FFI RAPPORT

EARLY AND RELIABLE DETECTION AND IDENTIFICATION: AN IMPORTANT ELEMENT FOR EFFICIENT BIODEFENSE PREPAREDNESS AND RESPONSE

BLATNY, Janet Martha

FFI/RAPPORT-2005/02581

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This report is based on the paper presented by the author at the International Bioterrorism Conference in Fürigen, Switzerland April 2005, "Meeting the Challenges of Bioterrorism: Assessing the Threat and Designing Biodefense Strategies". The paper has been modified for publication of this report.

The potential use of biological threat agents results in an urgent need for rapid and reliable detection and identification techniques of these agents in order to quickly respond to a biological attack. This paper highlights the importance of developing detection and identification methods of biological threat agents. A brief overview of some of these methods, with emphasis on molecular-based identification techniques, is presented. Also, some of the ongoing research projects at FFI regarding identification of biological threat agents and other closely linked projects addressing bioterrorism issues are briefly described.

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CONTENTS

EARLY AND RELIABLE DETECTION AND IDENTIFICATION: AN IMPORTANT ELEMENT FOR EFFICIENT BIODEFENSE PREPAREDNESS AND RESPONSE

1 ABSTRACT

The potential use of biological threat agents results in an urgent need for rapid and reliable detection and identification techniques of these agents. Traditional laboratory approaches include microbial cultivation, immunoassays, and nucleic acid detection methods. The design of perceptive and rapid detection and identification methods is necessary for an appropriate and fast response to release of biological threat agents and for medical counteractions to exposure of such agents. The sensitivity and the result of the identification assay are dependent on the type of threat agent, the sample, and the sampling processing. Forged alarms may be the result of either false negatives or false positives causing large economical impacts. This paper highlights the importance of developing rapid and reliable detection and identification methods of biological threat agents in order to quickly respond to a biological attack. Also, a brief overview of some of the detection and identification methods and devices used are presented. One of the main objectives at FFI is to develop and establish molecular-based techniques to identify biological threat agents in complex environmental samples. Some of these ongoing research projects at FFI, in addition to other projects addressing bioterrorism issues are briefly described in this paper.

This report is based on the paper presented by the author at the International Bioterrorism Conference in Fürigen (Nidwalden), Switzerland 22-23 April 2005, “Meeting the Challenges of Bioterrorism: Assessing the Threat and Designing Biodefense Strategies”. The paper has been modified to include some updates and further details for the publication of this report.

2 INTRODUCTION

Biological weapons are weapons containing biological materials and are regarded as weapons of mass destruction, or more appropriately as weapons of mass casualty (1, 2, 3). A biological weapon is more than the biological agent alone and implies a physical weapon usually consisting of a payload, munitions, delivery system, and a dispersion system, in which the payload is the biological material. The biological material is an infectious agent and the munitions maintain the potency of the biological agent during delivery (viability). The delivery system can be a missile, vehicle or an artillery shell to transport the payload. The dispersion system, such as a spray mechanism, ensures the dissemination of the biological agent at the target.

Biological threat agents are micro-organisms such as bacteria, rickettsiae, fungi, viruses, and toxins that cause infections leading to incapacitation or death. Toxins may be produced naturally by micro-organisms, plants or animals, and even synthesized chemically. The technologies for production and delivery of biological threat agents have been developed and perfected during the last century. It is generally believed that these agents are easily acquired and produced due to the "dual-use" purpose of the production. For civilian purposes such equipment is used for production of beer, yoghurt, vaccines, and antibiotics (4). However, terrorists will probably come across several barriers in implementing an effective biological attack. Two of the major hurdles are the development of a sufficiently virulent and infectious strain for the seed stock and the selection of the most appropriate dissemination method of the agent.

Even though biological threat agents have the potential to either kill or incapacitate a very large number of people, these agents may be used to kill crop plants and domestic animals damaging the national economy. Biological threat agents may be disseminated by food, water, insect vectors or by direct contact. Most experts believe that aerosolization is the main and most likelihood route for effective dissemination of these agents. The Centers for Disease Control and Prevention (CDC) has grouped the biological agents into three categories; A, B,

and C, according to public health importance. Category A poses the highest risk and causes the greatest impact on health and security, while many of the water and food pathogens belong to category B (Table 2.1). Most of the biological agents are zoonotic. Animals may show the first symptoms of a clinical infectious disease after a deliberate release of a biological agent. In such cases, veterinarians may be the first to encounter the disease caused by a zoonotic threat agent (1). Also, alerted and well-trained medical practitioners and physicians are crucially needed in recognizing human clinical symptoms derived from biological threat agents. Symptoms of those exposed to such agents may be non-specific and resemble common flu-like diseases. The World Health Organization (WHO) has prepared guidelines for strengthening the considerations to food terrorism, to ensure food safety, and to respond to preparedness for deliberate epidemics (5, 6).

Table 2.1. Potential biological threat agents.

