



Identification of biological warfare agents – recent progress and future trends



Defence Forces Research Agency DFRA, Finland

Tarmo Humpi

Tuuli Haataja

Sirpa Mustalahti

Centre for Military Medicine, Finland

Markos Mölsä

Forsvarets forskningsinstitutt FFI, Norway

Else Marie Fykse

Totalförsvarets forskningsinstitut FOI, Sweden

Torbjörn Tjärnhage



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Torbjörn Tjärnhage

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Approved by

Marius Dybwad

Project Manager

Janet Martha Blatny

Director

English summary

This report provides a brief summary of current and developing technologies available for identification of biological threat agents. In addition, some representative examples of bio-identification platforms are given. Discussions regarding the future trends and prospects in the field of bio-identification technologies are included.

The responsible nation for writing this report was Finland with assistance from the other nations. The Finnish report is enclosed to this FFI report and starts at page 5.

Sammendrag

Denne rapporten er en leveranse i NORDEFECO-samarbeidet mellom Norge, Sverige og Finland med tittelen “The biological identification and detection capabilities (BIDC)” (05.06.2015).

Hovedfokuset i leveransen er å beskrive hvordan dagens teknologiske tilnærminger og instrumentering kan brukes til å identifisere biologiske trusselstoffer i en kjemisk, biologisk, radiologisk og nukleær (CBRN) sikkerhets- og forsvarssammenheng.

Rapporten beskriver metoder og teknologier for identifikasjon av biologiske trusselstoffer. Den inneholder en kort oppsummering av teknologier som per i dag er tilgjengelige for identifikasjon av biologiske trusselstoffer, inkludert en diskusjon av fremtidige trender innenfor dette området.

Finland har hatt hovedansvaret for å skrive denne rapporten med innspill fra de andre nasjonene. Rapporten er i sin helhet vedlagt og starter på side 5.

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Preface

This report is a part of the NORDEFECO subgroup Biological Identification and Detection Capabilities (BIDC) (05.06.2015). The subgroup investigated the possibilities for co-operation within biological stand-off and point detection and identification, in order to improve biological defence. The aim of work was cost savings for the participating nations and enhanced capabilities. This is achieved by means of information exchange as well as identifying areas of common goals, needs, capabilities and harmonization. The objective is to seek possibilities to common procurement, education and training, standards, testing, burden sharing, resource optimisation and interoperability.

This report is a continuation of our previous paper “Detection of Airborne Biological Agents: Technological review and outlook. The scope of this paper is briefly to describe the current and developing technologies for identification of biological threat agents. In addition, a few examples of identification components and platforms will be given.

This report is aimed for military decision makers to aid in establishing and maintaining Nordic co-operation. The main focus of the report is describing current technological approaches and instrumentation being used for the identification of deliberate release biological agents in chemical, biological, radiological or nuclear (CBRN) security and/or -defence context.

1 Background and definition

Biological warfare agents (BWA) are harmful pathogenic microbes or toxins which are being used as a weapon to cause disease or death on target population. Although a large number of pathogens or toxins are suitable for BWA only twelve of those have been weaponized [1]. Today the use of BWAs as a means of warfare is extremely low. Weaponized BWAs are usually regarded as weapons of mass destruction or casualties (WMD). In the context of bioterrorism and BWAs as means of asymmetric warfare implication are often limited. Contrary to public believe establishment of offensive biological weapon capability is not plain and simple. There are many obstacles i.e. technological, political and economic. Modern microbiology and biotechnology have significantly advanced on the 20th century creating huge opportunities to develop highly sophisticated BWAs.

Nevertheless said before, biological warfare agents have remained a persistent concern for the people responsible the national security.

Besides prospects for BW weapons development modern technology provides tools to improve biodefence capabilities. Development of detection and identification methods for BWAs is an important component in our preparedness to protect troops and public health from the harmful effect of biological threat agents.

2 Units of measure for biological warfare agents

In microbiology colony-forming unit or CFU is gold standard to estimate the number of viable bacterial and fungal cells in a sample. In virology the counterpart of CFU is PFU or plague-forming unit. In the case of biotoxin mass based units such as μg or μmoles are being used. Among biodefence community for aerosol samples Agent Containing Particle Per Liter Air (ACPLA) is often the unit of choice [2], [3] to describe the number of particles contained in the air. It can be easy to measure for biological agents like bacterial spores with the use of slit-to-agar samplers. Measurement of ACPLA is robust and simple to perform and has been widely used, especially in connection of testing of bioaerosol detectors.

Unfortunately it provides little useful information about the nature of the bioaerosol particles. ACPLA doesn't reveal how many active microbial cells are present. A single particle or ACPLA may contain one or tens even hundreds of agents (Figure 2.1).

