Identification of biological threat agents in the environment and its challenge

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English summary

A release of pathogenic microorganisms may result in infections either in humans or animals, in which the development of clinical symptoms may be the first alert of a biological incident. Rapid, reliable and efficient identification methods must, thus, be in place for both clinical and environmental samples containing biological threat agents, also for forensic purposes. This report provides an overview of detection and identification methods of biological threat agents in the environment with emphasis on air, as well as the challenges posed to sampling and sample processing that may have an impact on the identification analysis. The persistence of the released biological agents may vary between the agents, and must be addressed when performing the identification analysis.

The Norwegian Defence Research Establishment (FFI) has focus on sampling, sampling processing and analysis of biological threat agents in the environment in which this work is based on the NATO Handbook for Sampling and Identification of Chemical and Biological Agents (SIBCA), also known as the AEP-10 document. FFI's involvement in biological preparedness is outlined in the last section of this report. Civilian authorities, institutes and defence laboratories involved in national preparedness, response and analytical capabilities may act as reach-back facilities and support SIBCA missions when needed. The AEP-10 guideline distinguishes between provisional, confirmed and unambiguous identification. FFI is capable of both provisional and confirmed identification of selected biological threat agents per June 2008.

An element in reducing the biological threat and improving biological preparedness is the capability in detecting biological threat agents prior to the development of clinical symptoms. Several detectors monitoring biological particles in air are commercially available or are currently at an R&D level. All biological detection and sampling equipment must be tested and evaluated in chamber and field trials prior to use in real-time situations. The lack of such trials will lead to commercialized unreliable equipment possessing an increased false alarm rate.

National biological preparedness and response depend on a combined joint effort among military and civilian agencies, institutes and organizations, including detection and identification of biological threat agents of both clinical and environmental samples. Regardless of an intended or natural outbreak of a disease caused by biological threat agents, preparedness needs to be established *prior* to such an event. Post-preparedness planning may in some cases be too late.

Sammendrag

Et intensjonelt utslipp av biologiske trusselstoffer, eller et naturlig utbrudd, kan være vanskelig å oppdage og i de fleste tilfellene vil utvikling av sykdomssymptomer hos de eksponerte være den første indikatoren på en slik hendelse. Dette krever årvåkenhet hos medisinsk personell. Et ledd i å forbedre den biologiske beredskapen, er å kunne raskt og pålitelig detektere biologiske trusselstoffer for å begynne den medisinske behandlingen så tidlig som mulig og for å kunne initiere de nødvendige beskyttelsestiltakene.

Etter et utslipp av biologiske trussetstoffer er det nødvendig med prøvetaking av de eksponerte (kliniske prøver) og av områdene som er blitt kontaminert (miljøprøver), etterfulgt av spesifikke analyser for å kunne identifisere de biologiske trusselstoffene. Ulike miljøfaktorer vil kunne påvirke overlevelsen av de forskjellige trusselstoffene deponert i miljøet. I tillegg er prosesseringen av komplekse prøvematrikser en faktor som kan være en utfordring for identifiseringsanalysen. FFI benytter bl. a. NATOs ”Handbook for Sampling and Identification of Chemical and Biological agents” som retningslinjer for analysering av biologiske trusselstoffer.

Denne rapporten beskriver ulike metoder for både deteksjon og identifikasjon av biologiske trusselstoffer, med fokus på luft, samt faktorer som kan påvirke mikroorganismens overlevelse i miljø og utfordringer som følger biologiske deteksjons- og identifikasjonsanalyser. Detektorer av biologiske trusselstoffer i luft er kommersielt tilgjengelige, men det er helt essensielt at slike testes og evalueres under kontrollerte betingelser i testkammer og i felt for vurderinger av evt. falske alarmer som kan oppstå. FFI har fokus på prøvetaking, prosessering av prøven og påvisningsanalyse av biologiske trusselstoffer i miljøprøver, og per juni 2008 har FFI mulighet til å utøve både foreløpig og bekreftende identifisering av enkelte utvalgte biologiske trusselstoffer.

Et effektivt nasjonalt biologisk beredskapssystem er avhengig av et koordinert samarbeid mellom departementer, etater, direktorater, institutter og instanser involvert i beredskapsarbeid, både militært og sivilt. Et godt gjennomført nasjonalt beredskapssystem er også formålstjenelig for beredskapssystemet i Europa og NATO.

Contents

Preface

This report is to provide the reader an overview of detection and identification, and the challenges, of biological threat agents in the environment. Although specific instruments and equipment for biological detection and identification are mentioned in this report, *these are not to be endorsed*. However, some of these are currently used for scientific research and have been tested at FFI. FFI can upon request test and evaluate biological detection and identification instruments. The report is a part of the ongoing FFI project “Detection and identification of biological threat agents” P1099.

1 Introduction

Biological threat agents are microorganisms causing infections leading to incapacitation or death of humans, domestic animals or destruction of crop plants (Hawley and Eitzen, 2001). The Centers for Disease Control and Prevention (CDC)[1], USA, has established a list of biological agents that are regarded as biological threat agents, and classified the biological threat agents in three categories, A, B and C.

Category A include high priority agents that i) pose a risk to the national security since they may easily be disseminated, ii) are transmitted from person to person, iii) may result in high mortality rates, and iv) may cause public panic and require special health prepardness. The agents *Bacillus anthracis* (anthrax), *Francisella tularensis* (tularemia), *Yersinia pestis* (plague), Variola major (smallpox), viruses causing viral hemorrhagic fevers and botulinum toxin (botulism) belong to Category A (Table 1).

In contrast, Category B agents consist of biological threat agents that are moderately disseminated and result in low mortality rates. This category includes *Coxiella burnetti* (Q-fever), *Brucella* spp. (brucellosis), *Burkholderia* spp. (glanders, melioidiois), viruses causing viral encephalitis, *Rickettsia prowazekii,* (typhus fever), and waterborne and food safety threats such as *Vibrio cholera* (cholera), *Shigella* and *Salmonella* spp., respectively, in addition to the toxins ricin, *Staphylococcus* enterotoxin B (SEB) and epsilon toxin of *Clostridium perfringens* (Table 1).

Category C includes agents causing emerging infectious diseases, exemplified by various viruses such as Nipah virus and hantavirus, and microorganisms that may be genetically engineered for mass dissemination. These agents are available, may be easily produced and may provide high mortality rates.

Table 1 provides a list of some biological threat agents and their features. The biological threat agents may be disseminated by food, water, insect vectors, as aerosols or by direct contact, but their ability to cause contagiousness and infectivity vary, as well as their incubation period and their effectiveness to medical treatment (Table 1) (for extensive reading see Franz et al., 1997, Mandell et al., 2005). Human pathogenic fungi are not listed as potential biological threat agents in CDC's select agent list, except for the *Coccidioidis* spp., in contrast to mycotoxins that are fungal products (Fierer et al., 2002, Casadevall and Pirofski, 2006).

Some biological threat agents may be deliberately dispersed in an easy matter, while others require technological and professional knowledge. One of the first hurdles to use these agents in illegal attacks, such as bioterrorism, bio sabotage, biological warfare, is to obtain a viable stock of the microorganism or a sample containing the biological threat agent of interest. Therefore, actions have been taken to reduce access of non-restricted persons/personnel to storages

[1] www.cdc.gov

containing potential biological threat agents (biosecurity). Also, control export/import regimes have been established, such as the Australia Group (AG)[2]. AG is an informal forum of 40 countries with the objective to use licensing measures ensuring that exports of certain chemicals, biological agents, and dual-use chemical and biological manufacturing facilities and equipment, do not contribute to the spread of chemical and biological agents. The AG list of biological agents for export includes 32 viruses, 4 rickettsia, 15 bacteria, 19 toxins and 2 fungi.

Table 1. Potential biological threat agents.

