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Current and Developing Technologies for Monitoring Agents of Bioterrorism and Biowarfare

Daniel V. Lim,* Joyce M. Simpson, Elizabeth A. Kearns, and Marianne F. Kramer

Department of Biology and Center for Biological Defense, University of South Florida, Tampa, Florida 33620

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* Corresponding author. Mailing address: Department of Biology and Center for Biological Defense, University of South Florida, 4202 E. Fowler Avenue, SCA110, Tampa, FL 33620-5200. Phone: (813) 974-1618. Fax: (813) 974-3263. E-mail: Lim@cas.usf.edu.

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INTRODUCTION

Recent bioterrorist events have emphasized the need to immediately detect and identify biothreat agents. Rapid, accurate identification of such agents is important not only to confirm that a bioterrorism event has occurred, but also to determine whether suitable measures should be implemented to protect public health. Clinical microbiology laboratories have access to the Laboratory Response Network, which is an organized system designed by the Centers for Disease Control and Prevention (CDC) to provide structured guidance for the detection, diagnosis, and reporting of biological threat agents (263; http://www.bt.cdc.gov/lrn/factsheet.asp).

The Laboratory Response Network is structured into four laboratory levels based upon testing capabilities and likelihood of handling biothreat agents. Level A laboratories are standard clinical laboratories which may handle specimens for likely biothreat agents as part of classic diagnostic analysis on hospital cultures. These laboratories are the first line of detection and need to be familiar with likely agents such as *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis*. Level A laboratories conduct diagnostic analyses as dictated by the CDC. Level B laboratories can confirm identification of suspicious isolates and are typically public health laboratories. Level C laboratories use molecular methods and typing procedures to further identify or confirm identification of species and strains of biothreat agents and are either typing laboratories or public health laboratories. Level D laboratories located at the CDC and U.S. Army Medical Research Institute of Infectious Diseases are able to do high-level characterization of biothreat isolates under tightly controlled conditions.

Various tests have been developed to detect and identify biothreat agents. Some of these tests were available before 11 September 2001; other tests have been developed since that time. Although many of these technologies claim to be rapid, accurate, and reliable, few have been extensively evaluated under field conditions. This review describes documented current and developing technologies for detection and identification of biothreat agents and addresses the challenges associated with detection in complex sample matrices.

BRIEF HISTORY

There have been many reviews written regarding the history, theory, and use of bioterrorism, biothreats, biological weapons, and biological warfare from the 14th century to today. The reader is referred to the following for additional information: Atlas (17), Christopher et al. (55), Hawley and Eitzen (115), Heden (117), Klietmann and Ruoff (160), van Courtland Moon (293), and Tucker (283).

One of the most well known early attempts to use biological agents was during the 14th century medieval siege of Kaffa (Feodosiya, Ukraine) (55, 305). The attacking Tartars (Mongols) catapulted dead and dying plague victims into the city in the attempt to spread the disease. There has been speculation that escaping Kaffa victims may have carried the plague (also known as black death, bubonic plague, or black plague) to other parts of Europe, thereby hastening the ensuing pandemic. An attempt was made in 1763 by the British at Fort Pitt in the Ohio River Valley to use blankets to transfer smallpox to Native Americans (55, 305). The fort had been afflicted with smallpox among the troops, and used linens from the infirmary were systematically dispensed to the neighboring Indian populations. There is also anecdotal reference of attempts to spread smallpox via infected British soldiers during the American Revolutionary War (1776 to 1781) and by contaminated clothing during the American Civil War (1860 to 1864) (55, 305).

The era of biological weapons was significantly advanced in the 20th century by modern microbiology and multiple international wars. The biological and chemical horrors inflicted during World War I resulted in the drafting of the 1925 Geneva Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases and of Bacteriological Methods of Warfare. However, many countries that signed the document did so with contingencies in the event of attack by a nonratifying entity and with the stipulation that the protocol did not prevent investigative research (http://www.state.gov/t/ac/trt/4784.htm).