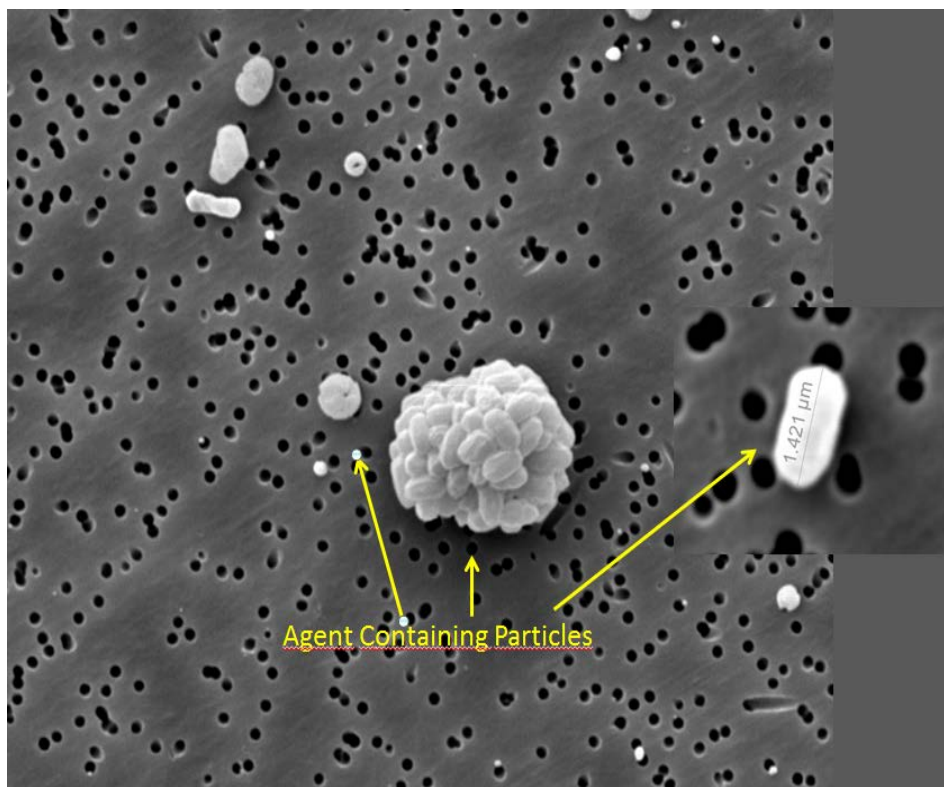


Figure 2.1 SEM image of aerosolized *Bacillus atrophaes* spores collected on polycarbonate filter. Spores are generated by nebulizing *Ba* suspension (10^8 spores/ml). Image Sirpa Mustalahti DFRA.

Moreover ACPLA particle size distribution remains unknown. It's well recognized that only bioparticles having particle diameter 1-5 μm are dangerous. Thus, to evaluate the health hazard from deliberate release of BWA the ACPLA unit is not useful. National Research Council in USA has suggested a new unit for the hazard prediction. The proposed hazard unit is Biologically Active Unit Per Liter Air or BAULADae, where Dae is the particle aerodynamic size. It measures concentration as a function of aerodynamic particle size. To be biologically active, it necessitates the presence of an active agent (infectious microbe or toxin molecule) in a particle. Aerodynamic particle size gives a rough estimate of the number of active agents in a particle. BAULADae provides useful information needed to estimate health hazard. However the proposed BAULADae is more complex to determine and for instance requires active particle counting as a function of aerodynamic particle size. In practice the determination of BAULADae is a challenging task. Biological activity of most aerosolized BWAs is not known and the methods for determining of agent characteristic such as viability, culturability, virulence etc. are challenging to measure. Moreover, the PCR based method currently being used for bio-identification cannot discriminate active agents from inactive agents. A new unit of measure ie "Total Agent per Liter Air with particle size distribution" or TALAp has been suggested [4]. It contains the two most important variables for evaluation of biodetectors and identifiers. The use TALAp provides significant improvements over ACPLA because it allows measurement of the particle size distribution and the amount of agent present.

A rough estimation of viable agents in ACPLA can be done by measuring concurrently aerosol concentration with the slit-to-agar sampler and air-to-liquid samples. In an aerosolization experiment the one ACPLA particle was found to contain three viable *Bacillus atrophaeus* spores (see Figure 2.2) [5].

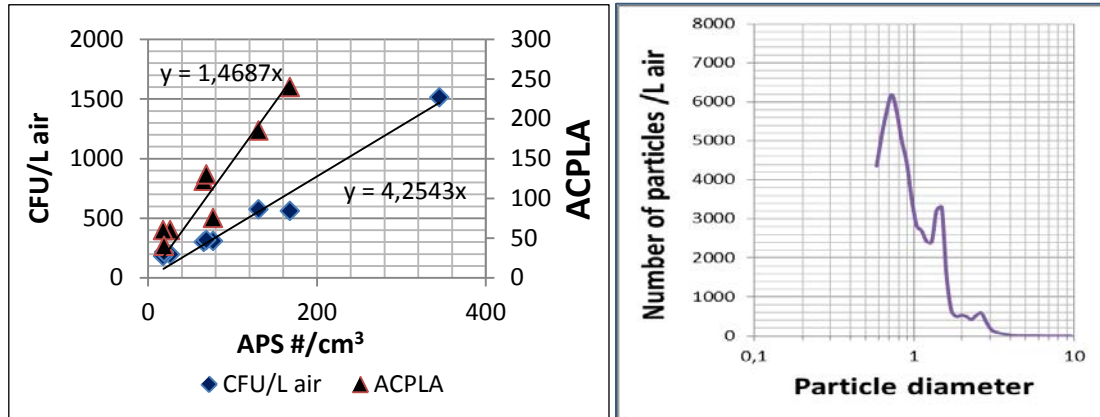


Figure 2.2 Correlation between total aerosol count (particles/cm³ as counted by an Aerodynamic Particle Sizer TSI APS) and viable particle count. One APCLA particle contains three viable spores. This is based on assumption that air-to-liquid-sampling (SKC BioSampler®) disperses any spore-to-spores agglomerates into single spores and collection efficacy of both sampling techniques are equal. Graph on the right shows the particle size distribution in the aerosol cloud generated by nebulizing from *Bacillus atrophaeus* spore suspension. Probably, peaks at 1.5 and 2.4 μm represent spore-to-spore agglomerates.

3 Evaluation of health risk from bioaerosol exposure

The health risk from deliberate release is a complex issue as shown in Figure 3.1. Risk assessment from bioaerosol exposure is out of the scope of this paper. However it would be worthwhile to discuss it to some extent. In recent study National Research Council has presented a framework for evaluating total health risk posed by bioaerosol exposure. The health risk consists of two components: health hazard posed by an agent and the physiological response of the individual exposed.