Micro-organism	Disease	Mortality untreated	Category CDC	Incubation time[c]
Bacillus anthracis	Anthrax	High	A	1-6 d
Yersinia pestis	Plagueb	High	A	2-3 d
Francisella tularensis	Tularemia	Low	A	2-10 d
Variola major	Smallpoxb	High	A	7-17 d
Clostridium botulinuma	Botulinum	High	A	1-5 d
Filovirus (e.g. Ebola, Marburg)	Viral hemorrhagic feversb (VHF)	High	A	4-21 d
Coxiella burnetii	Q fever	Low	B	10-40 d
Brucella spp.	Brucellosis	Low	B	5-60 d
Vibrio cholerae	Cholerae	Low	B	4 h-5 d
Shigella spp.	Shigellosisb	Low	B	1-7 d
Salmonella spp.	Salmonellose	Low	B	1-7 d
Escherichia coli O157:H7	STEC	Low	B	10 h-3 d
Ricin toxin		High	B	18-24 h

[a] The toxin is the biological threat agent.

[b] Contagious human-human.

[c] d, days; h, hours.

2.1 Assessment of threat and counteractions to biological agents need efficient detection and identification systems

In order to improve our understanding of the terrorist motivations to use biological and chemical weapons, a definition of the word "terrorism" has been proposed (7); "Terrorism is the instrumental use or threatened use of violence by an organization or individual against innocent civilian targets in furtherance of a political, religious, or ideological objective". This statement includes personal and apolitical motives, as well as the traditional intentions such as political or ethnical considerations, militant religious or apocalyptic groups, and the increasing interest and capability in the production of biological and chemical weapons (8). Many experts believe that biological agents may be more useful for obtaining panic and anxiety causing serious psychological impact instead of resulting in high numbers of casualties. The US senator Bill Frist stated at the World Economic Forum, Davos, January 2005, that "The greatest existential threat we have in the world today is biological" and that such an attack would occur at some time in the next ten years (9). The Canadian Press reported March 2005 that the military's intelligence arm has warned the federal government that avian flu may be a suitable biological threat agent. Newly emerging (e.g. SARS, Hendra, Nipah, avian flu) and re-emerging (e.g. West Nile, human monkeypox, multidrug-resistant *Mycobacterium tuberculosis*) pathogenic micro-organisms are of global concern and national preparedness plans are indispensable and some are already outlined (10, 11). Thus, there is a need for the development and production of vaccines, antivirals, and other therapeutics.

Reducing the threat from biological threat agents requires several actions including the improvement of preparedness and response. The design of efficient detection systems for early warning of potential threats is a central issue in the challenges posed by bioterrorism. The result of the avian flu outbreak in ten Asian countries killing around 50 million chickens has clearly showed the need for establishing rapid molecular diagnostics for mass screening of the virus flu carriers in order to improve public health responses to such an outbreak (12). Early detection to a release of biological agents will decrease the infectious rate and the people exposed (Figure 2.1). By the time the clinical symptoms have emerged, it might be too late for

treatment. However, in some cases, antibiotics may be effective as post-exposure prophylaxis, but this treatment needs to start before the onset of symptoms. Vaccines and other post-treatments are available but these must be administered promptly. Vaccination within 2-3 days of exposure to smallpox is required in order to be protective against the disease.

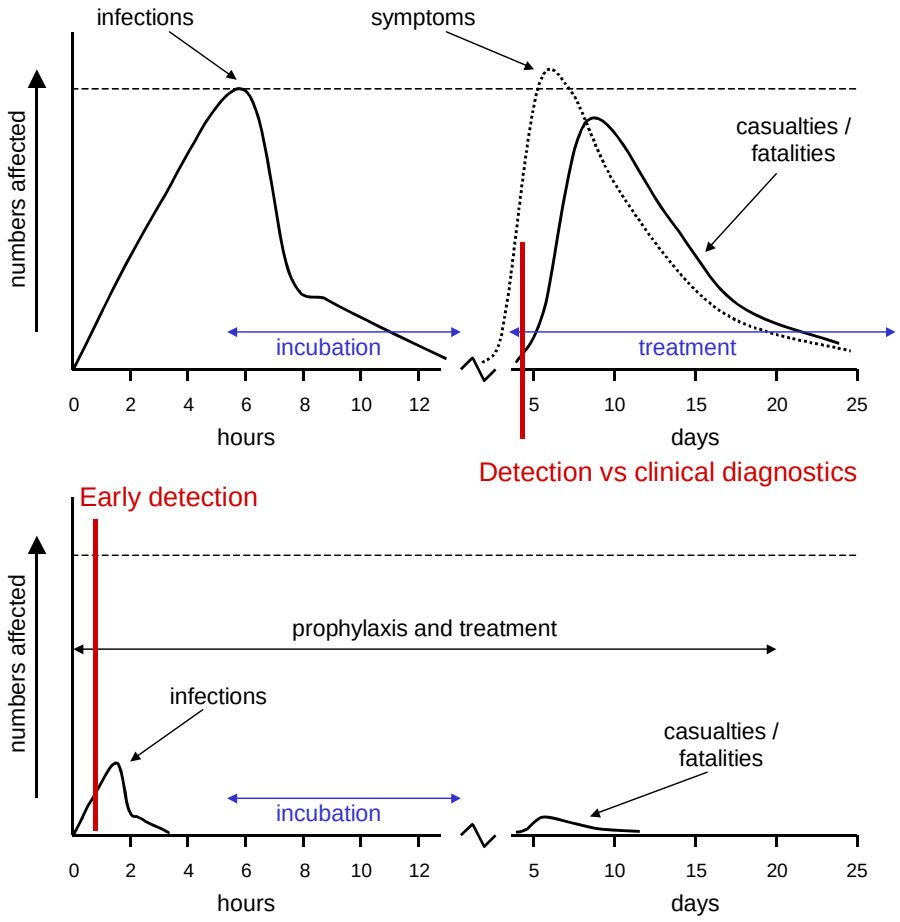


Figure 2.1. Early detection reduces the number of infected individuals and casualties. The figure is modified after reference 13.

