain reaction

PCR amplification of 16S rDNA target sequences was conducted using a pair of primers targeted at universally conserved sequences for bacteria/rickettsial organisms [6]. PCR analysis was performed directly on untreated SIBCA samples and on samples that were treated by two different DNA extraction methods, Puregene™ (Pur™) extraction method and Phenol/Chloroform (Ph/Chl) extraction method [5]. A summary of the PCR results obtained on SIBCA samples by 16S rDNA PCR is presented in Table 3. A 16S rDNA amplicon was observed for sample 137 and 160 suggesting these to be of bacterial or rickettsial nature. A more detailed account of the PCR results for SIBCA exercise I has been previously reported [5].

Table 3. *Summary of sample identification results based on 16S rDNA PCR*

Sample	Untreated	Purgene™	Phenol/ Chloroform
110	N.D.	N.D.	N.D.
137	N.D.	450 bp	N.D.
143	N.D.	N.D.	N.D.
157	N.D.	N.D.	N.D.
160	N.D.	N.D.	450 bp

N.D. – no amplicon detected

Comparison of DRES results to SIBCA exercise unknowns

The identification results obtained by DRES using LAPS, ICA, and PCR assays are summarized in Table 4 and are compared to the agents present in SIBCA samples [7]. Table IV, shows that *Bacillus anthracis* and *Yersinia pestis* were correctly identified, by both LAPS and ICA, in samples 137 and 143, respectively. Canada also correctly reported, based on the combined LAPS and ICA results, that samples 110, 157 and 160 did not contain *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*, *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, or Vaccinia virus. LAPS and ICA assays for Yellow Fever virus, VEE and *Coxiella burnetii* were not available at DRES, therefore the presence of these agents in SIBCA sample unknowns could not be assessed. 16S rDNA amplicons were correctly detected in two of the three samples that contained 16S rRNA gene (*Bacillus anthracis* and *Coxiella burnetii*), but were not detected for *Yersinia pestis*. 16S rDNA amplicons were not observed for the VEE or PBS blank as expected.

Table 4. Sample identification results based on LAPS, ICA, and PCR assays

Sample No.	LAPS Assay	ICA	PCR 16S rDNA amplicon	SIBCA Unknown[1]
110	Not detected: *Bacillus anthracis, Brucella melitensis, Burkholderia mallei, Francisella tularensis,* Vaccinia virus, *Yersinia pestis*	Not detected: *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Vibrio cholerae,* Vaccinia virus, *Yersinia pestis*	Not observed	PBS Blank
137	***Bacillus anthracis***	***Bacillus anthracis***	Yes - bacterial or rickettsial	*Bacillus anthracis*
143	***Yersinia pestis***	***Yersinia pestis***	Not observed	*Yersinia pestis*
157	Not detected: *Bacillus anthracis, Brucella melitensis, Burkholderia mallei, Francisella tularensis,* Vaccinia virus, *Yersinia pestis*	Not detected: *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Vibrio cholerae,* Vaccinia virus, *Yersinia pestis*	Not observed	VEE
160	Not detected: *Bacillus anthracis, Brucella melitensis, Burkholderia mallei, Francisella tularensis,* Vaccinia virus, *Yersinia pestis*	Not detected: *Bacillus anthracis, Brucella melitensis, Francisella tularensis, Vibrio cholerae,* Vaccinia virus, *Yersinia pestis*	Yes - bacterial or rickettsial	*Coxiella burnetii*

[1] Information provided by DPG, post-trial [7]

Technologies used by participating laboratories

This training exercise was designed as a learning experience, which provided the participating laboratories an opportunity to evaluate their own identification methods, as well as view the technologies used for BW agent identification in the other national laboratories. The most widely used approaches were immunological and genetic analyses; most laboratories used both immunological and genetic techniques for the assay of the SIBCA training samples. ELISA, immunofluoresence, and PCR were the most extensively used methods, with a number of countries using antibody-based, hand-held assays (immunochromatographic assays). The technologies used by each of the participant nations, as reported by Harper, 1999 [8], are provided in Table 5.