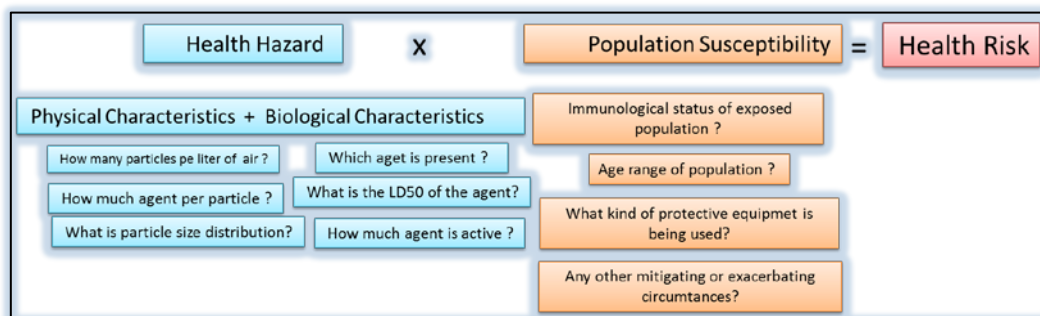


Figure 3.1 A framework for evaluating the health risk posed by aerosolized biological warfare agent exposure [6].

Physiological response is dependent on the population susceptibility. Smallpox virus is a hazardous only to individuals who are not immunized against smallpox virus. Health hazard includes physical and biological characteristics of the threat agent. In the context of the biodetection the former is related to characterizing of threat agents by physicochemical methods such as by detecting of innate fluorescence. The latter refers to the identification and characterization of the threat agent present.

4 Current technologies and established criteria for B-identification

The SIBCRA criteria for biological identification, as presented in NATO AEP 66, are a useful tool in biological defence communication and decision-making. The AEP 66 SIBCRA criteria for biological identification are presented in *Table 4.1*. These criteria can be applied to all classical microbes with the final goal in demonstration of viability of the agent used in the incident that lead to sampling and analysis. However, disease-causing and viability demonstration criteria for unambiguous identification do not apply for agents for whom there are no acceptable culture methods or agents that have been genetically manipulated to change their characteristics. Also novel agents, such as microencapsulated organisms, prions and infectious nucleic acids, do not fit well into these criteria.

The criteria are based on most commonly available technologies for achieving biological identification, and the document establishes ways how these technologies are combined in achieving the desired level of certainty. In this chapter of the report, some of the most common technologies for biological identification are briefly described before advancing to current identification platforms utilizing them. The techniques included are those that most readily lend themselves to military field point detection and identification at present; however, the area of biological detection is evolving rapidly and development of instrumentation is expected to bring new technologies to the battlefield in the coming years. Less emphasis is put on rest of the diverse field of biological identification as well as the national epidemiological surveillance and healthcare that also covers these issues and are an essential part of homeland security and biodefence in the Nordic countries.

Various methods have been developed for the rapid detection and identification BWAs. Most of the assays are based on detecting the:
whole organism, B) bacterial/viral toxins antigens, C) the nucleic acid or D) specific biomarker molecules.

The following example highlights five major methods for identification of *B. anthracis*.

Table 41 SIBCRA criteria for identification of biological agents (AEP 66)

Level of identification	In vitro culture/multi metabolic assay	Antigen demonstration (immunoassay)	Nucleic acid sequence demonstration (PCR)	Disease properties demonstration in animal model
Provisional	x	x	x	
Confirmed	Any two of the three - in presence of reference standards			
Unambiguous	All of the above - in the presence of authentic reference standards			

4.1 Conventional microbiological methods

4.1.1 Culturing techniques

Conventional culturing techniques have been regarded as gold standard for detecting, isolating and identifying biothreat agents. Culturing techniques used in clinical laboratories rely on selective nutrient mediums that inhibit the growth of non-target organisms as well as differentiate the target organism from others. Staining and other organism characteristics, such as organism motility are used in conjunction with the typical biochemical and metabolic profile of the culture to achieve identification result.

Culturing the agent readily enriches the organism for further characterization and examination, as well as demonstrates agent viability. Use of culture enrichment and selection typically takes time and may require up to several days for achieving even preliminary results. While these methods are reliable and their performance well known and understood, they are too time-consuming and laborious to be usable in the field and do not provide real-time or rapid detection of threat agents in the case of suspected use.

4.1.2 Immunoanalytical methods

Immunoanalytical methods are based on the specific affinity between microbial antigens and monoclonal or polyclonal antibodies that are produced in a cell culture or collected from test animals. Immunoanalysis can be applied in rapid detection and identification of bacteria, viruses and toxins. Antibodies and immunochemical methods can also be used to isolate or enrich these agents from environmental samples. When used alone without any other analysis method the immunoanalytical methods provide for the identification level of provisional identification.

Immunoanalytical method or immunoassays are rapid, sensitive and selective and generally cost effective. However, sensitivity and selectivity of the assay necessitates high quality antibodies.

4.1.3 Hand-Held immunochromatographic assays (HHIA)

The hand-held immunochromatographic assays (test strips) are commonly used for tentative or preliminary identification, both on-site and in laboratories [7]. In these tests a capture antibody is immobilised on a strip of nitrocellulose membrane, forming a line on the membrane. A second antibody is coupled to coloured particles. The liquid sample containing the analyte is then

allowed to mix with the antibody-coupled coloured particles. The analyte will be bound by the antibody-coupled particles and this complex will migrate by capillary action along the nitrocellulose strip until it meets the capture antibody where it is bound and immobilised. A visible coloured line is then formed and indicates a positive result whereas a negative result is when no line is formed. This type of assay takes only about 15 minutes to perform and the result can be read visually without any instruments, therefore this detection technique is especially suitable for on-site identification.

The HHIA have several limitations. First, only one agent can be detected per assay strip. Thus, several handheld assays must usually be run to obtain a presumptive identification. The second limitation is that each of the assays has varying sensitivity levels to their respective target agents. Assays for bacterial agents tend to be the most sensitive, able to detect from 2×10^5 to 2×10^6 CFU/ml while those for toxins have sensitivities ranging from 50 pg/ml to 50 ng/ml. Assays specific for viruses usually have the lowest sensitivities, ranging from 2×10^5 to 5×10^7 CFU/ml [8].