Biological agents may be difficult to detect and identify quickly and reliable. It is worth noting that there is a distinction between the terms “detection” and “identification”. The establishment of the presence or absence of a biological agent is termed detection. Identification is the determination of the precise nature of the biological agent. The identification system is usually

dependent on specific signatures of the micro-organism used. The Rajneeshee Cult had deliberately released *Salmonella typhimurium* at salad bars and supermarkets in Oregon, USA, 1984, causing an outbreak of salmonellosis where 751 people fell ill. Even though four days were used to identify *S. typhimurium* as the outbreak strain, it took more than a year to identify and confirm that only a single strain of *S. typhimurium* had been used (in addition to the confession by one of the cult members about the deliberate release) (reviewed in 2, 4). This illustrates that in some cases, identification may be time-consuming again emphasizing the need for rapid and reliable identification techniques.

3 DETECTION OF BIOLOGICAL THREAT AGENTS

Detection may be based on environmental and epidemiological monitoring. Some detection devices have been developed but the systems are often limited due to speed, sensitivity, and specificity. Many systems can only detect, and not identify the biological agent. Stand-off detection, as LIDAR (Light Detection and Ranging), is used for detecting potential threat clouds and is regarded as a detect-to-warn system (14, 15). The short range LIDARs can detect at a radius of approximately five kilometres from the instrument. Most LIDARs use UV (UltraViolet) radiation at wavelengths 266 nm or 355 nm. At these wavelengths biological material will fluoresce. The wavelength 266 nm excites fluorescence from the amino acids tryptophan and tyrosine in the bacterial cell, while 355 nm excites fluorescence from the co-factor NADH (Nicotine Adenine Dinucleotide, reduced form) associated with cell metabolism. UV excitation may also fluoresce fuel oils, diesel, and agrochemicals causing false alarms. LIDAR is not sufficiently operative during full daylight and needs good environmental conditions.

Fluorescence do not have the ability to identify the biological agent, it can only separate it from other materials in the atmosphere. Point detectors need to be surrounded by biological aerosols/particles in order to characterize their shape and size. MALDI-TOF-MS (Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy) may be used for detecting biological compounds in air (16). As the particles are collected and passed through scattering laser beams spectra based on the ionization results are achieved. The spectra for

each type of biological materials are unique based on the composition of various lipids, proteins, and oligosaccharides of the micro-organism. Other biodetection techniques are LSSPR (Light Scattering Surface Plasmon Resonance) and Raman spectroscopy in conjunction with nanoparticles (17, 18, 19). In general, different subunits, and specific biological compounds and molecular signatures (e.g. antibodies, DNA fragments), are combined in order to improve the selectivity and sensitivity of these nonspecific detection systems. In any case, further identification of the agent is usually needed. FFI has previously published an overview of various biosensors used for identification of biological threat agents and toxins (20).

Even though biological agents are detected, it is necessary to determine the living state and the viability of the agent. Biological agents may be present as vegetative cells, spores or in a dormant state (viable but non culturable state; VBNC). The substance adenosine 5'-triphosphate (ATP) is found in all living cells (not viruses) and may be detected by using a luciferin-luciferase assay. Such bioluminescence assays may be used for enumeration of the bacterial population. Further improvements of this assay have been outlined in order to separate bacterial ATP from nonbacterial ATP (yeast, somatic or free ATP), and to detect spores. Spores are deficient in ATP and a germination step is required before performing the bioluminescence assay (21, 22). Various growth medium and germinant molecules are able to promote spore germination and several such studies have been outlined for *B. anthracis* spores (23, 24). The bioluminescence assay has further been combined with specific phage associated lytic enzymes for specific identification of bacteria (25).

False positives (i.e. alarm, but no agent) may arise when the biological detector device respond to detect an interfering substance in the sample which is not the actual biological agent, such as contamination. Also, genetic engineering allows the construction of biological agents resembling biological threat agents causing false alarms. False positives may lead to a severe economic impact on the response system and needless panic. If a biological agent exists, but below an instrument's treshold value for detection, a false negative may occur. Thus, the detection experiments, including valuable internal controls, need to be carefully designed.

4 IDENTIFICATION OF BIOLOGICAL THREAT AGENTS

There are several methods available for identifying biological threat agents. Many bacterial threat agents occur naturally, and some may be closely related to other bacteria found in the environment. Thus, it is necessary to distinguish between terrorist events, naturally occurring outbreaks, and background levels. Various identification methods basically include conventional culture-based methods, immunoassays, and molecular methods such as nucleic acid amplification (reviewed in 26, 27, 28). The cultivation of bacteria in selective growth medium allows identification at the genus- and to a certain extent at the species level. This method can detect many agents at sub lethal levels, but toxins are not identified. Colony morphology, antibiotic sensitivity, and biochemical reactions are determined by these methods. However, the major drawbacks of these methods are that they are time consuming and require the presence of viable cells. Growth of cells in a dormant state may be triggered by specific environmental factors. Culture-based methods do not require substantial investments in technology or equipment, but trained personnel are needed.

