

Appendix E

Detecting and Monitoring Biological Agents

EXISTING TECHNOLOGIES

Microscopy

Microscopy is used to detect total microbial populations in a given sample without regard to the physiological state of the organism; both viable and nonviable organisms can be detected. Because classical microscopy relies on the recognition of morphology (size and shape), limitations of microscopy include lack of specificity and low sensitivity. The detection of submicroscopic viruses requires specialized instrumentation, such as epifluorescent or transmission electron microscopes (Stetzenbach, 1999).

Differential staining microscopic analyses are useful for segregating microorganisms into broad groups but cannot identify specific organisms. Differentiating target organisms from indigenous or background populations requires discriminating beyond the genus, species, and subspecies levels. By staining with fluorescent-labeled antibodies, target organisms can be detected, but the detection limits are generally greater than 10^4 cells/ml of liquid collection medium. Although microbial viability has been determined using special stains, the reliability of these methods has not been demonstrated. Multiple analyses combined with digital imaging can reduce variability, but interference from background particulates is a serious problem with all classical microscopic analyses.

Culture-Based Assays

Culture-based assays can only be used to detect organisms that will proliferate under the growth conditions of the analysis design. A successful culture depends on nutritional and environmental factors, the physiological state of the organism, and the absence of interfering substances. The best nutritional components in the culture media, incubation temperature, and atmospheric parameters (e.g., humidity and carbon dioxide) vary with the organism. Stresses on the organism during dispersal, transport, and collection contribute to the difficulty of detecting organisms using culture assays.

The analysis time depends on the organism, the growth medium, and the incubation temperature, but generally the appearance of classical bacterial colony formation requires about 18 hours. Fungal growth in culture often requires three to five days, and cell culture for viruses takes even longer. Once a colony forms, additional time is required for identification of the microbial populations. Detection limits vary greatly with the application of the sample to the growth medium. Filtration or centrifugation of the sample can improve detection, but they concentrate all of the organisms suspended in the sample, which can result in overgrowth of the targeted contaminant.

Biochemical and Immunological Assays

With biochemical and immunological-based analyses, the identification and enumeration of specific microbial contaminants in environmental samples has been improved. Generally, biochemical assays rely on substrates and computer-assisted analysis; immunoassays center on specific antigen-antibody recognition. When used sequentially with culture-based assays, specificity is increased. However, the analysis time is prolonged. Advances in nonculture-based immunoassays have improved specificity and sensitivity. Detection and identification of microorganisms has been improved by advanced biotechnology-based methodologies.

EMERGING TECHNOLOGIES

Polymerase Chain Reaction Amplification

Polymerase chain reaction (PCR) is an enzyme reaction that amplifies specific deoxyribonucleic acid (DNA) sequences to identify specific microorganisms or groups of organisms. This methodology does not depend on the physiologic state of the organism, but it requires that gene sequences specific to the targeted contaminant be known. PCR involves repetitive

cycles of amplification in which the gene sequences are copied, increasing the amount of DNA in the sample until it can be detected. The literature on PCR and its use in detecting biological organisms is growing quickly. The summary in the following paragraphs is based on studies conducted by Alvarez et al. (1995), Beyer et al. (1995), Buttner et al. (1997), Garner et al. (1993), Kuske et al. (1998), Rigler et al. (1998), Stetzenbach (1999), and Wu et al. (1997).

Nucleic acids have been extracted from microorganisms in a variety of ways. Hot detergent treatment, freeze-thaw, and bead mill homogenization have been used successfully to extract DNA from vegetative bacterial cells, endospores, and fungal conidia. Detection limits are affected by the physical condition and concentration of the target DNA, as well as by the presence and concentrations of background DNA in the reaction mixture. Pretreatment of samples may be necessary to minimize interference from biotic and abiotic material in the sample matrix.