Enzyme Linked Immuno-Sorbent Assay (ELISA)

Another commonly used immunoanalytical identification technique is the Enzyme Linked Immuno-Sorbent Assay (ELISA). In an ELISA the capture antibody is adsorbed to the plastic surface of a microtitre plate well and the signal is generated by an enzyme conjugated to the second antibody. If any analyte is present in the sample, the analyte will be bound by the capture antibody and the enzyme-coupled second antibody as well, forming a “sandwich” structure. After washing away any non-bound antibody, a substrate for the enzyme is added which is converted to a coloured product. ELISAs are relatively quick assays requiring a few hours to perform. However, they are restricted to fixed site or mobile laboratory use due to duration of the assay and several washing and incubation steps. The instrumentation involved (a spectrophotometer) is relatively inexpensive.

ELISA based methods are especially suitable for preliminary identification native biotoxins because they in pure state don't contain genetic material for PCR

4.1.4 Example of current immunoanalytical platform

ENVI Assay System

Immunochromatographic test kits are built on handy and compact immunoassay format in which BWA-specific gold- or fluorescence-labeled antibodies are utilized in capturing the BWA of interest from the sample, respectively. These rapid tests are suitable for both field and laboratory conditions with no requirement for special training, big investments or maintenance costs. In combination with a handheld ChemPro Reader Module, the ENVI Assay System (Figure 4.1) is all what you need for early indication of a biological threat [9].



Figure 4.1 *Envi Assay and ChemPro Reader module*

4.2 QTL Biosensor

QTL Biosystems is a portable, on-site instrument for rapid analysis, and identification of biological agents. Agent identification is based on immunomagnetic sandwich assay technology. Device allows five minute time to answer identification for many military important agents such as anthrax, ricin and botulinum toxin. Both wet and dry samples can be analyzed. This instrument is IP 67 certified and permanently housed in a sturdy, light weight and fully deconable Pelican case (Figure 4.2). QTL is produced by QTL Biosystems.



Figure 4.2 *The QTL Biosensor 2200 R handheld bio-detection instrument.*

A comprehensive list of commercially available bioidentification platforms are found on websites [10]. This guide summarizes technologies that can be used by first responders for collection and identification of biological threat agents.

Chemical Biological Medical Systems and Biosurveillance and the Defense Threat Reduction Agency (USA) have prepared the technology survey focusing on available detection and diagnostic systems and devices that can detect and/or identify the presence of biological, chemical, and radiological agents. This report reviews and scores vendor-supplied information about detection devices or systems [11].

4.3 Molecular biological methods

Identification methods based on molecular biology are used to achieve more rapid identification and characterization of the agent compared to conventional microbiology. These methods typically require the presence of 10-100 target biothreat agents. Interfering substances may complicate the analysis. In addition, these methods do not demonstrate, for example, neither agent viability nor infectivity. Molecular biology identification methods can be therefore combined with steps enriching the target agent or removing potential interfering agents from the sample matrix.

Real-Time Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a highly sensitive technique for identification of microorganisms. PCR is based on amplification of a specific nucleic acid sequence, followed by detection of the reaction product. Real-time PCR enables both amplification and simultaneous quantification of a targeted nucleic acid molecule. In multiplex real-time PCR, several DNA targets are detected simultaneously in the same reaction.

Real-time PCR uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the real-time PCR reaction. The amplified nucleic acid molecule is detected in real time as the reaction progresses, compared to a standard conventional PCR, where the reaction product is detected from an agarose gel after the PCR run. Detecting the amplified nucleic acid molecule in real time removes the need for manipulation of the reaction product after the amplification steps. Hence, reduced number of handling steps during real-time PCR provides good repeatability from sample to sample and minimizes the risk of contamination since less manipulation is required.

Positive identification is determined by identifying the cycle number at which the reporter dye emission intensity rises above the background noise. That cycle number is referred to as the threshold cycle (Ct).

When diagnosing viruses whose genomes are composed of RNA, such as influenza virus, an adaptation of real-time PCR called real-time reverse transcription PCR (qRT-PCR) is used. That is, RNA is transcribed into complementary DNA (cDNA) by reverse transcriptase enzyme prior to PCR. It is possible to carry out qRT-PCR as one-step qRT-PCR, in which both reverse transcription and real-time PCR steps take place in the same tube.

Real-time PCR requires purity of the template, primers, and probes used, as false positives are a problem that is common to the general application of real-time PCR. False positives occur mainly as a result of cross-contamination from either positive samples or reaction products. For detection of possible contaminations, different controls are used.

An internal positive control is a DNA or RNA control sequence that is amplified in the same reaction as the target sequence and detected with a different probe. The Internal control is generally used to test for the presence of PCR inhibitors which molecules are known to inhibit the

reaction. That is, if the internal positive control is detected but the target sequence is not, it indicates that the amplification reaction was successful, PCR inhibitors did not interfere with the reaction, and the target sequence was not present in the sample. Furthermore, DNA or RNA that is similar with the target sequence can be used as a positive control. This sample controls that the real-time PCR reaction is valid and the instrument settings are correct. In addition to positive and internal control, a “no template control” (NTC) is used. The NTC contains all essential components of the PCR reaction except the template.

Designing and validation are crucial parts of setting up real-time PCR identification assays due to the existence of numerous sources of variation, including template concentration and amplification efficiency. A valid real-time PCR assay is a reliable and rapid method for identification of pathogens.

Rapid identification of pathogens is essential for appropriate medical intervention in the event of suspected intentional release of severe biological threat agents. Reliable biological diagnosis allows early medical treatment before onset of severe symptoms.

Real-time PCR can be used as a reliable identification for field detection of selected B agents in the context of the Deployable CBRN Laboratory (CBRN reconnaissance team and SIBCRA team) and for point-of-care diagnostics.

The ideal real-time PCR assay delivers rapid, sensitive, specific and reproducible results while minimizing the requirements for specialized laboratory facilities and skilled technicians. The device software should include modules for result analysis.