Table 5. Technologies used by participating laboratories

Nation	Technologies
Canada	LAPS, ICA, PCR
France	IF, PCR
Germany #1	LM (Gram's stain/spore stain), IF, TEM, ELISA, PCR
Germany #2	ELISA, HHA, PCR
Hungary	LM (Gram's stain), IF
Italy	PCR
The Netherlands	LM (Gram's stain), ELISA, Immuno-dot blot, IF, PCR
Norway	PCR
Poland	PCR, ICA (Smart[R]), LM, TEM, DOT-ELISA
United Kingdom	ELISA, PCR
United States	ELISA, HHA

Legend: IF: Immunofluorescence; LM: Light Microscopy; TEM: Transmission Electron Microscopy; ELISA: Enzyme-linked Immunosorbant Assay; HHA: Hand-Held Assay

Identification achieved by participating laboratories

The identity of the agents in the training exercise samples and the results reported, by the host nation, for each participating laboratory for the two most commonly used methods (immunological and genetic analysis) are provided in Table 6. Nine out of the 10 laboratories participating in the SIBCA exercise correctly identified *Bacillus anthracis, Coxiella burnetii*, and *Yersinia pestis*. Four of these laboratories (including Canada) reported identification of *Bacillus anthracis* and *Yersinia pestis* at the NATO Provisional Level (identification by either immunological, genetic, or biochemical analysis) [9], while five laboratories reported identification of these agents at the NATO Confirmed Level (identification by two of the three technologies) [9]. Five laboratories identified *Coxiella burnetii* at the NATO Provisional Level while four laboratories reported identification of this agent at the NATO Confirmed Level. Most laboratories had problems with identification of VEE. Only two of the 10 participating laboratories correctly identified this organism and, in both cases, identification was achieved by PCR only (NATO Provisional Level). The host country speculated that problems keeping the VEE sample cool during shipping may have led to degradation of the RNA virus. This may in part explain the false negatives reported by six laboratories for VEE. While Canada reported no false positives for the training exercise samples, overall four false positives (generally for Vaccinia) were reported by the participating laboratories.

Table 6. *Comparison of identification results provided by participating nations‡*

Laboratory	*Bacillus anthracis*		*Coxiella burnetii*		*Yersinia pestis*		VEE		PBS Blank	
	Immuno	Genetic	Immuno	Genetic	Immuno	Genetic	Immuno	Genetic	Immuno	Genetic
Canada	Pos	Neg[1]	NT	Neg[1]	Pos	Neg	NT	Neg	Neg	Neg
France	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg
Germany #1	Pos	Pos	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg
Germany #2	Pos	Pos	Pos	Pos	Pos	Pos	NT	Pos	Neg	Neg
Italy	NT	Neg[2]	NT	Pos	NT	Neg	NT	Neg	NT	Neg
Netherlands	Pos	Pos	Neg	Pos	Pos[3]	Pos[3]	Neg	Neg	Neg	Neg
Norway	NT	Pos	NT	Pos	NT	Pos	NT	Pos	NT	Neg
Poland	Pos	Pos	Pos	Pos	Pos	Pos	NT	Vaccinia	Neg	Neg
United Kingdom	NT	Pos	Pos	Pos[4]	NT	Pos	Neg	Neg	Neg	Vaccinia
United States	Pos	NT	Pos	NT	Pos	NT	Neg	NT	Neg	NT

NT: Not Tested

[1] Positive for universal 16S rRNA
[2] Positive homology (90%) to *B. anthracis, B. cereus, B. thuringiensis and B. mycoides*
[3] PCR and Immunoblot positive for Vaccinia and *Y. pestis*
[4] Weak positive PCR for Vaccinia and *C. burnetii.*
‡ reproduced from Harper, B.G., 1999 [8]

Discussion

Canada's performance in the NATO international training exercise was satisfactory given that this was the first exercise of its kind held by NATO. The primary limitation was the lack of assays for VEE and *Coxiella burnetii* which meant that Canada could identify only two of the four agents present in the training exercise samples (i.e. *Bacillus anthracis* and *Yersinia pestis).* Canada correctly indicated that the remaining samples did not contain *Vibrio cholerae, Francisella tularensis, Brucella melitensis, Burkholderia mallei* or Vaccinia virus. This training exercise allowed Canada to assess its current capabilities and identify areas where problems exist in BW identification. In the following sections some of these areas and potential solutions are discussed.