Standard PCR involves using two unique primers to produce a single amplification DNA product. Multiplex PCR uses several sets of primers to produce multiple amplification products thereby increasing the specificity when the products are diagnosed. Reverse transcriptase PCR is used to detect ribonucleic acid (RNA) by generating a cDNA copy of the nucleic acids in a single-stranded RNA for the first cycle. The cDNA is then used as a template for successive PCR cycles.

PCR assay of soil has shown detection limits of 1 cell/g of soil. In milk, detection has been reported at 9 cells/ml, and in bioaerosol liquid collection, fluid detection was 10 cells/ml. A detection limit of 10^3 copies of plasmid carrying the *Bacillus anthracis* edema factor and 2×10^4 spores was improved by reamplification. Previously, post-PCR manipulation was required to detect the presence of amplified sequences, often by gel electrophoresis. Recently developed instrumentation has eliminated the need for post-PCR processing by the release of fluorescent-labeled probes to signal amplification. With computer-assisted comparisons to standard curves, microbial contaminants can be quantified rapidly. A detection limit of 1 to 10 template¹/PCR sample and 100 template for RT-PCR in a 96-well microtiter format has been reported within two hours of sample preparation. Combining PCR with immunological techniques has resulted in a rapid and efficient solution-phase hybridization of labeled targets and biotinylated capture probes. Results have been reported in two hours with a detection limit of 10 targets. Application of fluorescence correlation spectroscopy with PCR in microtiter plate format combines reagents for amplification and detection in a single tube or well. Double-stranded

¹ "Template" refers to the segments of nucleic acid being amplified.

target DNA is detected by two amplification primers 5'-tagged² with two different fluorophores (Rhodamine-Green and Cy5). This method has shown a detection limit of 10 to 25 initial copy number of template.

Microchips

Combining microchip technology and PCR improves detection (Belgrader et al., 1998; Ibrahim et al., 1998; Northrup et al., 1998; Waters et al., 1998; Wilding et al., 1998; Yershov et al., 1996). A microchip PCR array with 10 silicon reaction chambers, thin-film heaters, and solid-state optics provides real-time monitoring with low power requirements and no moving parts. Detections of *Erwinia herbicola* (vegetative cells), *Bacillus subtilis* (endospores), and MS2 (RNA virus) with a detection limit of 10² to 10⁴ organisms/ml within 16 minutes have been reported. Hybridization of fluorescent-labeled DNA on a microchip involves immobilizing an array of oligonucleotides into gel elements fixed on a glass plate. Several microchip elements are then analyzed simultaneously with a two-wavelength fluorescent microscope equipped with a charge-coupled camera.

Micromachined silicon high-efficiency reaction chambers (miniature thermal cycling chamber [MATCI]) with integrated heaters and simple electronics to control temperatures provides solid-state, diode-based detection for real-time fluorescence monitoring of product DNA. The MATCI, a briefcase-size instrument with rechargeable batteries, has detected single base-pair substitutions in orthopoxviruses (monkeypox, cowpox, camel-pox, and vaccinia viruses) and human genomic DNA and viral DNA.

With a combination of cell lysis, multiplex PCR amplification, and electrophoretic sizing on a monolithic microchip, amplified products were analyzed using a sieving medium for size separation and an intercalating dye for fluorescence detection. Electrophoretic analysis was accomplished in less than three minutes after PCR. A 4.5 μ l silicon microchip containing a series of 3.5 μ m "weir-type" filters spanning the flow chamber has been developed to minimize interference by separating target organisms from background media.

Molecular Beacons

The analysis of samples using nucleic acid probes that spontaneously undergo a fluorogenic conformational change when they hybridize with target fluorescent probes has been called "molecular beacons" (Tyagi and

² This has to do with DNA strand designations. 5' refers to one end of the nucleic acid.

Kramer, 1996). These beacons fluoresce only in the presence of a complementary target. Reactions are carried out in a sealed tube to minimize manipulation.