4.3.1 Current devices

4.3.1.1 ABI 7300 (Applied Biosystems)



ABI 7300 Real-Time PCR System from Applied Biosystems.

- Size and weight: 34x49x45 cm, 29 kg 96 sample wells
- Several detection channels and sample wells for multiplex detection of biological agents
- Analysis time app. 100 min

4.3.1.2 PIKOREAL (ThermoScientific)



Size and weight: 30 x 30 x 30 cm, 10 kg
Field usable, easily transportable by one person
24 or 96 sample wells
Pelican box protection case
Several detection channels and sample wells for multiplex detection of biological agents
Small power consumption
Analysis time 45 min

4.3.1.3 R.A.P.I.D (Biofire)



Size and weight: 50x36x27 cm, 22,7 kg
Hybridisation probe technology
Analysis capacity: 32 analysis/ 30 min
Protection case

4.4 Cellular fatty acid profiling (CFAP)

Cellular fatty acid profiling (CFAP) has become more practical method for identification of microbes of military and forensic interest. This development has contributed the recent progress of computer-controlled gas chromatography and data analysis, simplified sample preparation, and a commercially available GLC system dedicated to microbiological applications [12]–[14].

CFAP identification based on the converting cellular fatty acids from pure cultures of bacteria to fatty acid methyl esters. Samples are harvested, saponified, and methylated and then extracted and washed. The resulting organic phase is analyzed using a gas chromatograph for identification and quantification methylated fatty acids. Pattern recognition software is used to identify isolates from the chromatograph reading.

4.4.1 The Sherlock® Microbial Identification System (MIS)

Sherlock® Microbial ID System (MIS) is the product of MIDI Inc (MIDI, Inc., Newark, Delaware). Identification of BWAs based on the cellular fatty acid profiling employing fatty acid methyl ester analysis by gas chromatography (GC-FAME).

Today, The Sherlock MIS is the only commercially-available system that uses GC-FAME technology and is currently used in 35 countries in a variety of markets, including in 9 of the top 10 pharmaceutical companies in the world. FAME analysis is often specific enough to allow for strain or sub-species level comparisons [15].

4.4.2 FilmArray® Biothreat Panel

FilmArray® Biothreat Panel[16] is a PCR based identification platform utilizing a Lab-in-a-Pouch approach capable to identify 17 biothreat agents in a single sample in just over one hour. All reagents required for sample preparation, PCR reaction end point detection are lyophilized in a pouch. Simply, the biological sample containing agents to be identified are applied in to the pouch and then the FilmArray instrument does the rest. Recently FilmArray system has been successfully used for identification of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*[17].



Multi-Use: Used for BioThreat Detection and Pandemic BioSurveillance.

• **Easy-to-Use:** 2 minutes of hands-on time.

• **Quick Results:** Results in 1 hour.

• **Fully Automated:** Sample prep, amplification, identification, and reporting.

• **Single Instrument Integration:** Minimal equipment and consumables.

• **Freeze-dried Reagents:** Room temperature stable.

• **More BioThreat Targets:** Test 17 pathogens in one run.

• **More Sample Types:** Integrated sample prep removes PCR inhibitors and allows BioThreat detection in challenging environmental sample types.

4.5 Mass spectrometry

In addition to conventional and molecular biological methods there are many other options available to reliable identification of BWAs. Mass spectrometric techniques have been widely used for identification of chemical compound (such as chemical warfare agents). However most of those are best suited for highly sophisticated laboratory environments and require well skilled personnel to operate. Power requirement, laboratory gases, computers and other peripherals is needed for the running the system. These premises can be achieved only in deployable and/or semi-stationary field laboratories. Nevertheless, mass spectrometric techniques have become an important tool to identify microbial agents, toxins and biomarkes present in microorganism [18], [19]. The most important advantage of MS over other techniques lies in its capability to identify full spectrum of BWAs without the need of unique reagents such antibodies or PCR primers.

4.5.1 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS (Matrix Assisted Laser Desorption / Ionization mass spectrometry) has become an important technique to identify microbial agent. Identification based on comparison of the mass spectrum of an agent to that in the reference library. Reliability of identification is dependent on the contents and quality of library [20]. Currently MALDI-TOF-MS is widely used as diagnostic tool in microbiological laboratories. The advantages over conventional techniques are that MALDI-TOF-MS is fast, cost-effective and accurate. On the contrary to other identification techniques such as PCR and immunoassays which are using agent-by-agent approach, MALDI-TOF-MS rely on the library of reference spectra thus allowing the direct identification of wide range of microbial agents simultaneously.

A number of paper has been published on the identification of clinically important pathogens [21]–[23]. MALDI-TOF has been used for identification of anthrax spores [24] and specific biomarkers present in *Bacillus anthracis* bacteria [25]. MALDI-TOF MS has also been used for identification of airborne bacteria. The method is compared to 16rDNA sequencing and MIDI-MIS identification [26].

4.5.2 Pyrolysis- Gas Chromatography - Mass Spectrometry Py-GC-MS

Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) is a rapid analytical technique for characterizing polymeric materials. In Py-GC-MS the sample is heated to decomposition to produce smaller molecules that are separated by GC and detected using MS.

Microorganism are heat sensitive material and are readily decomposed on heating to smaller molecules derived from cellular component such as proteins, lipids carbohydrates. Agent specific biomarkers like di-picolinic acid is one of the constituents in pyrolysate of *Bacillus anthracis* spores and can be used for identification purposes.

Under EU Framework Programme 7 it has been developed a miniaturized GC-IMS (Gas Chromatograph - Ion Mobility Spectrometry) instrument able to identify the biological threat agent. The concept of BIO-PROTECT is the development of a fast-alert, easy-to-use device for detection and identification of airborne bacteria, spores, viruses and toxins. Identification of the agents is based on the recording the IMS fingerprint pyrolysis product and analysis that fingerprint spectrum against a database with fingerprint-data of several model-agents of bacteria, toxins and viruses [27].