Immunological assays: Problems and way ahead

Immunoreagents were available in the DRES reagent collection for development of LAPS assays for all SIBCA I agents except *Vibrio cholerae*, Yellow fever virus, and *Coxiella burnetii*. For DRES to develop assays for these three agents, a source of antigens and/or antibodies for each is required. Currently, limited quantities of antigens, as well as antibodies developed in two animal species, have been obtained for all three of the above-noted agents, for short-term use in subsequent SIBCA exercises. Larger stocks of antibodies for each agent are required for longer term use and mechanisms for acquisition of these materials are currently being developed.

The development of in-house immunoassay capability assumes that reagents incorporated in assays be evaluated for sensitivity (reactivity with homologous antigen) and specificity (non-reactivity with heterologous and unrelated antigens). In addition, selected immunoreagents must be optimized for concentration, orientation, and other assay variables on the platform of choice. While antibodies selected for use on the LAPS have been shown to be sensitive for detection of the homologous agent/antigen (Gail Thompson, personal communication), most have not been tested for specificity or non-reactivity in the presence of potential battlefield interferents. In addition, none of the LAPS antibody reagents has been assessed for reactivity with live agent. Further work is, therefore, required to demonstrate the specificity and live agent reactivity of immunoreagents incorporated in agent-specific LAPS assays at DRES.

ICAs used in this training exercise were available from one or both of two manufacturers, NHDC and MBI, for all target agents except *Burkholderia mallei*, VEE, Yellow fever virus, and *Coxiella burnetii*. Considerable problems were encountered in validating tickets for use in the SIBCA I trials. Many agent-specific tickets from NHDC were invalidated for reasons that included cross-reactivity with heterologous agents, non-reactivity with homologous agents, and non-specific reactivity with buffer or water. Some agent tickets from MBI reacted only with live homologous agent and were non-reactive with homologous killed (formalin-inactivated) agent. These and other problems experienced with the use of tickets from both manufacturers have been reported and discussed elsewhere [4]. In spite of the problems encountered in qualifying tickets for use in these trials, tickets from one or both manufactureres were validated for Vaccinia virus, *Yersinia pestis*, *Francisella tularensis*,

Brucella melitensis, *Bacillus anthracis*, and *Vibrio cholerae*. Currently, ICAs that were not available at the time of this training exercise are known to be under development, both by MBI (David Cullin, personal communication) and NHDC (David Trudil, personal communication). Furthermore, an initiative to have ICAs developed in Canada, using Canadian-sourced antibodies, has been undertaken.

Evaluation of LAPS and ICA results revealed an apparent relationship between the nature of antigen used to raise component antibodies and the reactivity of those antibodies with homologous antigen. For example, antibodies produced from cobalt-inactivated *Bacillus anthracis* could detect live or cobalt-inactivated *Bacillus anthracis* but could not detect formalin-inactivated *Bacillus anthracis*, either by LAPS or ICA. Similarily, antibodies produced from cobalt-inactivated *Yersinia pestis* could detect live or cobalt-inactivated *Yersinia pestis* by ICA but not formalin-inactivated *Yersinia pestis*; and antibodies produced from cobalt-inactivated *Brucella melitensis* could detect live *Brucella melitensis* by ICA but not formalin-inactivated *Brucella melitensis*. In the case of *Francisella tularensis*, there appeared to be an exception to this apparent trend, as antibodies produced from cobalt-inactivated *Francisella tularensis* could detect formalin-inactivated *Francisella tularensis* by ICA. Many of the antibodies developed by DRES have been produced from formalin-inactivated agents, thus it would be important to know whether antibodies produced from formalin-inactivated antigens could detect live agents. Unfortunately, there was little data generated from this trial to support an answer to this question, although antibodies produced from formalin-inactivated *Yersinia pestis* detected cobalt-irradiated *Yersinia pestis* in SIBCA sample 143 by LAPS. It is well documented that inactivation of proteins with formalin can result in damage to surface epitopes [4, 11, 12, 13]. The *Bacillus anthracis* positive agent control used in these SIBCA exercises was inactivated at DRES with 3.7% formalin (John Cherwonogrodzky, personal communication). Evidence suggestive that this material may have been damaged comes from anecdotal reports that the preparation exhibited properties of severe clumping, consistent with cross-linking of component proteins. Gamma irradiation of organisms as a means of inactivation of infectivity acts by fragmentation of component nucleic acids. This method of inactivation is believed to be the least damaging to the integrity of surface protein epitopes and, therefore, is the favoured method of inactivation for retention of antigenicity. The results of this study suggest that immunoassay performance may be influenced by the method of inactivation of the antigen used to produce the detecting antibodies incorporated in the assay. Further studies are required to determine the effects of various methods of inactivation on the antigenicity of specific biological agents and subsequent effects on immunoreactivity and biodetection capability of antibodies raised in response to these antigens. Until the results of such studies are known, it would be advisable that methods used for inactivation of agents for use as immunogens in the production of antibodies for biodetection, be those least likely to result in damage to surface protein epitopes. Present consensus in this regard is that gamma irradiation is the best procedure for inactivating the infectivity of microorganisms while, at the same time, retaining their integrity and antigenicity.