Microorganism	Disease	Mortality untreated	Infective dose[a]	Incubation time[b]
Bacillus anthracis	Anthrax	High	8000-5000 spores	1-6 d
Yersinia pestis	Plague[d]	High	100-500 cfu	2-3 d
Francisella tularensis	Tularemia	Moderate	10-50 cfu	2-10 d
Variola major	Smallpox[d]	High	10-100 pfu	7-17 d
Clostridium botulinum[c]	Botulism	High	0,003 μg/kg (LD_{50})	1-5 d
Burkholderia mallei	Glanders	Moderate	low	1-21 d
Filovirus	VHF[d,e]	High	1-10 pfu	4-21 d
VEE[f]	Encephalitis	Low	10-100 pfu	1-6 d
Coxiella burnetii	Q fever	Low	30-3000 cfu	10-40 d
Brucella spp.	Brucellosis	Low	10-100 cfu	5-60 d
Vibrio cholerae	Cholera	Moderate	10^3 - 10^6 cfu	4 h-5 d
Shigella spp.	Shigellosis[d]	Low	10-100 cfu	1-7 d
Salmonella spp.	Salmonellose	Low	10-100 cfu	1-7 d
Escherichia coli O157:H7	STEC	Low	$<10^3$	10 h-3 d
Ricin toxin	Ricin poisining	High	3-5μg/kg (LD_{50})	18-24 h
Aflatoxin	Aflatoxicosis[g]		Moderate/High	9000 μg/kg
Saxitoxin	Paresthesias[h]	Moderate		5 min-4 h

[a] Data from Franz et al. (1997), Granum (1999), Kortepeter et al. (2001), and Mandell et al. (2005).

cfu, colony forming units; pfu, plaque forming units

[b] d, days; h, hours

[c] The toxin is the threat agent

[d] Contagious human-human

[e] VHF, viral hemorrhagic fevers

[f] VEE, venezuelan equine encephalitis

[g] May cause chronic and acute hepatocellular injury, aflatoxin is a mycotoxin and a potent carcinogen produced by *Aspergillus flavus.*

[h] Saxitoxin is a neurotoxin produced by *Gonyaulax* spp. (shellfish poisining)

[2] www.australiagroup.net

In order to describe a biothreat agent's ability to pose a hazard to humans, its ability to survive, stay viable and cause infections need to be considered. According to Stuart and Wilkening (2005) survival is referred to the agent's ability to initiate i) growth in a given medium or ii) a disease in a susceptible host, while viability is referred to the survival of the agent outside a host regardless if it can cause a disease and is a prerequisite for infectivity. The term infectivity is defined as the agent's ability to replicate in a host generally causing disease symptoms.

Many biological threat agents are degraded when exposed to various environmental conditions, such as UV radiation. In some cases, food and water that can act as reservoirs of viable agents. Spores may survive for decades in the environment, while vegetative bacteria are commonly more sensitive to degradation and viability decay (Mohr, 1991, Mohr, 2002, Stuart and Wilkening, 2005, Sinclair et al., 2008). Introducing microbes into an environment different from its habitant/its origin will often result in inactivation of the microbial cells. Their survival depends on the temperature, humidity, radiation, acidity and oxidants of the environment (Mohr, 2002, Stuart and Wilkening, 2005, Sinclair et al., 2008). Even though viability decay studies of various bacteria and viruses subject to different environmental factors have been performed, limited detailed knowledge exists. Furthermore, it is difficult to compare various bioaerosol fate studies since different assay methods for sampling and analysis have been used, i.e. no standardized testing techniques have been established. Also, the stability profile of toxins varies among such threat agents (Loh, 2007).

2 The NATO Handbook for Sampling and Identification of Chemical and Biological Agents (SIBCA)

The NATO Handbook for Sampling and Identification of Chemical and Biological Agents (SIBCA), well-known as the AEP-10 document (STANAG 4329), provides procedures and guidelines needed to sample, detect and identify chemical and biological agents that NATO forces may be subject to on a battlefield, either by an attack or by hazards. This handbook has been delivered by the NATO Joint Capability Group (JCG) Chemical, Biological, Radiological and Nuclear (CBRN) Defence, SIBCRA Sub Group (AC/225) as a part of their Terms of Reference. AEP-10 is currently being revised to include sampling and identification of radiological agents (SIBCRA). Identification of radiological agents is outlined in the AEP-49 handbook (STANAG 4590). The AEP-10 handbook is intended for use by military and civilian personnel taking part in SIBCA missions and has two missions i) operational and ii) forensic.

The operational SIBCA is to allow the commanding and medical officers to make decisions necessary for protection, avoid contaminated areas, medical treatment and maneuver ability. During the first minutes to hours of an event, the most important factors to focus on are protection and reduction of health risks and handheld detectors may be used to estimate the hazard. During the following days beyond the incident it is necessary to identify the biological threat agent, reduce further exposure and initiate countermeasures and focus on sampling and measuring ambient exposure rates in addition to contamination of surfaces and air. Later, samples of food, water and agricultural products may be harvested to estimate the exposure.

The forensic mission is based on the use of chemical and biological agents by the enemy, and the important need for indisputable evidence when identifying international treaty violations. If the sampling, handling, sample processing, and storage of the samples are not correctly performed, the biological material for evidence may be lost (Budowle et al., 2005, 2006). One major difference between operational and forensic SIBCA is the *time* needed to obtain the identification requirements in which trained personnel, sophisticated instrumentation and possible containment facilities are needed.

The SIBCA handbook has defined three types of identification; i) provisional, ii) confirmed and iii) unambiguous in which certain criteria need to be fulfilled. In order to obtain provisional identification either the use of immunological methods, nucleic acid detection or *in vitro* culturing/metabolic assays is needed. For confirmed identification, at least two of the above criteria must be satisfied. Unambiguous identification requires the use of four methods, the latter including *in vivo* studies (animal models) in addition to the three methods stated above.

3 Biological threat agents in environmental samples

Biological threat agents may be deliberately dispersed by air both outdoor and indoor. However, many infections in humans and animals are caused by naturally occurring microorganisms dispersed in air and transported by the wind. Several bacterial pathogens commonly found in outdoor air may have a health impact, exemplified by the species *Escherichia*, *Legionella*, *Neisseria*, *Francisella*, *Burkholderia*, *Clostridium* and *Brucella*, illustrating the need for analyzing bacterial diversity in air (Kuske, 2006). Also, characterization of the microbial diversity in air will improve the development of reliable, specific, and sensitive detection of airborne biological agents. *Bacillus* spp. is commonly found in air and it is important to distinguish the threat agent *Bacillus anthracis* from other *Bacillus* species (Fykse et al., 2008a). An aerosol dispersion of biological threat agents may be deposited in the lungs by direct inhalation, by evaporation or by deposition on the ground and surroundings. These biological agents may then reaerosolize and enter agricultural products or livestock by forage. However, the risk from the deposited agents depends on their persistence in the environment (section 1).

Water sources, drinking water supply systems and treated drinking water can be contaminated with naturally occurring microbes such as bacteria (i.e. *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp), viruses (i.e. hepatitis A virus and human norovirus), protozoa (i.e. *Giardia* spp. and *Cryptosporidum* spp.) or different toxins (Hörman, 2005). The water distribution systems may be targets for bioterrorism, sabotage and intentional contamination, in which several of the potential biological threat agents shown in Table 1 can be used to contaminate the drinking water supply system. Scientific research at FFI has shown that *Salmonella* spp. is able to survive for long time periods in supply and drinking water (unpublished results FFI). *V. cholerae* is naturally occurring bacteria found in fresh, brackish and salt water, and is the etiological agent of cholera. In some cases, it may be difficult to distinguish *V. cholera*e from the natural background of *Vibrio* spp. in sea water since *Vibrio* spp. may constitute up to 4 % (10^8 cells/ml) of the total bacterial background in sea water (Heidelberg et al., 2002, Thompson et al., 2003).

Biological threat agents may be deposited on the ground after a release. Soil is a heterogeneous matrix with a mixed microbial community under fluctuating local conditions. The viability of the microorganisms is affected by the soil type and the content of water, temperature and UV-radiation. Non-common habitants of soil such as human or animal pathogens, will probably have a short lifetime in natural soil environments compared to indigenous soil bacteria. *C. botulinum* is a natural soil inhabitant that most probably will be part of the natural soil fauna in considerable time after an artificially dispersion of this agent. In general, microorganisms will adsorb to the soil particles causing a challenge to sample processing (section 4) and less than 1 % of all bacterial species represented in the soil is assumed to be cultivable (Torsvik et al., 1990). These findings illustrate the difficulties in identifying the presence of biological threat agents in soil samples.

B. anthracis spores survive for decades in soil (Manchee et al., 1981), water and air, and are not affected by open air factors. It is still an open question whether *B. anthracis* has an environmental vegetative life or not in soil (Ramisse et al., 1999, Jensen et al., 2003). The decay rate of *F. tularensis* in air is proportional to sunlight and increases as the relative humidity decreases. However, *F. tularensis* may survive in unsterile water up to 60ºC at approximately 9ºC while the survival time decreases as the temperature increases (unsterile water spiked with *F. tularensis*). The survival time of *Brucella abortus* may be several hundred days at beyond freezing temperatures when added to water and soil (Mitscherlich and Martin, 1984, and Mohr, 1991 and references therein).

In addition to water, soil and air, environmental samples may include smoke, liquids, wastes, solids, surfaces, vegetation, snow, street and gutter dirt. The survival and viability of biological agents depends on their reservoir and matrix, illustrating the need for detailed knowledge of environmental factors that may have an impact on obtaining efficient sampling and successful identification.