4.1 Immunological methods

Immunoassays include the use of specific antibodies targeted against a toxin or a particular antigen at the surface of a bacterial cell or spore. Immunological methods usually provide quick results and are suitable for fast screening of a large number of samples. However, the method is less specific and sensitive, and the detection limit may be a 100 –1000 -fold higher than the infectious dose (28, 29). A false positive may occur due to cross-reactivity of the antibody to similar antigens on the surface of other related bacterial cells. Antibody specificity and affinity are the limiting factors of immunoassays. Some immunological devices are commercially available such as the BioVeris detection system (30), Meso Scale Discovery Sector PR (31), and Luminex 100 (32). In general, immunoassays are good for presumptive detection but more specific tools are needed for further verification.

4.2 Molecular-based methods

The use of molecular-based methods (nucleic acid detection), in particular real-time polymerase chain reaction (PCR), enhances the monitoring strategies by increasing the sensitivity, specificity and reproducibility, and decreases the analysis time. Various nucleic acid detection assays and devices have been reviewed (26, 27). PCR is a common method of creating copies of specific DNA fragments and amplifies the DNA target region within a few minutes in a thermocycler. The PCR amplification process requires available DNA, thermostable DNA polymerase enzyme (e.g. *Taq* polymerase), nucleic acid primers, dNTP (deoxyribonucleotide triphosphate), and specific PCR buffers containing Mg^{2+}. The concentrations of primers, DNA, and Mg^{2+} usually need to be optimized for each PCR assay. One PCR cycle consists of three steps (Figure 4.1). This amplification technique is frequently used in diagnostic assays and can be used to detect single nucleotide polymorphisms. PCR may also be used for measuring DNA concentrations at very low levels (Figure 6.1).

The increased sensitivity of PCR assays also makes the system sensitive against impurities causing false positives. Therefore, well-designed PCR experiments including internal controls are needed to overcome such false positive signals. Internal controls may consist of either a plasmid or a DNA fragment in which the amplified DNA sequence is unique in the assay (33, 34, 35).

Detection and identification of the DNA target region in a PCR assay is commonly obtained by using a specifically designed fluorogenic nucleic acid probe. Fluorogenic 5’ nuclease TaqMan probes, molecular beacons labeled with different colored fluorophores, and fluorescent labeled hybridization probes (FRET-probes) are frequently used for real-time PCR analysis. Such probes are suitable for multiplexing PCR. However, probes are not necessarily needed for detection. The fluorescent dye SYBR Green I, which binds to the minor groove of the DNA double helix, can be used to measure the increasing amount of PCR amplified DNA. The particular amplicon may also be identified by its specific meltingpoint which is determined by the GC-content of the nucleotide sequence. Several real-time PCR assays have been outlined for a number of biological threat agents, and commercial kits containing the specific reagents are available. The target genes/regions for PCR identification are specifically chosen for each

micro-organism. Parts of the chromosomal *16S rRNA*, *rpoB, gyrA,* Ba813, and *vrr* genes/regions located on the virulence plasmid pXO1, and the *lef*, *cya*, *pga* toxin, and *cap* capsule genes located on the other virulence plasmid pXO2, are frequently used for identification of *B. anthracis*. *B. anthracis* cells lacking these plasmids will not be identified if the PCR assay is designed for targeting only plasmid-encoded regions. Thus, complete identification of *B. anthracis* requires the identification of both plasmids and a chromosomal marker since the plasmids may be lost from the bacterial cells or transferred between *Bacillus* spp.. Five unique regions of the *B. anthracis* chromosome have been identified suitable for PCR identification (36, 37). A new unique chromosomal marker has also recently been identified for real-time PCR identification of *B. anthracis* (tested on 45 *B. anthracis* strains and 62 other *Bacillus* spp. strains) (38). FFI has also identified and designed unique targets for *B. anthracis* identification (see section 6.1).