4.6 Biosensor

Biosensor is an analytical device where the agent identification based on the interaction of an analyte (ie BWA or it's cellular component, toxin molecule) and biological recognizing element. The device comprises of a transducer and a biological element that may be an enzyme, an antibody, a nucleic acid or are receptor molecule. The recognizing element interacts with the analyte being tested and the biological response is converted into an electrical signal by the

transducer. There are several type biosensors platforms. They are classified according to transducer being used (such as acoustic, potentiometric and piezoelectric) or according to recognizing element (such as immunosensor) reflecting the construction of the sensor. Biosensors have several advantages like sensitivity, low cost and ease to miniaturization. However, quite a few biosensors, if any commercially, are available for identification of biological threat agents. In the laboratory environment they may performs well but in real world there are several obstacles for successful utilization. Need of consumables may cause a significant logistic burden.

A number a very good review articles are now available from the usage of biosensors for BWA detection and identification [28]–[31].

4.7 Integrated systems

Integrated detection and identification systems usually consist of several components. They usually consist of a bioaerosol collector coupled to a pathogen identifier.

The Lawrence Livermore National Laboratory (USA) has been developed and tested a fully autonomous pathogen detection system (APDS) capable of continuously monitoring the environment for airborne biological threat agents. The APDS is completely automated, offering continuous aerosol sampling, in-line sample preparation fluidics, multiplexed identification immunoassays, and nucleic acid-based polymerase chain reaction (PCR) amplification and identification [32].

Another example of an integrated systems is TIGER (Triangulation Identification for the Genetic Evaluation of Risks). Identification relies on mass spectrometric analysis of base composition signatures obtained from PCR amplification of broadly conserved regions of the microbial genome [33].

More recently Battelle laboratories has introduced an all-inclusive chemical-biological-explosive detector at the size of a microwave oven that can detect airborne pathogens in minutes [34]. The Resource Effective Bioidentification System (REBS) are based on raman spectroscopy. Open scientific literature doesn't mention the REBS technique and independent studies about the performance are still missing.

4.8 Sampling

Sampling is an important and sometimes very difficult process of BW detection and identification. The purpose of sample collection is to capture a BWA into solid or liquid medium for identification. Today, a number of collection methods are available for collection airborne and surface settled BWAs. Most widely used methods for airborne BWAs are impaction onto solid surface [35], impingement into liquid [36]–[38], filtration [39] and cyclone scrubbing [40], [41]. Wipe [42], wet/ dry swab and vacuum sock [42]–[45] has been developed and validated for sampling agents from porous and non-porous surfaces. A reasonable high volume of air has to be collected in order to measure an agent concentration from 1 to 100 particle/m³.

Slit-to-agar samplers are being used for reference measurements when testing BWA detectors. Interestingly, the same apparatus can be used for effective surface sampling¹. By connecting a Tygon tubing into air inlet of the sampler it can be used a kind of a vacuum cleaner for picking microbe particles directly onto rotating nutrient agar plate (Figure 4.3).

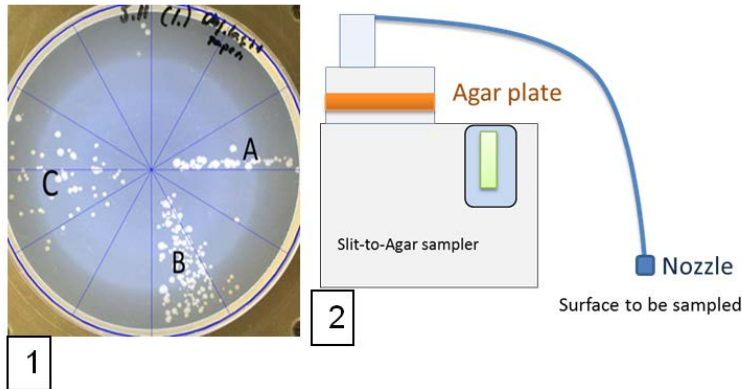


Figure 4.3 1) *Bacillus atrophaeus* colonies on Slit-to Agar nutrient plate. Letters A, B and C represent spores hovered up surfaces contaminated of aerosolized *Bacillus atrophaeus* spores.
2) Sampling arrangements.

Environment agency (USA) has recently reviewed current methods for bioaerosol sampling and made recommendations of usage of them [46]. Recently, FFI has tested and evaluated nine different air samplers, which are meant for the use of collection of airborne microbes [47].

4.9 Deployable CBRN field laboratories

Field identification of biological warfare agents may be a daunting task. Due to the lack of all-purpose technology capable to detect and identify variety of biological threat agents, one must rely on multi-tier detection architecture (Figure 4.4). Today there is no single platform to identify all possible agents in the field condition. A lot of technologies and platforms have been developed. Various commercial apparatus utilizing immunochemical, biochemical or PCR assays are available to identify BWAs. However, the operation with the sophisticated instrumentation outside the laboratory environment may be a difficult task. Thus, many countries have put a deployable field laboratory into operation. The EU FP7-Security project MIRACLE (Mobile Laboratory Capacity for Rapid Assessment of CBRN threats Located within and outside EU) has published major recommendations to the European commission regarding deployment and operation of a CBRN laboratory within and outside EU (www.cbrnlab.eu/miracle/).