4 Sample processing and its challenge

One prerequisite to efficiently detect and identify biological threat agents in environmental samples is the need for well-defined procedures describing sampling and analysis. This need is outlined in the AEP-10 NATO Handbook on Sampling and Identification of Biological, Chemical Agents (SIBCA) (section 2). One hurdle in order to obtain efficient identification analysis is the sampling process, in which decision-making of the area and objectives to be sampled must be addressed. Expertise in performing sophisticated and consistent sampling is a must to sample processing and further analysis, and therefore, executive training is needed[3] (Fig. 1).

Figure 1. FFI participates in the BIOTECT sampling exercise, 2006, in Denmark.

The first step to detect and identify a biological threat agent is to collect a sample containing the agent of interest. This may be a challenge if the concentration of the agent is low compared to other related and non-related agents in the specific environment often resulting in the need for a concentration step during sample processing.

Efficient sample preparation is crucial in order to obtain successful detection and identification of the biological threat agent in environmental samples. Well-defined methods for sample preparation of biological agents in clinical samples have been established, and several of these protocols have been adjusted and established for environmental samples. Environmental samples can include a wide variety of complex matrixes exemplified by soil, wastes, liquids, vegetation and dirt, in contrast to less complex matrixes as air, smoke, water, solids, surfaces, and snow. The former matrixes need to be processed prior to identification analysis. In addition, contaminated water and water containing humic acid need extensive processing.

[3] http://www.mil.no/felles/ffi/start/article.jhtml?articleID=160509

The need for sample preparation is of course dependent on the analytical methods chosen e. g. immunological methods, nucleic acid detection or *in vitro* culturing assays. In some cases, harsh methods are used to obtain sufficient and efficient extraction of nucleic acids. The extraction of nucleic acids from air samples is usually easier, compared to contaminated water and soil samples. A protocol to lyse Gram-positive bacteria and spores might be needed to enhance the detection and identification signal. This can be achieved by sonication or bead-beating (Fykse et al., 2003, Fykse et al., 2008a). In some cases air samples can be analyzed directly without any time-consuming sample preparation (Fykse et al., 2008a) except lysis of the microorganism. However, if the agent of interest is present at very low concentrations a sample preparation is needed (Blatny et al., 2007b and 2008). In general, if the biological agent is present at low concentrations, it is necessary to concentrate the sample before sample preparation. Devices for automatic sample preparation from environmental samples have been to a certain extent developed (Liu and Zhu, 2005). Still, even manual preparation protocols of complex matrix samples may pose a challenge to professional trained personnel.

Standard procedures for control of drinking water have been established. In Norway, no coliform contamination is accepted in a water volume of 100 ml. This is a limit of detection easy to handle because no coliform bacteria is expected to be present in lakes used as drinking water. However, if this detection limit should be introduced for i.e. *V. cholerae* in water volumes imported to Norwegian waters by ballast water from tankers, the analytical work would be highly challenging because *V. cholerae* may be difficult to discriminate from the natural *Vibrio* spp background in sea water since i) *Vibrio* spp may constitute up to 4 % of the total bacterial background in sea water (Heidelberg et al., 2002, Thompson et al., 2003), and ii) highly *V. cholerae* selective/differentiating growth medias have not yet been developed (Farmer and Hickman-Brenner, 1991). Also using polymerase chain reaction (PCR) (section 7.2.1), which is a sensitive and selective method, it would probably in some cases be difficult to achieve. Sample preparations from sea water as well as fresh water not containing humic acid are rather easy to perform and several commercial kits can be used for extraction of nucleic acids from water. In many cases, a filtration method for concentration of the sample will increase the concentration of the potential biological threat agent.

Conventional methods for testing and analyzing water samples imply culturing and enrichment of bacteria. Several bacterial species has shown the ability to enter a viable-but-nonculturable (VBNC) state, which means they are viable but are not able to grow on conventional growth agar (Oliver, 2005, Alam et al., 2007). This phenomenon has been demonstrated for several bacterial genera such as *Vibrio* (Chaiyanan, 2001), *Escherichia* (Wang and Doyle, 1998, Makino et al,. 2000, Grey and Steck, 2001, Bjergbaek and Roslev, 2005, Oliver et al., 2005), *Salmonella* (Oliver et al., 2005) and *Legionella* (Hussong, 1987) among others. The presence of these bacteria may escape culture based detection methods and supplementary methods are therefore required to ensure reliable test results.

Microorganisms will generally attach to each other as well as to other particles in the vicinity. They can adsorb to soil particles challenging efficient extraction of their nucleic acids when

molecular analysis needs to be performed. There are several commercial kits available for DNA extraction from soil, as well as published literature describing various procedures for DNA extraction. However, since no single method is optimal for DNA extraction from all different soil types, each method needs to be tested on the soil of interest. In general, removal of inhibiting contaminants as fulvic and humic acids to obtain acceptable amounts of the nucleic acids DNA seems to be a challenge in all extraction methods. The majority of the commercial kits use a mechanical disruption method of the sample, such as bead-beating, combined with a chemical detergent (Schneegurt et al., 2003). Since humic aids will often coextract along with the nucleic acids it is often a prerequisite to purify the nucleic acids prior to molecular analysis (Schneegurt et al., 2003, Dong et al., 2006).

Waste waters, sewages and heavily contaminated or dirty water are liquids that in general need a clean-up step before performing identification analysis. Biological agents deposited on surfaces may be sampled using either dry or liquid swabs. Such samples are usually easier to process and identify (Buttner et al., 2004).

5 Detection of biological threat agents

The establishment and implementation of detection and identification methods of biological threat agents is an important element of biological preparedness. A natural outbreak, an intended or accidental release of biological threat agents will result in infections either in humans or animals in which clinical diagnosis may be the first alert of a biological incident. Thus, rapid, reliable and efficient identification methods must be in place for both clinical and environmental samples containing biological threat agents, also for forensic purposes. Detection and in particular identification methods need to be tested, evaluated and implemented at several laboratories responsible for public health preparedness and biological defence.

In contrast to (reference) laboratories, deployable and mobile laboratories may be involved in sampling of a contaminated region, sample processing/preservation and to provide provisonal identification. In some cases, confirmed identification may be obtained at such laboratories, depending on the equipment available. The sample is then transported to the responsible laboratory for further identification analysis (Fig. 2). One critical factor is the time needed for analysis in order to provide sufficient information to initiate medical treatment of those exposed and to start a decontamination process.

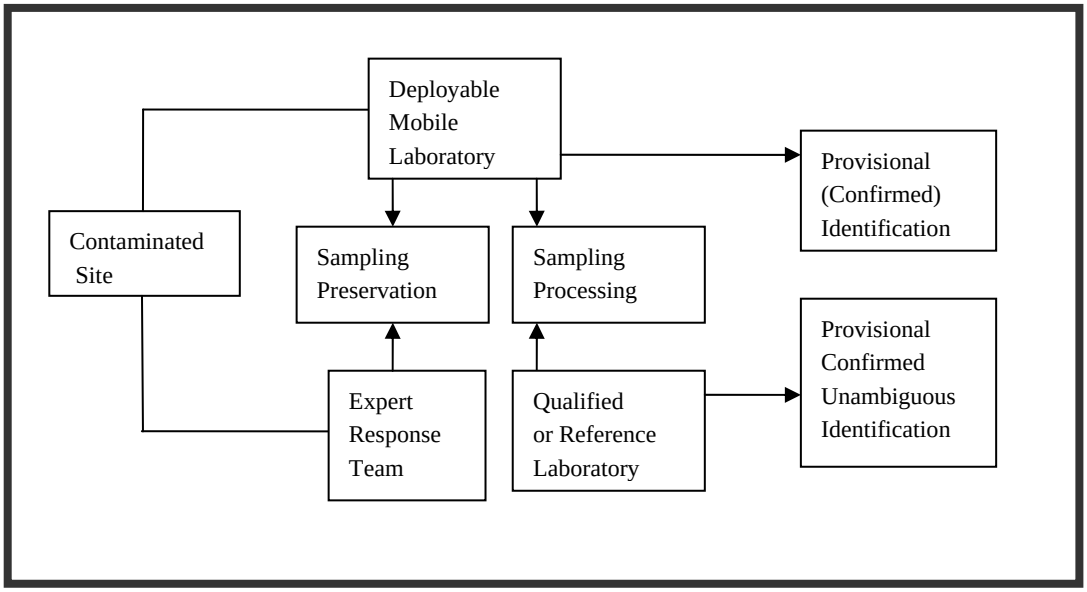

Figure 2. A general scheme involving sampling and identification of biological threat agents from a biological contaminated site.