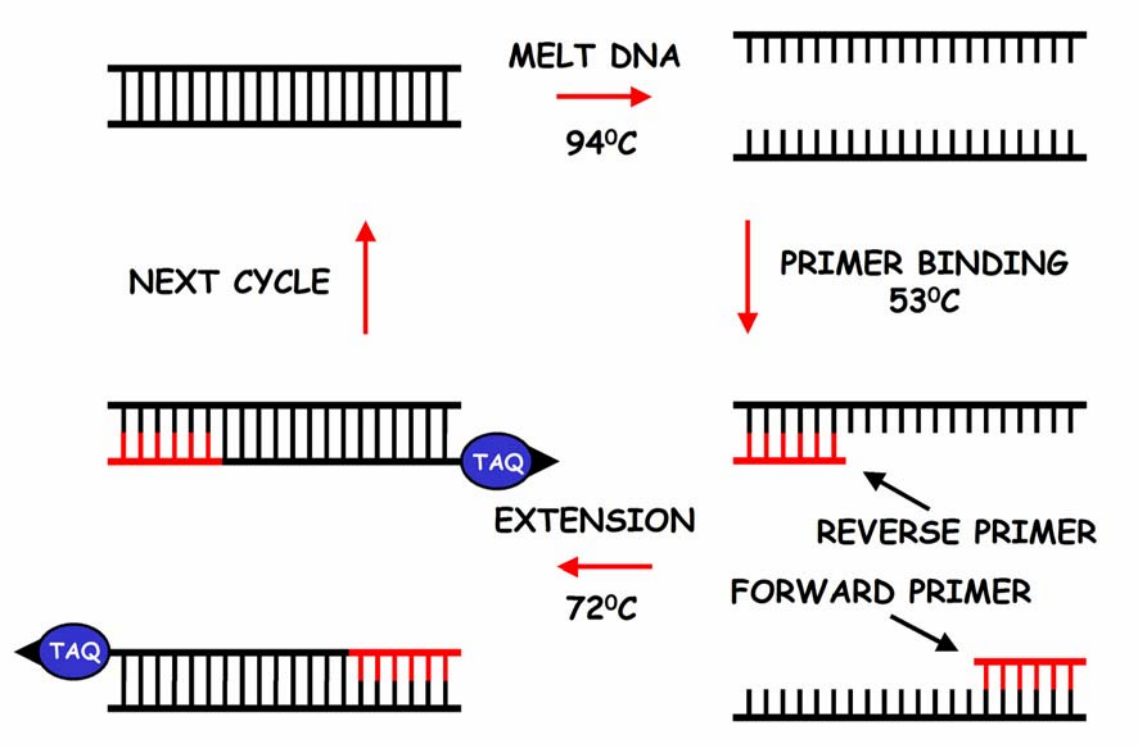


Figure 4.1. The scheme of PCR amplification. One PCR cycle includes three steps; i) Double-stranded DNA is denaturated by heating (94°C), ii) The primers are annealed to

the single-stranded DNA at a lower temperature (50 – 55°C), iii) The Taq DNA polymerase synthesizes a new DNA strand by adding nucleotides to the primer and makes a complimentary copy of the template strand (72°C).

Unculturable micro-organisms may be detected and analyzed by using PCR. It is generally believed that only 1-10% of the micro-organisms in the environment can be cultivated. PCR can detect DNA from both viable and dead cells, but culture-based methods are needed for confirming the presence of viable cells. NASBA (Nucleic Acid Sequence-Based Amplification) is a method in which RNA instead of DNA is amplified. In contrast to PCR, the reaction assay is performed at one single temperature (41°C) and employs three enzymes; reverse transcriptase, RNaseH and T7 RNA polymerase (reviewed in 39, 40, 41). NASBA is able to detect very low levels of RNA and is more sensitive than reverse transcription PCR (RT-PCR). A 10^9-fold amplification level after five cycles is usually obtained with NASBA compared to 10^6 after 20 cycles with RT-PCR. NASBA can be used to detect viable cells since mRNA is detected and amplified. The design of specific primers, in which one of them contains the recognition sequence site for T7 RNA polymerase, is crucial for the NASBA assay. A similar careful design is needed for the molecular beacons containing a flourophore and a quencher at the 5' and 3' end, respectively. NASBA has been widely used for virus diagnostics, and only few reports describe the use of this technique for bacterial detection. This method seems to be well-suited for use in conjunction with liposomal-based biosensors, which has been developed for *B. anthracis*, *E. coli*, and *Cryptosporidium parvum* (42, 43, 44).

Viruses are commonly identified by PCR. An integrated virus detection system (IVDS) has been developed at Edgewood Chemical and Biological Center (ECBC), USA. Based on the physical characteristics (e.g. size and shape) of the virus, both known and unknown viruses are detected in a liquid sample (buffer). The IVDS detects the viruses by a gas-phase electronic mobility molecular analyzer consisting of an electrospray unit, a differential mobility analyzer, and a condensate particle counter (45, 46).

A.

B.

Figure 4.2. PCR devices at FFI. A, Smartcycler^{R} (47); B, Lightcycler^{TM} (49).

Several real-time PCR devices are commercially available, such as the Smart cycler^{R} (47), RAPID (48), and the LightCycler^{TM} (49) (Figure 4.2). Idaho Technologies and Smiths Detection have developed RAZOR and Bio Seeq, respectively, which are hand-held PCR based biological detection devices suitable for field tests (48, 50). PCR is suitable for automation and mass screening analysis. Miniaturization into a "lab-on-a-chip" system, i.e. microarray systems, containing specific gene or protein targets for certain biological threat agents have been developed to a certain extent. A microarray immunoassay for detecting ricin, cholerae toxin, staphylococcal enterotoxin B, and *B. globigii* (a model organism for *B. anthracis*) has been reported (51). ECBC and ANP Technologies have developed a multiplex hand-held protein microarray system (52), while Bruker Daltonik GmbH has constructed a microarray system using PCR (53, 54). There have been many attempts to develop biosensors based on electrochemics, micro-fluidics, high frequency, and optics, combined with immuno- and molecular-based assays, but only few are commercially available (reviewed in 20, 55).

A complete and reliable identification method of biological threat agents requires simultaneously use of several assays. The results obtained by immunoassays are often confirmed by real-time PCR analysis (and *vice versa*). In order to detect viable cells, the samples are generally plated out on agar plates containing selective growth medium for the biological agents and incubated at a given temperature optimal for growth. The PCR assay usually requires disruption of the bacterial cells and spores in order to make the DNA available for amplification. Spore disruption is often obtained by chemical, mechanical, enzymatic, or thermal treatments. Thus, an efficient sampling preparation may often be needed prior to the PCR analysis.