Genetic assays: Problems and way ahead

Since gamma irradiation is known to fragment nucleic acid, 16S rDNA PCR was used in the training exercise in order to determine whether PCR technology could be successfully applied to gamma-irradiated material. Results from DRES and other labs, indicated that PCR amplification of gamma-irradiated (killed) organisms was possible and revealed areas where further effort is needed in order to improve and expand DRES's genetic identification capabilities for future trials.

The 16S rDNA PCR assay employed in this exercise failed to amplify 16S amplicons from one of the three organisms containing 16S rRNA genes (the same result was reported by two other laboratories). By contrast, a third laboratory was able to correctly detect 16S amplicons for all three prokaryotic organisms in the panel using a different 16S rDNA PCR assay, indicating the need for DRES to adopt a better 16S rDNA assay.

The current method used by DRES (size analysis of the 16S PCR amplicon by agarose gel electrophoresis) provided no information that could be used to identify the agent. One way to obtain more information from the 16S amplicon would be to sequence the amplicon. Nucleotide sequence analysis would provide discrimination of genetic sequences to the primary nucleotide level, providing the ultimate in definitive identification of genetic sequences. Reproducible sample processing methods that provide quality template nucleic acid for PCR are needed. DRES failed to obtain reproducible results for two sample processing methods used in the exercise and was unable to observe any amplicons from neat (unprocessed) samples. Other laboratories were successful in obtaining PCR amplicons from samples processed by various extraction methods and at least one laboratory correctly amplified 16S and agent-specific amplicons from neat samples. Having an in-house sequencing capability would provide quick and easy access to this data and provide for self-sufficiency. 16S amplicon sequence data would provide a clearer indication of the identity of the organism in the sample. However, this data is limited to prokaryotic threats (bacteria/rickettsia) and may be not provide enough information to provide identification to the species level (thus incapable of distinguishing harmful pathogens from harmless organisms in the same genus). Consequently, it is necessary to develop PCR assays that target agent-specific sequences. Agent-specific PCR would address not only bacterial and rickettsial threats but viral threats as well.

Contamination problems were experienced which revealed, first-hand, the potential for false-positive identifications by PCR and the need for developing methods to overcome this problem. It is interesting to note that three countries reported the presence of vaccinia-specific amplicons in supposedly, non-vaccinia-containing unknowns, in this exercise, albeit in different unknowns (*Y. pestis*; VEE; *C. burnetii* & PBS blank, respectively). In one lab, vaccinia was detected in the *Y. pestis* sample, not only by PCR analysis, but by immunological analysis as well. This raises the question as to whether vaccinia was present in the samples prior to shipping or whether it was introduced within the individual laboratories post-shipment. Several methods to overcome this problem have been published [14, 15, 16, 17]. Investigation of methods that are effective and suited to operational requirements should be conducted.