5.1 Methods for biological detection

Several methods and equipment for detection of biological threat agents are available, especially for monitoring airborne agents. Figure 3 provides a schematic overview of an optimal biological detection surveillance and warning system and the following identification steps.

Detection is nonspecific and does not provide identification, but rather an indication, of the biological agent present. Generally it is difficult to distinguish between the natural bacterial background and those that have been deliberately illegally introduced, and in many cases, it is not possible. Biological detection methods may be suitable as warning systems, for first responders and commanding/medical officers in battlefields for decision-making regarding protection and medical treatment. Such detection is usually reliable if the biological agents are present at high concentration levels. In general, air and waterborne biological agent samples need to be concentrated for sufficient detection. Several overviews of various biological detectors are found in the literature (NATIBO, 2001, Wästerby et al., 2003, Fatah et al., 2005, Emanuel and Fruchey, 2007). In order to select the most suitable detector, strict testing of the detector system is needed (section 8).

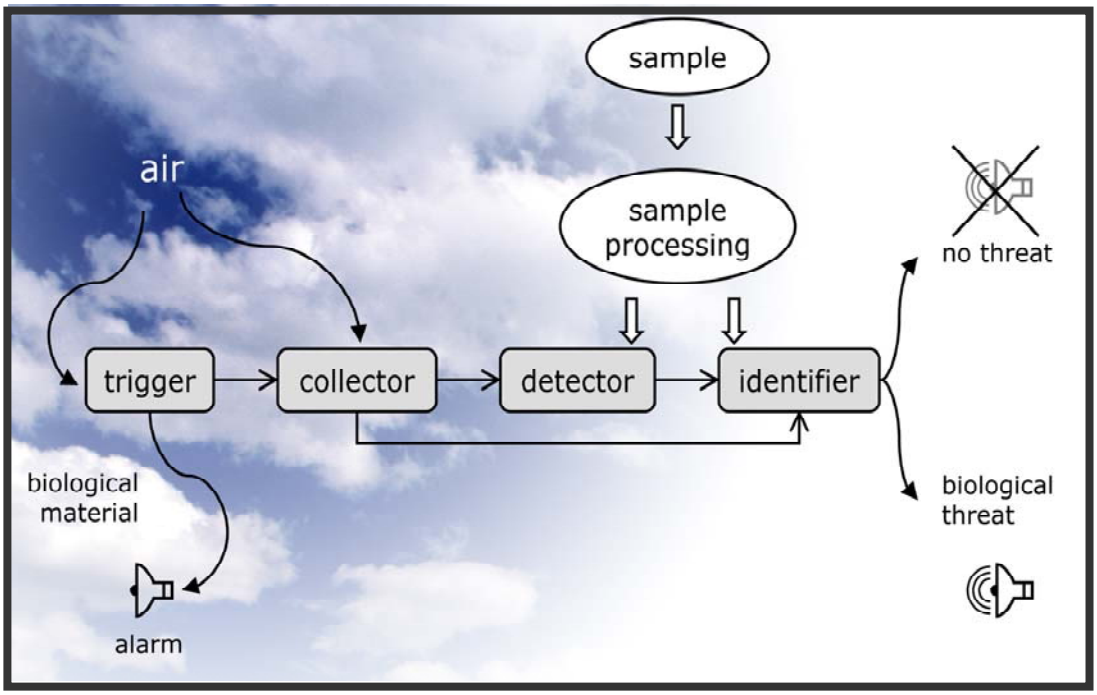

Figure 3. An optimal biological detection warning system and the following necessary identification process of airborne biological agents.

Table 2 summarizes the technologies that may be used for detecting and identifying biological threat agents mainly in air. The ultimate future approach is to obtain an integrated system containing all four components that will provide sensitive, specific, fast, reliable detection and identification.

5.1.1 Light detection and ranging LIDAR

The use of light detection and ranging (LIDAR) for stand-off surveys of airborne biological agents is a developing technology, and much effort is put into the improvement of stand-off detectors among the NATO member countries. Stand-off detection may be done by ground or by air using an unmanned air vehicle (UAV). Stand-off detectors still have a limited approach due to their high level of false alarm rates caused by naturally occurring biological material that commonly fluoresce, such as oils, diesel, agrochemicals as well as limitations regarding distance (Baxter and Clark, 2004). UV or IR lasers are frequently used in LIDARs. Biological material usually fluoresce when radiated with UV. At wavelengths of 266 nm and 355 nm the amino acids tryptophan and tyrosine and the cofactor nicotinamide adenine dinucleotide (NADH), respectively, will excite fluorescence. These biomolecules are commonly found in all living microorganisms. However, simulants of bacterial and viral airborne threat agents may be distinguished from interfering particles (Tjärnhage et al., 2001, Sivaprakasam et al., 2004, Jonsson et al., 2007), but the use of UV fluorescence in biological point detection requires complex instrumentation and spectral analysis. Still, one benefit of using UV fluorescence based optical methods is the fast response time.

5.1.2 Aerodynamic particle sizing APS, counting and UV fluorescence

Aerodynamic particle sizing (APS) is a useful approach to determine the concentration and size of bioaerosols. APS may be combined with UV fluorescence and used as a trigger detection technology. When the detected bioaerosol concentration has reached a certain level the trigger system will respond by alerting signals. If an air collection unit is connected to the trigger, bioaerosol sampling may be initiated upon the alarm signal. Dycor Technologies has developed a robust warning system, C-FLAPS, suitable for field use, based on the fluorescent laser aerodynamic particle sizing technology developed by the Defense Research and Development Canada – Suffield. The high volume aerosol particle concentrator, XMX/2A (Dycor), may be connected to the C-FLAPS for air sampling on the incident of a biological alarm.

Various particle counters are available that determine the number and to a certain extent the size of airborne particles present in indoor and outdoor environments (<usually 1- 20 µm in diameter), exemplified by the TSI Aerotrak optical particle counter. Particle counting is often integrated into automated or semi-automated biological detection systems.

There are also several commercially available systems, stated to be used for monitoring biological agents indoor, exemplified by EnviScreen Bio100 (Environics) and AirSentinel® (ICX Mesosystems). These instruments trigger an alarm based on an increase of airborne biological particles compared to a threshold value using particle counting and UV fluorescence. Smiths detection has recently launched a portable biological detector designated the Smart BioSensor (SBS) enabling detection of biological aerosols as well as classification of the agent into bacteria, bacterial spores, toxins and viruses. Biological detection technologies are continuously being improved and developed. The choice of a biological detector depends on its use, operating personnel, sensitivity and specificity, and thus, needs to be tested and evaluated at the site of interest before use.

5.1.3 Immunoassay tickets

Handheld biological detectors that may be suitable for first responders and soldiers are commercially available. Still, some of the corresponding assays may be difficult to perform by non-trained personnel. Examples of such commercially available immuno assay tickets are SMART (sensitive membrane antigen rapid test) (New Horizons Diagnostic Corp.) and the BioThreat Alert test strips (Alexeter Technologies). The reader is referred to Blatny et al. (2006) for an overview of various commercialized detector technologies. Usually a liquid or a wetted swab sample is taken and applied on the test strip for analysis, but the sensitivity of these tests may in some cases be limited. These assays may be used to test suspicious powder (Tims and Lim, 2004), but confirmed and unambiguous identification of the biological agent detected by these bioassays must be performed at a qualified (reference) laboratory.

Table 2. Biological point detection for airborne contaminants and biological agents.

Trigger	Detector	Collector	Identifier
-UV fluorescence - Aerodynamic particle sizing -Particle counters	- UV fluorescence - Bioluminescence - Flow cytometer	- Cyclone - Impactor - Virtual impactors - Impingers	- Genetic methods - Immunologic methods - Microbiological and biochemical methods (viability) - Microscopy - Microarray - Optical systems combined with specific identification - Mass spectrometry
Can be combined			
Integrated systems			

5.1.4 ATP assay

The ATP (adenosine 5'-triphosphate) bioluminescence method, based on the luciferin-luciferase reaction, is a non-specific detection method of bacteria and germinating bacterial spores in a sample. ATP is found in all living microorganisms and, thus, it is necessary to eliminate ATP contamination from the non-target agents present to obtain reliable detection. This detection system seems to be useful in analyzing food samples for the presence of biological threat agents and for suspicious powders, in which some commercial test systems are available (Fujinami et al., 2004, Lee and Deininger, 2004, Lim et al., 2005).