Electrochemiluminescence Immunoassay

This technology integrates equilibrium immunoassay with electrochemiluminescence (Grimshaw et al., 1997). The format involves a biotinylated antibody sandwich with a labeled N-hydroxysuccinimide ester of a ruthenium (II) tris-bipyridine chelate for detection. Streptavidin-coated paramagnetic beads capture the antibody-antigen-antibody sandwich complex. Detection ranges for human protein sequence are from 2.5 ng/ml to 2,000 ng/ml with an accuracy and precision of less than or equal to 15 percent; for mice, the detection range was 0.5 ng/ml to 200 ng/ml.

Biosensors

Immunoassay in conjunction with a flexural plate wave transducer membrane has been used for the detection of bacteria (Harteveld et al., 1997; Pyun et al., 1998). Current detection limits are relatively high (3.0×10^5 to 6.2×10^7 cells/ml). The incorporation of a 20 MHz piezoelectric quartz crystal sensor in a flow injection system with a polyclonal antibody detected 0.1 $\mu\text{g/ml}$ of staphylococcal enterotoxin B. However, inhibition was noted at concentrations greater than or equal to 10 $\mu\text{g/ml}$.

Mass Spectrometry

Gas chromatography-ion trap tandem mass spectrometry (GC-MS-MS) and conventional quadrupole GC-MS have been used to detect 3-hydroxy fatty acids (e.g., endotoxin and bacterial lipopolysaccharide in gram-negative cells), muramic acids (e.g., peptidoglycan in gram-positive and gram-negative bacterial cells), and ergosterol (fungal biomass) as indicators of the presence of microbial contamination (Kaufmann, 1995; Koster et al., 1996; Kraemer et al., 1998; Larsson and Saraf, 1997). Endotoxin and bacterial lipopolysaccharide present in gram-negative cells is diagnosed by 3-hydroxy fatty acids. The detection of muramic acids indicates peptidoglycan, which is present in gram-positive and gram-negative bacterial cells. Ergosterol is an indicator of fungal biomass. Electrospray ionization mass spectrometry can detect proteins as indicators of microbial contamination.

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry can identify gene sequences not readable using gel electrophoresis. MALDI has also been used for the detection of quasimolecular ions of

large organic molecules (up to several 100 kDa molecular mass), such as biopolymers (peptides, proteins, oligosaccharides, and nucleotides in the subpicomolar range) with an accuracy of 0.1 to 0.01 percent.

Flow Cytometry

Flow cytometry uses simultaneous measurements of light scatter to determine cell size and structure (Davey and Kell, 1997; Fouchet et al., 1993; Lange et al., 1997; Perez et al., 1998; Seo et al., 1998). The incorporation of fluorescence increases the capabilities of this technique to include quantitation of cellular components, antigen detection, and estimations of cell physiology. Instrumentation permits the measurement of 500 to 5,000 objects/sec with the results displayed in bivariate histograms. Staining techniques used with flow cytometry include fluorescent brighteners. An ultraviolet-excited fluorescent whitening agent (Tinopal CBS-X) with ethanol used as a stain for both vegetative cells and endospores was able to discriminate the target substance from background material.

The combination of flow cytometry and fluorescent *in situ* hybridization (FISH) increased detection by two orders of magnitude over culture-based assays, but detection below 10^2 cells is beyond the capabilities of currently available detectors. Immunomagnetic separation with fluorescent antibody-labeled beads and flow cytometry have also been used. This methodology has shown a detection of less than 10^3 colony-forming units for pure culture suspensions and 10^3 to 10^4 colonies of *E. coli*/ml in a mixed suspension with a one-hour analysis time. Immunomagnetic separation and flow injection analysis with amperometric detection has advantages over enzyme-linked immunoassay (ELISA) methods because only viable cells are measured rather than total bacterial concentrations, although detection limits can be high (10^5 cells/ml).