Subsequently, Germany, Japan, the Union of Soviet Socialist Republics, and the United States initiated research programs to develop and refine biological weapons (55, 293). In 1969, under President Richard M. Nixon, the United States began dismantling its offensive biological weapon programs. Henceforth, all biothreat agent research programs in the United States were of a defensive nature, and the 1972 Geneva Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological and Toxin Weapons and on Their Destruction was developed and ratified (http://www.state.gov/t/ac/trt/4718.htm#treaty). However, several countries that signed the convention (notably the Union of Soviet Socialist Republics and Iraq) continued offensive research and production of biological agents as recently as the mid-1990s. Additionally, there have been increasingly more subnational terrorist and radical groups that have independently worked on offensive use of biological weapons since the mid-1980s to today (283, 285).

The Chemical and Biological Weapons Nonproliferation Program maintains an open-source database containing information on all known incidents worldwide involving chemical, biological, or nuclear materials since 1900 (285). This database is searchable, and annual summary reports of activity are available; however, large amounts of biological weapons material remain unaccounted for and, thus, there is a critical need to

develop methods for rapid, accurate detection of biothreat agents.

CHALLENGES TO DETECTION

The prospect of biological terrorism presents many challenges for detection platforms. Some challenges are unique to bioterrorism and others are common for all testing situations. Ideally, detection platforms should be capable of rapidly detecting and confirming biothreat agents, including modified or previously uncharacterized agents, directly from complex matrix samples, with no false results. Furthermore, the instrument should be portable, user-friendly, and capable of testing for multiple agents simultaneously. Although several detection platforms exhibit many of the desired characteristics, no one system satisfies all of the criteria.

Detection assays must be sensitive and specific, capable of detecting low concentrations of target agents without interference from background materials. Although many chemical detectors can detect chemical agents at levels that pose a risk to human health, biological detectors can only rarely detect microorganisms directly from samples at or below human risk levels because of their lack of sensitivity.

In general, nucleic acid-based detection systems are more sensitive than antibody-based detection systems. The PCR assay can detect 10 or fewer microorganisms in a short period of time (23, 95, 133). However, PCR requires a clean sample and is unable to detect protein toxins and other non-nucleic acid-containing analytes such as prions. Furthermore, cultures of the target organism are not available for archiving and additional tests after PCR analysis.

Specificity is as important as sensitivity in the detection of biothreat agents. High specificity is important to minimize background signals and false-positive results from samples that are often complex, uncharacterized mixtures of organic and inorganic materials. Specificity can be affected not only by humics and other background particles, but also by high concentrations of competing antigens and DNA. In the case of PCR, its high sensitivity can also be a major weakness because contaminating or carryover DNA can be amplified, resulting in false-positive results.

In addition to sensitivity and specificity, reproducibility is an important requirement for detection platforms. Detection platforms that do not provide reproducible results are unreliable and may exacerbate a terrorist event. Many factors can affect the repeatability of bioassays, including the stability and consistency of reagents and differences in assay conditions. These variations can often be reduced by standardizing assay conditions and procedures.

Detection platforms must be capable of detecting a variety of biothreat agents in samples. This multiplex capability is vital because suspect samples may contain toxins, bacteria, viruses, or other types of analytes. In some instances, known biothreat agents may have been deliberately altered through genetic, antigenic, or chemical modifications or may represent new or uncommon variants of known microorganisms. Such modifications can make detection of a biothreat agent difficult.

Even without modifications, conventional biothreat agents are difficult to detect in complex sample matrices. Samples such as human specimens (for example, blood and stool), powder, food, water, and even air present challenges for detection assays. Anticoagulants, leukocyte DNA, and heme compounds in blood inhibit PCRs (97, 206). Lipids in ground beef and high numbers of background bacteria in stool specimens interfere with immunoassays. For this reason, target analytes usually must be isolated or purified from such samples prior to analysis and identification. These steps can add hours or days to detection protocols and often cannot be performed in the field. A further complication is that some viable microbes may not be culturable or may require specific nutritional requirements for culture.