5.1.5 Flow cytometry

Flow cytometry uses the principle of light scattering to detect particles between 0,5 – 40 µm in diameter. As particles and cells pass by a laser beam, they scatter light depending on their size, and they may fluoresce due to autofluorescence or fluorescent binding dye (specific antibodies) bound to the cells. The light impulses are converted to electrical pulses proportional to the scattering or fluorescent emission and measured by optical detectors. Flow cytometry may be suitable for used for particle counting, sorting and sizing (mass), and to characterize subpopulation of cells. Flow cytometry seems to be a promising tool for analyzing biological agents in air and may be a part of integrated biological detection systems. Commercial portable flow cytometry instruments for field use are available, such as MICROCYTE (BioDetect) (Gran et al., 2002) and Miniflow (Lawrence Livermore National Laboratory). At reference laboratories more sophisticated flow cytometry instruments are used, exemplified by the FacsCalibur flow cytometer from Becton Dickinson.

6 Air collectors

When an alarm has been triggered due to an increased level of biological material, that may contain biological threat agents posing a threat to humans, a sample is to be collected for further identification analysis. Various air collectors are available, and the choice of a suitable air collector depends on its use and features (Table 3) in addition to costs, consumables, power requirement and if the collector may run continuously as well as being ruggedized. In general, air collection of microorganisms is usually impaction or impingement. Impaction samplers collect the particles on an agar plate that is used for cultivation analysis, while impingement usually collects the bioaerosols into liquid. Air samples often need to be concentrated, especially when the concentration level of the biological agents is low. Some of the air samplers perform a concentration step prior to sampling, but high concentration levels can be obtained using high flow rate and long sampling time. However, high flow rate air samplers may cause a reduction of viable microorganisms sampled, which are identified by culturing, which is one of the methods used for unambiguous identification.

Table 3. Some features of bioaerosol samplers commercially available.

Features	**Variables**
Sampling methodology	impaction, impingement, virtual impaction, wetted-wall cyclone
Sampling time	5 minutes - 12 hours
Sampling volume	2 mL– 15 mL
Flow rate	3 L/min - 1000 L/min
Collection type	liquid, filter, cartridge
Particle size range	submicron – micron (0.2 – 10 µm)
Weight (portable)	0,2 kg – 17 kg
Decontamination	vaporized hydrogen peroxide, bleach solution, surface wipe, paraformaldehyde

Several bioaerosol samplers are commercially available, and a list of various bioaerosol samplers for collection into liquid is summarized in CBRNe World (summer issue, 2007). FFI has experience in using the SKC Biosampler (SKC), SpinCon (Sceptor Ind.), OMNI 3000 (Sceptor Ind.), SASS 2000^{PLUS} (Research international), XMX/2L (Dycor Technologies), MAS-100 (Merck) and STA-204 (New Brunswick), in which the latter two are impactors (Fig. 4) (Blatny et al., 2007a, b and 2008, Fykse et al., 2008a and b). The wetted-wall cyclone SASS 2000^{PLUS} is very well-suited for sampling viable airborne bacteria (Blatny et al., 2007a, b and 2008). In order to evaluate various air collectors standardized tests are needed, which are still lacking in the scientific community.

Figure 4. Various equipment for air sampling all tested by FFI.
A: STA-204 (New Brunswick), B: SKC Biosampler (SKC), C: SASS 2000^{PLUS} (Research international), D: XMX/2L (Dycor Technologies), E: SpinCon (Sceptor Ind.), F: OMNI 3000 (Sceptor Ind.) and G: MAS-100 (Merck).

7 Identification of biological threat agents

A review of several identification techniques and sample processing methods of biological threat agents in various kinds of samples, i.e. environmental, clinical, suspicious powders etc. is provided by Lim et al. (2005). Also, Firmani and Broussard (2003) provide an overview of various diagnostic techniques for identification of the Category A biological threat agents with focus on clinical samples. The majority of these techniques may be utilized for biological identification on environmental samples, following a sufficient sample preparation step. In this section, some of the most commonly used identification techniques are described.

7.1 Cultivation and microscopy

Classical microbiological cultivation techniques are widely used for analysis of viable microorganisms. However, an important characteristic of natural environment is that only a proportion (0.1 to 10 %) of the bacteria present can be cultured by traditional methods (Pickup, 1991). Culture methods are time-consuming (days-weeks) compared to molecular methods (sections 7.1 – 7.2). Culture methods are often combined with microscopic analysis and serological testing. To obtain the highest level of identification in the SIBCA system, identification of live agents is required (unambiguous). Therefore culture methods are needed for the final identification purposes. This is also valid for civilian epidemiological analysis of environmental samples when investigating an outbreak of disease, exemplified by the outbreak of Legionnaires disease in Norway in 2005 (Blatny et al., 2007b and 2008). VBNC cells are frequently found in the environment and are now recognized as a common phenomenon of many bacterial species. These microorganisms may escape detection if only culture methods are used (Huq and Coldwell, 1996). This illustrates the need for supplementary identification methods.

Light microscopy may be used to classify a bacterial group by shape (such as cocci, rods) and is commonly combined with use of culture methods. Electron microscopy (EM) is an efficient tool to analyze any presence of viruses and bacteria, but can only be used for classification purposes. EM may determine the virus group facilitating the following molecular analysis, such as real-time PCR, and thus may be an efficient first-hand tool indicating the presence of bacterial and viral particles in a contaminated sample. Staining techniques may be used with microscopy to differentiate between Gram-positive and Gram-negative bacteria. EM is suitable for visualizing the morphology of viral particles.

In addition to cultivation, biochemical and metabolic tests may be used as one of the methods described in the AEP-10 guidelines (section 2). Biochemical and metabolic methods can assist in the classification of bacteria, exemplified by the catalase- and oxidase tests, and analysis of oxidation/fermentation, hydrolysis and amino acid degradation.

7.2 Molecular methods

7.2.1 Polymerase chain reaction PCR

Polymerase chain reaction (PCR) has become a frequently used identification method in microbiology and confirms the presence of specific genetic regions in the target micro organism. PCR has become an outstanding method for a rapid identification. Rapid detection is important for initiating protection and medical treatment in the field. Subsequently, the samples can be analyzed with more time-consuming methods, such as culture methods, at a reference or a mobile laboratory. Several PCR instrument are commercially available in which some are suitable for field use (Blatny et al., 2006).

PCR methods use a pair of primers designed to hybridize to the target DNA, and each hybridized primer forms a start point for the synthesis (DNA polymerase) of complementary strands (amplification). The amplification is taking place during a temperature cycling and the product is called an amplicon. PCR assays have undergone significant change over the last decade. In real-time PCR the amplicon is detected as it accumulates using a fluorescent dye or a specific fluorescent probe binding to the amplicon. Real-time PCR is rapid and can be performed in less than one hour. The high sensitivity of real-time PCR makes the system vulnerable for impurities causing false positive or negative analyses. Using an internal amplification control is needed to avoid this, especially performing real-time PCR of environmental samples.

PCR has become a frequently used detection method and it has been used for about one decade to detect and characterize bacterial species in environmental samples (Alvarez et al., 1995, Kuske, 2006, Kuske et al., 2006). Several real-time PCR methods for analysing bioterrror agents including *B. anthracis, F. tularensis, Y. pestis,* C. *burnetii* and *Brucella* spp. have been described (Tomaso et al., 2003 and 2007, Wilson et al., 2005, Klee et al., 2006, Skottman et al., 2006, Olsen et al., 2007). Bacterial biological threat agents can be detected by real-time PCR within one hour in a liquid air sample containing high concentration levels of commonly found airborne bacteria and potential airborne interferences. In that case no time-consuming DNA extraction was necessary since the potential interferences did not have a large impact on the detection limits (Fykse et al., 2008). FFI has established real-time PCR assays for specific detection of various biological threat agents, including *B. anthracis, B. cereus, Bacillus* spp., *Brucella* spp., *C. burnetii, Y. pestis, F. tularensis, C. perfringens, C. botulinum, E. coli* O157:H7, *V. cholerae, Salmonella spp., L. pneumophila, Campylobacter* spp. *and Leishmania* spp. (Fykse et al., 2004, Olsen et al., 2006). This list is continuously being updated and increased (Table 4).

7.2.2 Nucleic acid sequence based amplification NASBA

The nucleic acid sequence based amplification assay (NASBA), is a sensitive, transcription-based amplification system specifically designed for detecting RNA (Compton, 1991). In contrast to PCR in which a thermo cycler is used, the NASBA method is isothermal (41°C) and only a heating block is needed for the amplification. NASBA is specifically designed for the detection of RNA targets. However, in some NASBA systems DNA may be amplified (Deiman et al., 2002, Rodríguez-Lázaro et al., 2004). The DNA amplification is ineffective and occurs only in the absence of RNA targets or in the case of a 1000-fold excess of target DNA. Generally at 41°C genomic DNA is double stranded and therefore not a substrate for NASBA.