CURRENT DETECTION EQUIPMENT

Current biological detection systems are not as mature as chemical detection systems in terms of reliability, sensitivity, selectivity, speed, and portability. Current techniques for the detection of biological agents are based on the analysis and/or collection of aerosols. Point samples of soil or aerosol must undergo microscopy and culture methods for a definitive identification and count of the biological agent organisms present. The following subsections briefly describe the capabilities and limitations of these systems. The information for this review was provided by a number of sources: Ali et al. (1997), DoD (1997, 1998, 1999), IOM (1999), Jane's Information Group (1999), JSMG (1999), U.S. Army (1992, 1994),

U.S. Army and U.S. Marine Corps (1996), U.S. Army SBCCOM (1998, 1999), and U.S. Navy (1999).

Biological Integrated Detection System

The biological integrated detection system (BIDS) is a collection of components that provides mobile detection capability. The flexible BIDS design is intended to be easily updated as new technologies become available. The current BIDS includes a sampler that passes ambient air through a two-stage virtual compactor that concentrates aerosol particles. The BIDS consists of five major components: a vehicle, a shelter, auxiliary equipment, a power source, and a biological detection suite.

Interim Biological Agent Detector

The interim biological agent detector (IBAD) is a point detector system used to detect background changes indicative of human-made biological warfare attacks. IBAD is designed to provide point detection capability onboard combat ships as a near-term solution to the current lack of detection, identification, and warning devices. The IBAD is composed of a particle size sorter/counter, a wet cyclone sampler, a manual identifier, and an improved membrane colorimetric ticket (flow-through assay). The system is linked to visual and audible alarms located locally and in the ship's Damage Control Central. IBAD automatically detects real-time changes in environmental background for initial sample collection and alarm and provides agent identification within 20 minutes.

XM94 Long-Range Biological Stand-off Detection System

The XM94 long-range biological stand-off detection system provides long-range, large-area aerosol cloud detection and ranging and tracking capability. XM94 can detect aerosol clouds out to a range of 30 km, sometimes even 50 km. The XM94 uses light detection and ranging (lidar) backscatter to detect clouds but does not explicitly detect biological agents.

Nuclear, Biological, and Chemical Reconnaissance System

The nuclear, biological, and chemical reconnaissance system (NBCRS) is a lightly armored wheeled vehicle capable of detecting, identifying, marking, sampling, and reporting NBC contamination on the battlefield. The three-person NBCRS crew uses a sophisticated suite of nuclear and chemical alarms and detectors that have been integrated into the vehicle chassis. The crew can also collect samples for laboratory analysis.

EMERGING DETECTION EQUIPMENT

One critical component of effective defense is real-time, pre-exposure detection, discrimination, and identification of a biological threat. To address this requirement, agencies such as the Defense Advanced Research Projects Agency are focusing on the development of detection systems that are robust, unattended, real-time (less than one minute), highly sensitive (2 to 10 particles), as well as sensors that are small (less than 5 pounds) and low cost (less than \$5,000 per unit). The goal is to enable soldiers to detect biological agents on the battlefield in real time with no false alarms. However, no technology currently under development will meet these needs in the next five years.

BIDS Update

The chemical biological mass spectrometer (CBMS) might be integrated into the BIDS in the future. In this system, samples of air are passed into an infrared pyrolyzer where small particles are trapped and heated. Off gases are then analyzed by tandem mass spectrometry (Berry, 1998).

Joint Biological Point Detection System

The Joint Biological Point Detection System (JBPDS) Program is developing a common point-detection capability for individual service platforms. The detection suite will integrate an identifier, trigger, sampler, and detector for real-time detection and identification of biological agents. In less than 15 minutes, the suite will detect biological agents at levels below the level that would affect combat effectiveness. The JBPDS will increase the number of agents that can be identified by the BIDS and IBAD systems; decrease detection time; increase detection sensitivity; provide automated, knowledge-based, real-time detection and identification; and provide a first-time point-detection capability to the Air Force and Marine Corps.