4.3 Microbial forensics

Genomic sequencing of many biological threat agents (56, 57, 58) has provided important data about unique regions that may be used as specific targets (molecular signatures) for PCR identification. This progress has thereby improved the techniques for microbial forensics. For many bacterial agents, the genome of several strains of the same species has been sequenced providing insight into the genetic variation and global distribution of species and strains.

Classification of bacterial strains is often based on the identification of DNA polymorphisms. When the genetic diversity within a bacterial species is high, it is often adequate to sequence only a few number of DNA fragments in order to classify the strain. In contrast, strains belonging to more homogenous species, in which little sequence divergence has occurred, require the sequencing of very long DNA regions or the analysis of several loci with high mutation rates. VNTR (Variable Number of Tandem Repeats) is a linear arrangement of multiple copies of short repeated DNA sequences that vary in length and are highly polymorphic. The size of the DNA fragments containing VNTRs is measured by PCR. Most bacterial genomes contain several VNTRs and multi locus VNTR (MLVA) analysis has now proven to be a suitable tool for strain typing and for tracing back to the origin of the bacterial agent (59, 60). It has been proposed that VNTRs may provide a mechanism for environmental adaptation of the bacterial cell. The finding that many bacterial membrane protein-encoding genes contain tandem-repeats supports this hypothesis (61).

VNTR analysis was used to identify the *B. anthracis* Ames strain used in the October 2001 anthrax attacks in the US (62, 63). The Federal Bureau of Investigation (FBI) is now implementing the use of such molecular signatures in their molecular forensic studies (64, 65). The future construction of the US biodefense facility "National Biodefense Analysis and Countermeasureures Center" (NBACC) will include a new Bioforensics Analysis Center (NBFAC) which will be located in Ft. Detrick, MD. NBFAC will operate as a joint federal effort including Department of Homeland Security, FBI, and the Army.

5 ENVIRONMENTAL SAMPLING

One of the lessons learned from the October 2001 anthrax attacks in the US, was the need for adequate and standardized procedures for sample collection methods (66). There is a lack of knowledge in collecting environmental samples, in contrast to clinical samples that are often easier to process due to the routine analyses of such samples. The First National Conference on Environmental Sampling for Bio-Threat Agents was held in January 2005, Baltimore, USA, where important issues addressing processing of environmental samples were discussed. Efficient sampling is necessary in obtaining reliable detection and identification assays of

biological threat agents. The sampling method and the type of sample influence the collection efficiency, while the biological agents affect the sensitivity of the detection and identification methods. Studies including the use of various swab materials and *B. anthracis* spore recovery from non-porous surfaces have now been outlined (67, 68). Environmental samples, such as soil, contaminated water, and powder, often need a clean-up step before PCR is used for further identification. An effective sample preparation may reduce the presence of false negatives since impurities (left overs from the sample) may inhibit the PCR assay.

5.1 Bioaerosols

Bioaerosols are collections of biological material in ambient air. The microbial diversity in air is complex, consisting of infectious agents (such as fungal and bacterial spores), mycotoxins, and other non-infectious agents. In order to monitor and characterize these aerosols, an efficient air sampler device is needed. In particular, the particle size and the air flow rate determine the choice of sampling device. Bioaerosols with aerodynamic equivalent diameters of 5 μm or less usually remain in the air for extended periods of time. The settling rates of bioaerosol particles depend on physical (particle size, density, shape) and environmental (temperature, humidity, electrical) factors. With a flow rate of 450 l/min, the impinger SpinCon® air sampler collects particles in the range of 0.2-10 μm, thereby including viruses, into a liquid. OMNI 3000 is an improved air collector based on the SpinCon® technology but with a slightly lower flow rate (300 l/min) collecting aerosols above 1 μm (Figure 5.1). Impaction samplers deposit the bioaerosols onto a solid agar surface used for culture-based analysis. The detection and identification of airborne micro-organisms is commonly performed by PCR analysis (reviewed in 70). An autonomous detection system that performs aerosol collection, sample preparation, multiplex immunoassay, and real-time PCR assay for *B. anthracis*, *Y. pestis*, and botulinum toxin has recently been described (71).

A.

B.

Figure 5.1. Air sampler collectors. A, SpinCon®; B, OMNI 3000 (69). (Note that pictures are not in scale).

6 ONGOING PROJECTS AT FFI

Various research projects regarding detection and identification of biological threat agents at FFI are described in sections 6.1 and 6.2. Section 6.3 provides a brief overview of other closely linked projects addressing different bioterrorism issues.

6.1 Molecular-based identification methods

FFI has developed and established real-time PCR assays for identification of the biological threat agents *B. anthracis*, *Y. pestis*, *C. burnetii*, *F. tularensis*, *B. melintensis, V. cholerae,* and Vaccinia using either the fluorescent dye SYBR Green I or fluorescent labelled DNA probes using the LightCycler ™ (Figure 4.2). The templates used were inactivated agents from Dugway Proving Ground, USA, and the DNA probes were specifically designed for each biological agent. The real-time PCR assays were optimized for amplifying the *16S rRNA* gene and specific target genes/regions for each agent. This work has recently been published (72). The *16S rRNA* gene is highly conserved among bacterial species, but can be used to discriminate between Gram-negative and Gram-positive bacteria (73). However, species-specific primers and probes may be designed for targeting definite regions (intergenic spacer, variable region) of the *16S rRNA* gene (74). At FFI, the Ba813 chromosomal region was used as one of the targets for identifying and quantifying to a certain extent *B. anthracis* (Figure 6.1) (see reference 72 for extensive reading).