In NASBA, single-stranded RNA amplicons are produced, which can easily be detected by hybridization with a molecular beacon. NASBA has been extensively applied in clinical microbiology in detecting RNA viruses (Deiman et al., 2002), and for detection of microbial pathogens in food and environmental samples (Cook, 2003). NASBA has among others been used for the detection of *Vibrio cholerae* (Fykse et al., 2007), *Campylobacter* spp. (Uyttendaele et al., 1995), *Listeria monocytogenes* (Blais et al., 1997), *Salmonella enterica* in various foods (Cook et al., 2002), for *Cryptosporidium parvum* (Baeumner et al., 2001) and *Escherichia coli* in water (Min and Baeumner, 2002). It has been shown that as few as ten viable spores of *Bacillus anthracis* could be detected by NASBA (Baeumner et al., 2004). FFI has established NASBA assays for *Bacillus* spp., *Vibrio* spp and *Salmonella* spp. (Fykse et al., 2007 and 2008c) (Table 4).

In principle, the presence of RNA in bacterial cells may serve as an indicator for viable cells (Keer and Birch, 2003). RNA species are supposed to degrade rapid when are cells are dieing. Therefore, NASBA as a potential method to detect viable cells is interesting (Fykse et al., 2007) in contrast to PCR that only detects DNA and, thus, can not distinguish between viable, VBNC and dead cells.

Table 4. Methods used for identification of biological threat agents at FFI per June 2008. References are provided as footnotes to the table, otherwise unpublished in-house procedures (FFI).

Microorganism	**Real-time PCR[a]**	**Geno-typing[b]**	**NASBA**	**Immuno-assay**	**Growth analysis[c]**	**BSL-level[d]**
Bacillus anthracis	+	+[e]	-	-	Per June 2008, growth analysis has been established for some agents.	3
Baillus cereus	+	+[e]	(+)	-		2
Bacillus spp.	+	(+)	(+)	-		2/3
Brucella melintensis/abortus/suis	+	-	-	-		3
Coxiella burnetii	+	-	-	-		3
Francisella tularensis	+	-	-	-		3
Yersinia pestis	+	-	-	-		3
Burkholderia mallei/pseudomallei	+	-	-	-		3
Clostridium botulinum	+	+[f]	-	-[g]		2
Clostridium perfringens	+	-	-	-		2
Vibrio cholerae	+[h]	+[i]	+[j]	-		2
Escherichia coli O157	+	-	-	-		3
Salmonella typhimurium	+	-	+[k]	-		2
Legionella pneumophila[l]	+	-	-	-		2
Campylobacter jejuni/lari/coli	+	-	-	-		2
Leishmania spp.	+	-	-	-		2

[a] Fykse et al. (2004), Olsen et al. (2006), Tomaso et al. (2008)

[b] Methods for genotyping (section 7.2.3), not established at FFI, are available to FFI through the EDA collaboration "Database of B agents" (section 10).

[c] Growth analysis will be established for all BSL-3 microorganisms when the BSL-3 laboratory is operative (January 2009).

[d] BSL; BioSafety Level

[e] Olsen et al. (2007)

[f] Tveten (2008)

[g] Performed at the Norwegian School of Veterinary Science

[h] Koskela et al. (2008)

[i] Pedersen (2008)

[j] Fykse et al. (2007)

[k] Fykse et al. (2008c) and Strømhylden (2008)

[l] Blatny et al. (2007b and 2008)

7.2.3 Genotyping

Genotyping refers to the process of determining the genotype of an individual organism with a biological assay. This process is usually more time-consuming and work-demanding compared to the PCR and NASBA methods described above. Genotyping will be performed at a reference laboratory as part of the identification process using sophisticated instruments for PCR analysis, electrophoresis, DNA sequencing including complex software for bioinformatic analysis.

Genotyping can be performed by AFLP (amplified fragment length polymorphism) (Masiga et al., 2000), PFGE (Pulse Field Electrophoresis) (Römling et al., 1992), RAPD (random amplification of polymorphic DNA) (Busse et al., 1996), MLST (multilocus sequence typing) (Maiden et al., 1998), MLVA (multilocus VNTR analysis) (van Belkum, 2007) and SNPs analysis (single nucleotide polymorphism**) (**Keim et al., 2004**)**. These methods are used to identify variations in the organisms nucleotide sequence, allowing identification of the microbe at its subspecies level. Genotyping is well established in forensic medicine and used regularly in affiliation cases. Genotyping is used for molecular epidemiology or forensic microbiology such as determining the source of an outbreak or a microorganisms origin (van Belkum, 2007).

Genotyping was used to identify the *B. anthracis* Ames strain used in the anthrax letters in the US, 2001 (Hoffmaster et al., 2002). Databases of the genetic fingerprints of bacterial threat agents are currently being established, facilitating the analysis of a potential new strain, classification of the strain and determination of the origin of the bacterial strain. The MLST genotyping method has been used for characterizing the strains of *Legionella pneumophila* isolated from biological treatment plants (Blatny et al., 2007b, Blatny et al., 2008).

It has been shown that *B. cereus*, an opportunistic human pathogen, may contain the *B. anthracis* like virulent plasmids pXO1 and pXO2 (Hoffmaster et al., 2004, Hoffmaster et al., 2006, Klee et al., 2006), demonstrating transfer of genetic material between these species. In the recent years, *B. cereus* strains containing these plasmids have caused human infections resembling anthrax symptoms (Hoffmaster et al., 2004, Hoffmaster et al., 2006, Klee et al., 2006). A survey and a genotyping scheme has been outlined for the chromosomes of the various *Bacillus* species in the *B. cereus* group with a special focus on strains with close genetic similarities to *B. anthracis* (Valjevac et al., 2005, Olsen et al., 2007). Figure 5 shows the genetic distribution of 104 various *B. sereus* group members based on a comparison of the DNA sequenses from seven different housekeeping genes (MLST) (Olsen et al., 2007). Methods for genotyping (MLVA, MLST, CRISPR) of several biological threat agents have been established at FFI (Table 4).

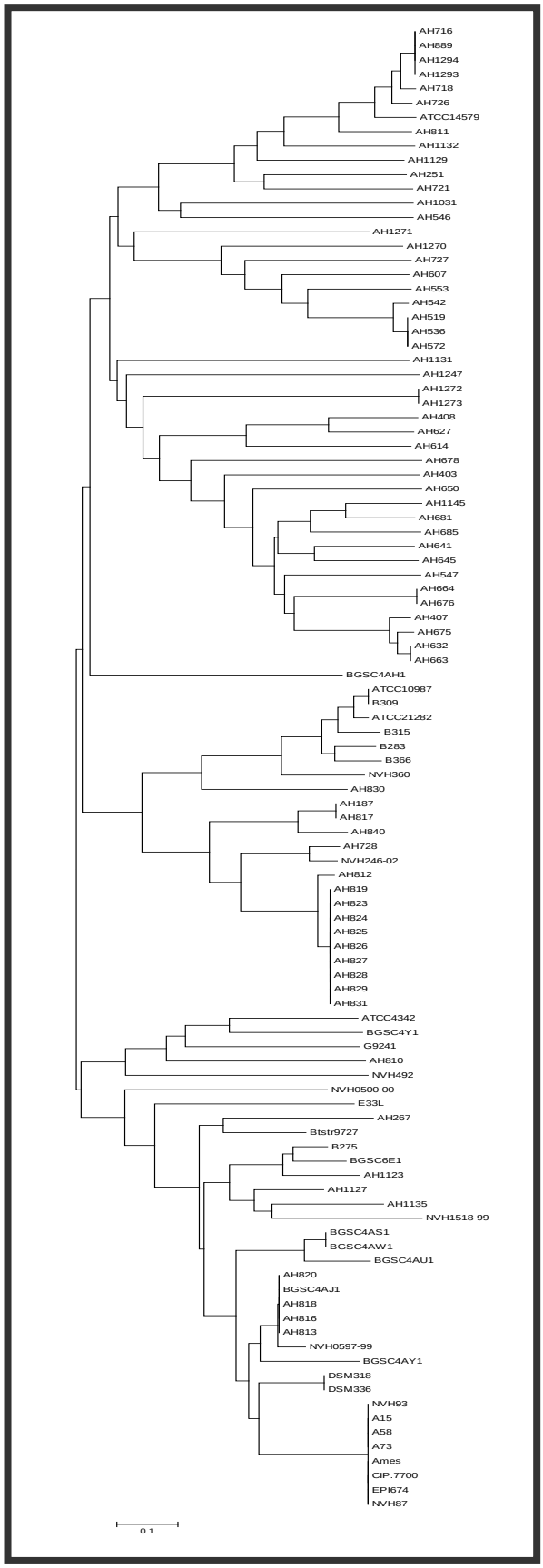

Figure 5. Phylogenetic analysis of 104 B. cereus group members based on MLST (Olsen et al., 2007).