¹ Unpublished work of Finnish Defence Forces Research Agency 2015

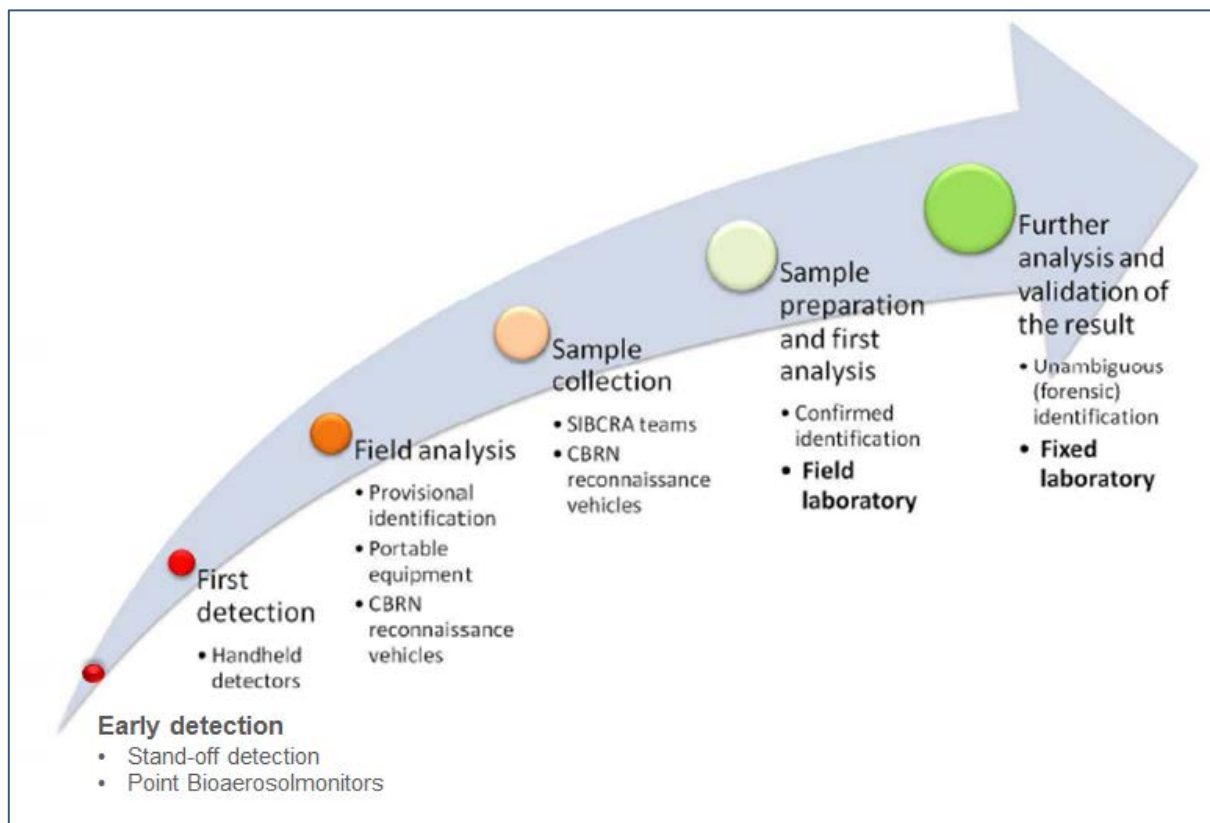


Figure 4.4 Timeline of BWA detection and identification.

4.9.1 The Finnish mobile laboratory

The Finnish mobile diagnostic CBRN laboratory, the “Field Laboratory”, can be used in all three major tasks of the Finnish Defence Forces: national defence, supporting other authorities and international crisis management operations. The laboratory contains four laboratory modules, namely the chemical analysis (C) laboratory, biological (B) laboratory, radiological/nuclear (RN) laboratory and the laboratory of field hygiene (FH) (Figure 4.5). The C and B laboratories are located inside a semi-trailer whereas RN and FH laboratories are located in two collective protection tents that are connected to the trailer. These tents are stored at the semi-trailer roof during transportation and are lowered in place when the laboratory is set up.

The Field Laboratory platoon consists of the field laboratory personnel and a four-member forensic sampling (SIBCRA) patrol. The SIBCRA patrol has a leader and three specialists, one for C, B, and RN each. These specialists have academic degrees and special training for forensic CBRN sampling according to the NATO standards. The field laboratory personnel includes eight people: the Field Laboratory platoon leader as well as two scientists in C and B laboratories each and one in RN and FH laboratories each. In addition, a mechanic is responsible for technical issues and maintenance. The Field Laboratory platoon is a part of a Deployable CBRN Laboratory detachment which comprises a command element and further two platoons: the HQ and Logistics platoon that contains e.g. the supply and medical squads, and the CBRN platoon which is responsible for tactical CBRN tasks such as reconnaissance and decontamination. The Deployable CBRN Laboratory, including the Field Laboratory, has been evaluated by NATO in the Operational Capability Concept (OCC) program.

Analytical instruments:

Bacteria and viruses:

- real-time PCR: Applied Biosystems ABI and Idaho technologies RAPID

Toxins

- QTL Biosystems immunoanalyzer
- 96 well ELISA



- Autoclave, double-ended
- 3 Door Airlock with access to the outside for ingress of sample
- Class III Glovebox
- Dunk Tank
- Class II biosafety cabinet
- VHP Decontamination Unit
- Refrigerator/Freezer

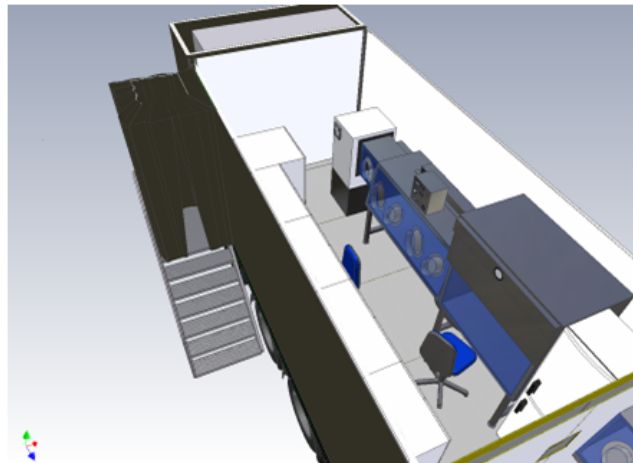


Figure 4.5 The Finnish deployable CB laboratory.

Real Time PCR is used for identification of viral and bacterial threat agents.

The protocols which are being used are developed in our facilities or we use commercially available reagents.