FFI has developed a rapid sonication method for lysis of Gram-positive bacteria without the need for additional lysis reagents. This method reduces the time needed for sample preparation prior to real-time PCR assays (75). In these studies, *B. cereus* was used as a model organism for Gram-positive bacteria. Results showed that maximum yield of DNA was obtained after 3-5 minutes of sonication and that the yield of DNA was dependent on the growth phase of the bacterial cell culture.

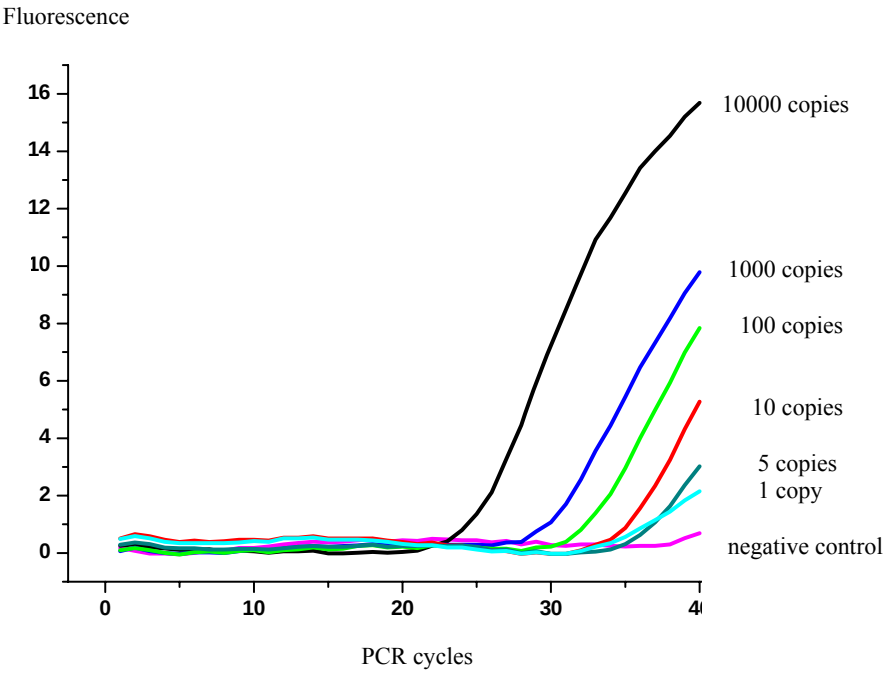


Figure 6.1. Identification of B. anthracis by real-time PCR (LightCycler™) using Ba813 specific primers and probe (72).

One of the ongoing projects at FFI is to characterize a set of strains belonging to the *B. cereus* group by using the molecular methods MLVA and MLST (Multi Locus Sequence Typing) (see section 4.3). This work is within the frame of WEAG/CEPA13 involving six nations; Sweden (lead nation), France, Germany, Italy, the Netherlands, and Norway. The major goals are to develop molecular markers and assays for bacterial strain typing of various biological threat agents (*F. tularensis, C. botulinum*, *Y. pestis*, *C. burnetti*, *Burckholderi* spp., *Brucella* spp., *B. anthracis*, and *B. cereus*), and to construct an internet-based database to easily compare new data with existing reference data. Our results show that *B. anthracis* strains can be grouped separately from closely related *B. cereus* strains (unpublished). Furthermore, current research work using MLVA for characterization of *V. cholerae* is in progress.

FFI is also using NASBA (see section 4.2) in order to detect and identify viable *B. anthracis* and *V. cholerae* cells. Specific primers and molecular beacons have been designed for both conserved and unique chromosomal encoded genes. Bacterial cells are harvested in exponential phase and the nucleic acids are extracted by Booms method using the NucliSens (Organon Teknika) isolation kit. Booms method includes the use of silica particles that binds to nucleic acids in the presence of guanidine isothiocyanate. Preliminary results show that both *B. anthracis* and *V. cholerae* can efficiently be identified in both laboratory and environmental samples by NASBA (unpublished).

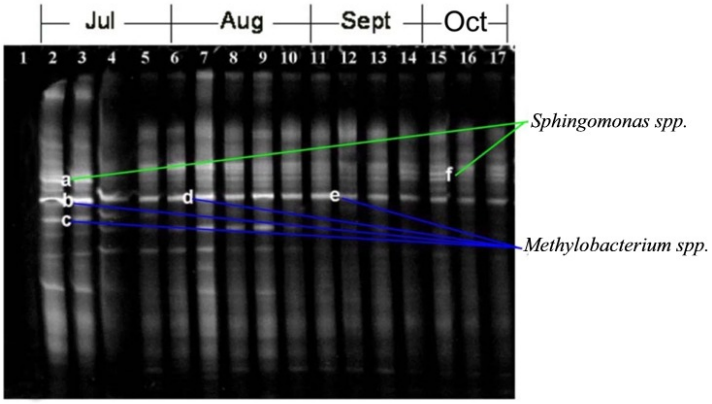
FFI is establishing and implementing molecular PCR-based methods for identifying pathogens in food and water samples. This project is in collaboration with the Norwegian Armed forces.