General issues: Problems and way ahead

Import permits

The importation of live Risk Group 2 or 3 microorganisms is normally a straightforward procedure. Applications are submitted to the appropriate agencies (Canadian Food Inspection Agency (CFIA) and/or Health Canada (HC)), depending on the microorganisms to be imported, and a permit is issued in one to two weeks. In this exercise the microorganisms to be imported were killed, rather than live and at the outset it was felt that importation would be a non-issue. This was not the case.

The permit application process was initiated 02 December 1998. Both CFIA and HC were reluctant to issue a permit. The main concern centered around proof that the samples in question were, in fact, killed materials. The US organizers of the exercise made use of ^{60}Co irradiation to kill the microorganisms and issued a "Certificate of Non-viability". Considerable discussion between the agencies and DRES was required and the permits were finally issued on 03 March 1999 (HC) and 19 March 1999 (CFIA).

Identification technologies

DRES needs to increase the number of pathogens that can be identified by immunological and genetic methods. Genetic methods should be expanded beyond 16S rDNA PCR to include agent-specific PCR and nucleotide sequencing capability so as to provide a more definitive analysis of genetic sequences.

Gamma irradiation

Successful identification of gamma-irradiated pathogens by immunological and genetic methods in this exercise has important implications for the BW research program at DRES. Having ready access to gamma irradiation capability would allow for the safe handling, transport and testing of samples within and between non-biocontainment laboratories (e.g. DRES laboratories, industry, universities, Health Canada) and outside Canada (e.g. Center for Disease Control, NATO countries) for both detection/identification as well as prophylaxis/therapy research. In addition, it could provide a means to identify mixed chemical/biological threats on site. DRES should seriously consider the need for ready access to this capability.

Biocontainment issues

Inactivation of the unknown pathogenic samples in this exercise allowed DRES to participate in the training exercise as these samples could be handled in a biocontainment level (BL)2 laboratory with no threat of infection to DRES personnel. Had the exercise required live agent analysis or had CFIA or HC refused to allow the importation of these ^{60}Co-inactivated materials unless they were used within the BL3 suites, DRES would have had to perform the analyses in containment. While DRES has the capability to handle organisms and perform microbial analysis in BL3 (e.g. culture, gram stains, differential stains, antibiotic resistance,

bacterial typing for live, culturable bacteria), there is no immunological or genetic analysis identification capability in the BL3 containment laboratories. In future, however, the capabilities and equipment available in BL2 containment should be reproduced within the BL3 suites. Having this capability would allow for a more comprehensive and rapid response.

It is also important to note that DRES BL3 suites are not normally available year round. There are significant periods of time (normally May-June) when the suites are unavailable due to annual re-certifications mandated by HC and CFIA. If DRES is to establish a credible biological identification capability BL3 facilities must be available year round. This will require additional BL3 capability so that the annual re-certification periods can be staggered, allowing a BL3 laboratory to always be available. The resources required to acquire this capability must take into consideration not only the cost of construction of the labs, but also the life cycle management costs of the resources needed to run the laboratory over a 15-20 year period.

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In March 1999, the NATO Subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the first international training exercise on the identification of biological agents. Eleven laboratories representing 10 nations: Canada, France, Germany (two laboratories), Hungary, Italy, Netherlands, Norway, Poland, United Kingdom, and the United States participated in the international training exercise. Each participating laboratory was sent five "unknown" samples, four of which contained ^{60}Co (Cobalt-60)-inactivated biological material and one blank containing phosphate buffered saline (PBS).

The United States, as the host nation, distributed PBS, *Bacillus anthracis*, *Coxiella burnetii*, Venezuelan Equine Encephalitis (VEE) virus, *and Yersinia pestis* as the samples for the training exercise. Canada correctly identified *Bacillus anthracis* and *Yersinia pestis* and correctly indicated that the remaining samples did not contain *Vibrio cholerae*, *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei* or Vaccinia virus. As no assays were available at DRES for Yellow Fever virus, VEE or *Coxiella burnetii*, the presence of these agents could not be determined in the remaining samples.

This report summarizes the results obtained by Canada and compares the performance of DRES to the other participating international laboratories. Areas requiring improvement at DRES for the in-house identification of biological agents are discussed.

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