7.2.4 Microarray

DNA microarray is a powerful tool for analyzing and detecting several hundreds of DNA fragments. The probe, which is usually a fluorescence-labelled single-stranded (ss) DNA (oligonucleotides), is spotted on a plastic, glass or silicon chip. Upon specific binding (hybridization) to the complementary ssDNA harvested from the sample, a fluorescence signal is achieved and optically monitored (Schena et al., 1998). DNA microarray may also be used for gene expression analysis and for identifying SNPs. Antibody microarrays have been developed for toxin detection, such as *Staphylococcus enterotoxin* B (SEB), tetanus toxin, anthrax toxins and the cholera toxin (Rucker et al., 2005).

The bacterial content of urban aerosols has been analyzed using DNA microarrays, thereby classifying 21 bacterial groups and more than 1800 bacterial types in the air (Brodie et al., 2007). The NanoChip Electronic Microarray (Nanogen) and the Assay Processing and Specific Identification System APSIS (Bruker Daltonics) are commercially developed instruments using microarray for detecting various biological threat agents. As for other identification techniques, a comprehensive sample processing step prior to the successful detection of biological agents by microarray analysis is needed.

7.2.5 Additional molecular methods

Fluorescence *in situ* hybridization (FISH) is frequently used for detecting and quantifying various bacterial agents simultaneously in the environment. In FISH, fluorescent microscopy is used to detect bacterial cells identified by their ribosomal RNA, which attaches to specific fluorescent probes (Souza et al., 2007). Denaturating gradient gel electrophoresis (DGGE) is based on electrophoresis of PCR-amplified 16S rRNA gene fragments in polyacrylamide gels in which the DNA fragments of the same length are separated due to their different GC/AT-content (Muyzer et. al, 1993). DGGE has been used to analyze the bacterial diversity in various liquids, such as waste waters, marine environment/sediments and at biological treatment facilities (Blatny et al., 2007b and 2008).

7.3 Immunoassay

Several immunological methods have been developed for biological identification. They are based on the use of specific antibodies (monoclonal), varying in sensitivity and specificity, against the biological threat agents. In general, fluorescence molecules (probes) conjugate to the antibodies that specifically bind to the biological threat agent. Several commercial biological identification systems are available, both for provisional and confirmatory identification. For the former type, test strips (section 5.1.3) are suitable. However, such immunoassays may be limited in their sensitivity (> 100 000 cfu/ml), and improvements are continuously developed, such as the use of magnetic field to enhance the detection signal of the immunoagglutination reaction (Rouzeau et al., 2007). In this case, the antigen (the agent) binds to an antibody on a coated magnetic particle (bead), which forms chains when the magnetic field is turned on. When the particles are in close vicinity, an increased probability in obtaining an antibody-antigen agglutination complex of is achieved. The use of antimicrobial peptides for identification of toxins, such as the botulinum toxin ,and toxoids has recently been demonstrated by Kulagina et al. (2007).

For confirmatory analysis, the BioVeris instruments (M-series) provides to be satisfactory for identification, but these instruments are not field applicable due to their large size. They are suitable for reference and mobile laboratories BioVeris uses specific antibodies immobilized on the surface of magnetic beads that binds to the antigen. The beads attach to an electrode surface providing a signal for optical analysis. In general, detection limits of immunological techniques are in the range of 100 –1000 cfu/ml for bacteria. Storage of antibodies may cause challenges in the field, thus, there has been a development of freeze-dried reagents.

7.4 Mass spectrometry

Mass spectrometry (MS) is a well-known method for identification of chemical agents. However, MS may also be used for identifying biological toxins. MALDI-TOFMS (matrix associated laser desorpsjon/ionization time of flight MS) is a developing tool for identification of bacteria and viruses, and identification of different *Bacillus* spp. strains, such as *B. globigii*, *B. cereus, B. thuringiensis*, has been achieved, showing that MALDO-TOFMS may be used to classify bacteria belonging to the same genus (Kedney et al., 2007). Electrospray (ESI) is frequently used as an ionization technique coupled to TOFMS, and may be used for classification and analysis of viruses (Bothner and Siuzdak, 2004, Thomas et al., 2004). ESI and MALDI-TOFMS has recently been used to detect and characterize biological threat agents based on specific PCR fragments amplified from the microbes. So-called “intelligent PCR primers” are selected and mass spectrometry is used for analyzing the amplicon (Hofstadler et al., 2005).

7.5 Biosensors

The call for rapid methods detecting biological threat agents has revealed the interest for biosensors, in which electrochemical, high frequency and optical transduction modes are used. Ligler et al. (2003) and Deisingh and Thompson (2004) describe the technology of biosensors and its use in detecting bacteria and toxins. Evanescent wave fluorescence technology seems to be a good approach in analyzing "dirty" samples real-time. An evanescent wave biosensor uses fiber optic waveguides to transmit and receive light information generated by the detection signal. Usually, antibodies labelled with fluorescent probes are attached to the fiber, and a detection signal occurs when the antibodies bind to the target analyte (Lim et al., 2005). The evanescent wave fiber-optic portable biosensor Analyte 2000 has been used for identifying *B. anthracis* spores at concentration levels of 3,2 x 10^5 spores per mg of powder (Tims and Lim, 2004). The Analyte 2000 has also been mounted on an UAV designed for aerosol collection (Anderson et al., 1999). The biosensor RAPTOR (Research International) uses evanescent wave optics for detection and the detection limit may reach 100 cfu/ml and 1 ng/ml for *B. anthracis* and ricin, respectively, according to the manufacturer. The NASBA method (section 7.2.2) for identification of *B. anthracis* spores has been successfully implemented into a membrane-strip-based biosensor (Baeumner et al., 2004). A brief overview of various biosensors has been provided by Blatny et al. (2006).

8 Field and chamber trials

Biological detectors for monitoring airborne microorganisms must be tested and evaluated in field prior to use, or at least at similar circumstances as planned for use (Fig. 3). The need for biological detection and identification equipment, also among the civilian society, is increasing allowing a growing marked for commercializing scientific products. Efficient reliable biological detectors must possess a low false alarm rate, also requiring technologies and software (algorithms) that distinguish natural microbial background from the released agent.

Field trials using non-pathogenic biological simulants are performed at several biological defence research institutes with expertise in bioloigcal detection, such as the Defence Research and Devolopment Canada (DRDC), Porton Down (UK), and the Swedish Defence Research Agency (FOI) (Fig. 6). The Czech Republic, Germany and the US are also carrying out field trials for biological defence studies. Such trials are crucial in order to obtain information about the dispersal of biological agents in air as well as testing and evaluating biological detectors. However, aerosol chamber trials are generally performed prior to field trials, but are executed under controlled closed containments and may not resemble real-life situations.

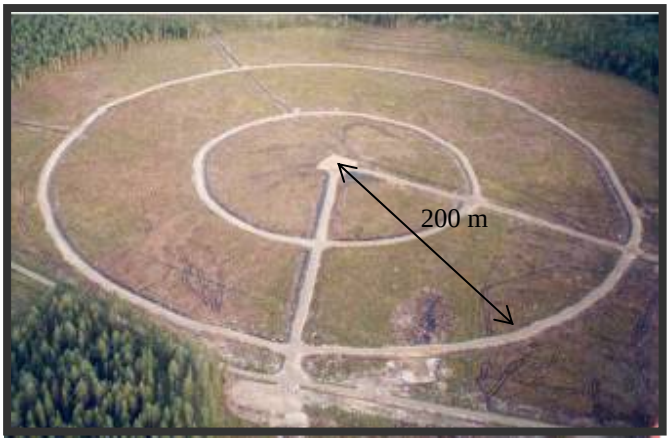

Figure 6. The region used for biological field trials located near the Swedish Defence Research Agency FOI, Umeå. The biological detection equipment is located in the center of the field. The release of the biological simulants takes place at a 100 or 200 m distance in radius from the equipment.