6.2 Identification of bioaerosols

The threat of an airborne release of biological threat agents has increased the importance of detecting and identifying bioaerosols. FFI is involved in monitoring the microbial diversity in outdoor air to characterize various micro-organisms present during seasonal variations, and to develop reliable identification methods of biological threat agents in complex air samples. The knowledge of the biological atmospheric background is important in turn of constructing specific primers and probes for molecular identification of biological threat agents. Some primers and probes may cross-react with other similar microbial strains resulting in false positives.

A.

B.



C.

Figure 6.2. Detection of bioaerosols. A, Air sampling at FFI using SpinCon®; B, DGGE analysis of the PCR amplified 16S rRNA fragments of bacteria in air; C, Growth of bacteria (left) and fungi (right) on R2A and MEA growth medium, respectively.

Air samples were collected with SpinCon® (Figure 6.2 A). The bacterial diversity was characterized by amplifying the *16S rRNA* gene from the microbial community of the sample using general *Bacteria* primers and the PCR products were separated by DGGE (Denaturing Gradient Gel Electrophoresis) (Figure 6.2 B) (76, 77, 78, 79). Aliquots of the air samples were also plated out on growth medium for bacteria and fungi (Figure 6.2 C). The *16S rRNA* gene is frequently used to identify bacteria at the genus level and is often used to study bacterial communities in different environments (76). Furthermore, the air samples were spiked with inactivated biological threat agents and successful specific real-time PCR analysis was obtained directly on crude samples (i.e. no lysis reagents/methods and DNA extractions were performed) (80). FFI will in short time receive the OMNI 3000 air collector (Figure 5.1) from Sceptor Industries, Inc., for testing, performance of comparison studies, and evaluation regarding future research activities including air sampling.

6.3 Additional projects addressing bioterrorism

FFI is a national center for research in protection against biological, radiological and nuclear weapons, and *the* center for research in counter measures of chemical weapons. FFI is involved in various national and international collaboration projects regarding CBRN (Chemical, Biological, Radiological, Nuclear) issues, in particular within WEAG (Western European Armament Group), NATO (North Atlantic Treaty Organization), and ANNCP (Anglo Netherlands Norwegian Collaboration Project).

In addition to the projects described in sections 6.1 and 6.2, FFI is also working with bioterrorism issues including i) assessment of biological threat perspectives, ii) biodefense and counterterrorism, iii) analysis and validation of defined biological scenarios, iv) risk analysis and assessments, and v) modelling and simulation. FFI provides consultancy and recommendations for improvements of preparedness and response for the Norwegian Armed Forces and the civil sector. Future activities will include further detailed analysis of the dispersion of biological weapons and transmission of biological threat agents. FFI also develops and tests modern protective clothing and equipment for soldiers that may be exposed to chemical and biological agents.

FFI is heavily involved in supervising students (graduate/MSc/PhD), giving lectures, teaching, and training as a part of the military CBRN education and for different civil sectors (e.g. police, civil force, fire fighters, first responders). FFI is also involved in arranging workshops and conferences, such as "the annual Norwegian International Defence Seminar (NIDS). In 2004, the purpose of NIDS II was to address the threat from CBRN materials and weapons, and how to best optimize the security benefits from international cooperation, disarmament and national preparedness (81).

7 HOAX LETTERS IN NORWAY

After the 2001 anthrax attacks in the US, hoax letters containing white powders were sent to both public and private offices in Norway in October and November 2001. The city hall in Notodden received one of the letters, which resulted in seven people being sent to the hospital for check-up and prescribed antibiotics due to being in contact with the letter. The Norwegian Institute of Public Health analyzed the powder for the presence of *B. anthracis* with negative results. Since then, approximately 450 million NKr (~ 50 mill euro) have been used in supporting the Norwegian national preparedness to terrorism. In December 2004, the Norwegian embassy in Colombo, Sri Lanka, received a letter containing white powder. It has been stated that the powder did not contain *B. anthracis*. Norway is involved in the peace negotiations between the Tamil guerrilla and the Sri Lankan government. The Norwegian peace mediators have been criticized for being biased in this case.

8 CONCLUSION

Several detection and identification methods for biological threat agents have been developed, but there is still a need for sensitive, reliable, and fast-in-use instruments. Also, portable and hand-held devices are essential for use in fields. No current systems yet fulfil all the addressed requirements since there is generally a trade-off between sensitivity/specificity and speed of detection. Future challenges include the construction of consistent and prompt systems promising both sensitivity and specificity in order to respond more rapidly to reduce health risk and collateral damage. Culture-, immuno-, and molecular (PCR)-based methods are supplementary techniques needed in order to verify the presence or absence of a biological threat agent in a sample. The results obtained by one of these methods are usually confirmed by performing one of the other methods. Today, culturing of biological threat agents is the only way to detect viable cells, which is very time-consuming. Thus, other methods are needed for addressing this issue. Efficient sample preparations are usually compulsory for successful identification when using immuno- and molecular-based assays. An optimized identification assay requiring a minimum of sample processing is therefore highly desired. Future work includes developing sample protocols to include identification methods that reduce false positives and false negatives to avoid forged alarms and unnecessary panic.

FFI takes part in several of these actions to improve national preparedness and response, by establishing and improving detection and identification techniques for biological threat agents and being strongly involved in various issues concerning biological threat assessment. FFI is currently increasing its expertise in the dispersion of biological weapons and the transmission of biological threat agents. Without enough knowledge and understanding about these important concerned topics, the development of an appropriate and well-defined biodefense strategy may not be sufficiently obtained.

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