Portal Shield Advanced Concept Technology Demonstration

This program is being set up to demonstrate the military use of an air base/port biological detection capability and develop operating concepts for that capability. It is anticipated that the program will also demonstrate biological agent identification and will develop three increasingly automated systems. The air base/port biological detection system should automatically detect biological aerosol attacks and generate NBC warning reports. The biological sensor will use Navy technologies and components

(e.g., Naval Medical Research Institute's hand-held assay tickets and several Navy IBAD components).

MAGICChip (Micro-Array of Gel-Immobilized Compounds)

The MAGICChip (micro-array of gel-immobilized compounds) detector is planned to simultaneously identify a vast number of biological threat agents, including bacteria, viruses, fungi, and toxins. Both pathogenic microorganisms and plasmid-associated toxin genes that might be inserted into otherwise innocuous microorganisms will be detected. Microorganisms will be identified via unique, microbe-specific sequences of (rRNAs) and other RNAs, as well as microbe-specific gene sequences. Future detector capabilities will include probing for bacterial virulence factors. Viral microchips will identify the type of virus and the viral strain and will discriminate pathogenic from nonpathogenic strains, which can differ by only a few nucleotides in specific genes.

ANALYTICAL METHODS AND A MASS SPECTROMETER LIBRARY

The mass spectrometry approach to the classification and identification of biological threat agents is another system that offers robust capability for speed, signature bandwidth, and specificity. Two universities are collaborating on the design and execution of signature measurements of threat simulants by mass spectrometry. The objective of this investigation is to develop the experimental chemotaxonomic methods and analytical strategy for the determination of biomarkers from simulant threat microorganisms and their constituents. Specifically, simulants such as *E. coli*, *B-subtilis*, *E. herbicola*, and MS-2 capsid protein will be characterized using single and tandem mass spectrometry systems and soft ionization techniques. However, none of these is operational and, even if a system becomes operational, it will not be available in the next five years.

Phosphor-Diode Laser Technology for Biological Agent Detection

The goal of this project is to develop a new reporter material for biological agent identification and incorporate this technology into hand-held and flow cytometer instruments. The approach uses submicron microspheres of upconverting phosphor material (upconversion is a two or three photon absorption process in the phosphor to produce emission frequencies in the visible region of the spectrum upon excitation with near-infrared light), as the reporter system and a single near-infrared diode laser as the excitation source in immunoassay formats. This system

has the potential for zero optical background, and hence improved sensitivity, because nothing in nature upconverts. The compact, reliable, electrically efficient laser source, combined with the availability of many spectrally unique phosphor colors, allows for a greater degree of multiplexing (simultaneous detection of multiple antigens) than can be achieved using conventional fluorescent reporters and more complicated detection systems. Compared to commercially available biosensors for clinical diagnostics, at least a two order-of-magnitude increase in sensitivity with very rapid response (less than five minutes) has been demonstrated in a prototype hand-held biosensor. In addition to increased sensitivity, false alarms caused by nonspecific binding, typically encountered in current devices, are expected to be reduced. Independent test and evaluation, significant gains in multiplexing, and transitioning to manufacturers for field applications are in progress. A fieldable, hand-held unit is expected in the next two to three years (Carrico et al., 1998).

Spore-Specific Phosphorescence

The focus of this research is on bacterial agents, especially on the spores of *Bacillus anthracis* and *Clostridium botulinum*. The objective is to investigate a novel technical approach involving the generation of bacterial spore-specific phosphorescence that would constitute a basis for detecting the viability and quantity of the bacterial spores of simulants of toxic biological agents (i.e., *Bacillus anthracis* and *Clostridium botulinum*). A phosphorescence-based sensor could be integrated with one or more inert matrices suitable for on-site and/or remote sensing of biological agents in liquid and aerosol modes in the sensitivity range of 100 spores or less.

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