FFI has participated in both field and chamber trials at FOI and Porton Down, respectively, in 2006 and 2008, in which the air collectors SASS 2000^{PLUS} and XMX-CV was tested and evaluated. During the field trials at FOI, *Bacillus subtilis* var *niger* (previously and now designated *Bacillus globigii* and *Bacillus atrophaeus*, respectively) and *Erwinia herbicola* were used as simulants of *B. anthracis* spores and vegetative bacterial cells, respectively. Environmental samples of soil and water were collected and analyzed using real-time PCR at FFI successfully gaining experience in sampling, sample processing and identification of complex matrixes. Results showed that optimized method are needed for efficient DNA extraction of the bacterial simulants deposited in soil, while analysis of both air and water samples are less challenging, and in some cases DNA extraction from bacteria in air samples can be neglected (Fykse et al., 2008a). As a follow-up, FFI has performed limit of detection tests using three commercial DNA extraction kits for soil (Olsen et al., 2008). Specific detection using real-time PCR relies also on well-designed primers and probes targeting distinct regions in the bacterial genome.

Figure 7. Sampling campaign of airborne bacteria outdoor at FFI 2007. A: C-FLAPS biological detector, B: Tone Aarskaug (FFI) and Jim Ho (DRDC) preparing the slit-array for air collection, and C: location and equipment in place for the sampling campaign.

In May/June 2007, a sampling campaign of ambient air was performed at FFI together with Defence Research Development Canada (DRDC)[4] (Fig. 7). The aim of this campaign was to gain expertise in air sampling and biological detection using a slit-array, the air collector XMX-CV and the C-FLAPS (Dycor Ind.), in addition to test and evaluation of an air collector prototype (XMX/2L-102, Dycor Ind.). A slit-array was established collecting airborne bacteria on R2A growth agar medium during day and night, and the bacterial colonies were analyzed by 16S rDNA sequencing (unpublished results, FFI). The C-FLAPS is an off-the-shelf system for

[4] http://www.mil.no/felles/ffi/start/article.jhtml?articleID=143316

biological detection based on fluorescent laser aerodynamic particle sizing technology developed by DRDC. Fluorescence and scattered-light signals are excited using a 405 nm laser diode detecting and distinguishing biological airborne particles from inorganic particles, thereby, acting as a trigger for biological detection. The C-FLAPS has been implemented on naval ships and military vehicles in various NATO and Partnership for Peace (PfP) nations. FFI has purchased the C-FLAPS for testing and evaluation purposes for military and civilian use and we are currently analyzing the airborne background at various locations. False alarm rates may occur, and, thus, it is crucial gaining information about which environments may trigger false alarms.

9 Integrated systems

Both for military and civilian use, an integrated system containing all components that provide fast, sensitive, specific, reliable detection and identification of biological threat agents would be a "gold standard". Equipment containing integrated systems has been developed, especially for military use (see also Blatny et al., 2006). The experience and knowledge provided by the defence institutes and Ministries of defence is of significance for the civilian society.

The Joint Biological Point Detection System (JBPDS, USA) (General Dynamics, USA is a stand-alone robust, man-portable automatic detection and identification instrument, which can be mounted on ships and trailers, suitable for analyzing airborne biological threat agents in operations. It contains a trigger based on laser-fluorescence for monitoring the biological background in air, a bioaerosol sampler and concentrator collecting the particles into liquid, and immunoassays for identification.

General Dynamics has provided the Canadian Forces with the Canadian Integrated Biochemical Agent Detection System (CIBADS) (General Dynamics, Canada including the 4WARN system, the VP Bio Sentry System, and instruments for detection and automatic collection of bioaerosols into liquid upon an incident followed by identification.

The Integrated Biological Detection System (IBDS) (Biral) is used by the British Armed Forces, in which the sensor characterizes the size, shape and concentration of biological particles in air to distinguish biological threat agents from the natural background, in addition to fluorescence measurements (Clark et al., 2006).

For civilian use, the Department of Homeland Security has developed the BioWatch program which monitors urban bioaerosol using the Autonomous Pathogen Detection System (APDS) (Lawrence Livermore National Laboratories). The integrated system monitors the concentration of biological agents in the air and triggers air sampling when the particle concentration has reached a threshold value. The APDS uses both immuno- and DNA based methods for identification.

10 Norwegian Defence Research Establishment (FFI)

The biodefence work at FFI includes

- establishing methods for sampling, sampling preparation, detection and identification of biological threat agents in environmental samples
- evaluation of biological scenarios as basis for biological threat assessments
- technology watch of biological detection and identification devices
- development of a biolidar system for stand-off detection
- modelling and analysis of the dispersion of biological threat agents in air

Reducing a biological threat requires several actions, including a well-defined and efficient national preparedness and response system for both military and civilian societies. One of our major aims is to understand the biological threat and provide technological advice to the Chief of Defence and Ministry of Defence regarding biological defence issues. FFI plays an active role in national biological preparedness and response, not only for military purposes but also civilian, when needed.

FFI takes part in several international collaboration projects to improve biological preparedness and response, exemplified by the NATO SET097 RTG54 Biological Point Detection group, consisting of six participating nations (Canada, the Netherlands, Norway, Germany, Spain, USA) in which Norway (FFI) is the lead nation. The aim of this work is to "Work towards standardization of protocols, methods and analysis procedures for point detection of BW (biological warfare) materials, based on environmental sampling of air, soil and water, and work towards a common reference for reporting same." FFI participates in an European Defence Agency (EDA) project call "Establishment and management of a common database of B-agents" (2007-), which is a follow-up of the WEAG (Western European Armament Group) CEPA13 project "Identification of B-agents". The objective of this project is to establish a strategic European biodefence laboratory network to increase the European preparedness and protection against biological threat agents. Twelve different European countries take part to improve their laboratories capability for forensic analysis by constructing a database containing genotyping data obtained by MLVA, MLST, SNPs and MS (section 7). Furthermore, FFI plays an active part in the Anglo-Norwegian-Netherland-Germany project collaboration (ANNGCP) "Protection against Biological Weapons" and the Nordic Armaments Co-operation (NORDAC) "Biological Warfare Detector Demonstrator".

FFI is currently establishing a laboratory capability to analyze samples potentially contaminated with biological, chemical and radiological agents (mixed samples) and has recently participated in the first NATO Laboratory Exercise for mixed samples (Sweden lead nation) (Nygren, 2008, Breivik et al., 2008). The objective of this exercise was to establish laboratory protocols and methods for sample preparation from receipt to final analysis.

Also, FFI takes part in the National Biological Preparedness Committee, and has close collaboration with several institutes and agencies involved in biological preparedness, such as the Norwegian Institute of Public Health, Norwegian School of Veterinary Science, National Veterinary Institute, Directorate for Civil Protection and Emergency Planning (DSB), Norwegian Food Safety Authority, Ullevål University Hospital, as well as the First Responders, i.e. Police and the Fire Brigade.

The biological identification methods established at FFI includes provisional and confirmed identification according to the AEP-10 guidelines (Table 4). FFI has not yet established immunological assays for the so called ”dirty dozen” biological threat agents, but preliminary tests of immunoassay kits have been, and are currently being, performed.

11 Conclusion

The first sign or alert of a biological incident may be the appearance of clinical symptoms in humans or animals. An efficient biological preparedness and response system able to rapidly implement necessary counter measurements includes several actions, such as biological crises management, protection, strategies and technologies for biological detection as early-warning systems, sampling performance, sample processing methods and identification methods of both environmental and clinical samples, as well as medical countermeasures and an awareness of clinical symptoms caused by biological threat agents. Regardless of an intended or natural outbreak of a disease caused by biological threat agents, preparedness needs to be established *prior* to such an event.

The Norwegian Defence Research Establishment (FFI) has focus on sampling, sample processing and analysis of biological threat agents in the environment which contain complex matrixes having an impact on the identification analysis. The persistence of the various biological agents deposited in the environment may also have an impact on the analysis. The NATO Handbook for Sampling and Identification of Chemical and Biological Agents acts as a guideline for establishing identification methods of biological threat agents, and distinguishes between provisional, confirmed and unambiguous identification. FFI is capable of both provisional and confirmed identification of various biological threat agents per June 2008.

Rapid, reliable and efficient identification methods must be in place for both clinical and environmental samples containing biological threat agents, also for forensic purposes. Such methods may include microbiological, biochemical, metabolic, immunological and molecular methods in addition to microscopy and mass spectrometry.

Several biological detectors and collectors, acting as early warning systems, are commercially available or are currently at an R&D level monitoring the level of biological particles in air. In order to rely on their performance in real-time (life) situations, testing and evaluation in chamber and field trials is an utmost prerequisite for their use.

A national biological preparedness and response system requires an extensive collaboration between ministries, directorates, governmental and private agencies, academia, institutes and defence (military and civilian) laboratories to obtain a national efficient response to any kind of incident where biological threat agents have been used or accidentally released. An efficient national biological preparedness system will also take part in improving the European and NATO’s preparedness against biological threat agents.

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