5 Future trends in biodetection

How the identification technologies of BWAs will develop and what kind of identification platform available depends on how the threat evolves. The destruction of World Trade Center in September 2001 by the terrorist attack and subsequent anthrax letter mailings awed incredible fear of bioterrorism. The response of scientific community was increased interest in biodefense related research topics. Number of publication per years rocketed and numerous papers concerning BWA identification have been published (Figure 5.1). Nevertheless, true breakthrough hasn't been achieved in the performance of BWA field identifiers.

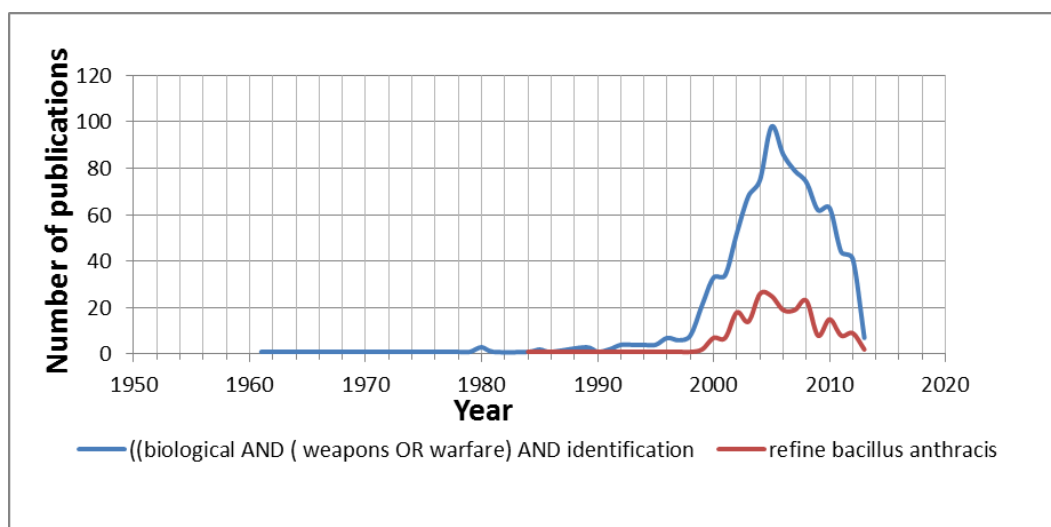


Figure 5.1 Number of publication per year found in SciFinder database using the search term ((biological AND (weapons OR warfare)) AND identification (blue line) and search refined to “bacillus anthracis” (red line).

Since 9/12 anthrax letter we haven't encountered any major biological terrorist attacks. This has influenced significantly on research and development as Figure 5.1 shows publishing activity has declined rapidly after 2005.

The driving force in developing BWA identification is the needs of general microbiological diagnostics. Early diagnosis of infectious diseases demand rapid and accurate detection and identification of the causative agents with high sensitivity. Conventional diagnostic techniques are cumbersome, expensive, time consuming, insensitive and not suitable for the use in the field. Recent achievements in developing of molecular diagnostic tools such as real-time PCR, DNA fingerprinting and DNA microarrays provide new prospects for point-of care diagnostics and BWA field identification. Biosensor technology enables miniaturization of complex devices.

Nanomaterials having high surface to volume ratio greatly enhance bimolecular interactions and hence enable the construction of more specific and sensitive optical, electrical and electrochemical biosensors.

There has been an explosive research in new nanomaterials and –structures such as nanoparticles, nanotubes, nanowires and graphenes [48]. In future, nanodiagnostic assays will be commercialized and became for the use in early detection and identification of microbial agents.

The threat posed by biological warfare agents and biological weapons has changed considerably over the past decades. 1960s and 1970s were characterized by biological and chemical weapons race, whereas towards the end of the last century the advances in medical science, international treaties and the development of nuclear weapons made the chemical and biological weapons irrelevant as a part of any advanced state's military deterrence and doctrine and they have lost their weapons of mass destruction status. However, radicalization, terrorism and the changes in

the concept of war towards asymmetry have increased the overall likelihood of the use of biological agents and their respective improvised dispersal methods.

The biothreat landscape continues to evolve. In the future, naturally occurring epidemics and pandemics are a more likely threat. However, the advances in biotechnology and molecular biology as well as the transfer to information society where virtually all information is available in the network has brought detailed information of the various biothreat agents available to all who wish to seek it. Manipulating the genetic material of pathogens has become possible, even creating novel agents from molecular building blocks (synthetic biology). With technological advances this can in the future be possible by a lone individual, without significant input of money or specialized instrumentation. A dual nature of the threat can therefore be perceived - whereas low tech traditional crude preparations and agents with improvised dispersal might still prove to be more effective in causing actual casualties, demonstrating capability for design and use of novel biological warfare agents would guarantee the responsible organization the unlimited worldwide attention of the media and public.

Concerning the deliberate release of biological threat agents, the key issue is the fast detection of the incident and identification of the agent in question. Rapid identification makes countermeasures such as quarantine, medication and decontamination more timely and effective. Advances in molecular biology, bioinformatics and optical and spectroscopic methods will make biodetection considerably faster in the future. Exact planning and design of specific molecules for recognizing elements of various sensors is becoming a reality. Instrumentation will continuously be miniaturized and more affordable. The future of biodetection technology is characterized by automation and robotics. False positive indications are no longer tolerated.

Sequencing the organisms' genetic material and the primary structure of its molecules will be in use in everyday bedside clinical diagnostics within the next decade. This will shorten the time required for identifying known pathogens in a clinical setting to more minutes-hours. This technology will replace in general the conventional slower techniques currently in use and will also lend itself to the detection and identification of deliberate release biothreat agents on the field. Bioinformatics and development of data transfer will, together with the advances in computer simulation, even allow the recognition of previously unseen organisms or molecules as potentially harmful or pathogenic. As sensor technology is miniaturized, it will be taken in use in everyday biological identifiers for personal monitoring of surroundings, food, water and health.

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