An important consideration in biodetection is the collection and handling of samples. Issues associated with sampling include the type of material to be tested, the collection procedure, and sample transport. Air-borne and water-borne samples generally must be concentrated from large volumes to detect low levels of target analytes. Air-borne samples must also be extracted to a liquid because most detection platforms process only liquid samples. The efficiency of recovery from concentration and extraction procedures can vary and affect detection limits. Sample size, number, and distribution should be considered, as well as the transport time and method, particularly for fastidious, living microbes that may require specific environmental and nutritional conditions for survival. In some instances, confirmation of microbe viability may be important to ascertain whether the microorganism poses a health threat.

SAMPLE MATRIX PROCESSING

Sample Processing

Conventional culture and staining techniques are currently the gold standard for isolation, detection, and identification of target biothreat agents. These culture and isolation methods are based on the ability of healthy bacterial cells to multiply in nutrient-rich medium containing selective and differential agents that inhibit the growth of nontarget organisms and differentiate target from nontarget organisms. Use of culture enrichment and selection, however, results in lengthy assays, which can take days for preliminary results. Rapid detection methods replace the selective and differential culturing steps with DNA hybridization, nucleic acid amplification, antibody agglutination, and/or enzyme immunoassays. The majority of these rapid detection methods are suitable only when the biothreat agent is present in high numbers and/or in the absence of interfering substances.

In most cases, rapid detection methods require steps to grow or concentrate the target biothreat agent and/or purify the target analyte from the sample matrix prior to rapid detection. For example, PCR and nucleic acid sequence-based amplification (NASBA) enrich a single specific DNA or RNA sequence up to 10^6-fold in 20 min to a few hours and theoretically have a sensitivity of a single bacterial cell. These methods give rapid, specific detection but are limited by small sample volumes (e.g., 5 μl). In addition, substances such as bile salts, polysaccharides, heme, and humic acids in sample matrices inhibit enzymatic reactions required for nucleic acid amplification (236). Low levels of target analyte in samples require concen-

tration and/or cultural enrichments to provide sufficient target for amplification.

Methods for Sample Processing

Ideally the method to separate, concentrate, and purify the target biothreat agent should be universal, utilizable for all samples for all types of target analytes. In addition, the sample preparation method should be capable of rapidly removing the sample matrix that could inhibit detection capabilities and concentrating the analyte. The majority of current sample preparation methods can do neither of the above. The preparation procedure is usually limited to specific types of samples and is generally time-consuming and sometimes labor intensive. In addition, the concentration/purification method should ideally maintain cell viability so that, upon positive detection, the target organism can be cultured for confirmation of viability, further characterization and archived as evidence in the case of a criminal investigation.

Many sample preparation methods are currently under investigation. These methods include chemical, physical, and biological manipulation of the sample and are described elsewhere (25, 236, 272). Many of these methods have been developed for foods because of the historic need to test food for the presence of pathogens. Therefore, the majority of the methods described in this review will be for food sample preparation, but it would be possible to modify many of these methods for other types of sample matrices. This discussion will focus on rapid and/or automatic sample preparation methods for various solids, liquids, and aerosols and will include methods that are commonly used for detection of biothreat agents in samples such as blood, powder, and air.

Centrifugation. Centrifugation is a method that has conventionally been used to concentrate and recover microorganisms from liquid samples. Centrifugation times can vary from 30 seconds to 1 hour depending on the type of sample and the number of wash steps. Undesired sample debris may also be concentrated during the process and the centrifugation process cannot be easily automated. *Escherichia coli* O157:H7 cells have been recovered directly from a ground beef/buffer suspension by a 5-minute differential centrifugation step that separated the suspension into three distinct layers (69, 71). The middle layer containing the majority of the target cells was used for rapid detection of *E. coli* O157:H7 with an evanescent wave fiber optic biosensor. The efficiency of *E. coli* O157:H7 cells recovery from the ground beef by this procedure was not determined. Buoyant density gradient centrifugation has been used to separate and concentrate *Yersinia enterocolitica* in meat fluids from pork (311). This centrifugation procedure removed dead cells, and the concentrated samples contained only viable cells that were then used directly for PCR. Buoyant density centrifugation has also been successfully used to separate and concentrate bacteria from food in a one minute procedure (184, 185).

Filtration. Filtration can be used to separate microorganisms on the basis of cell size. Although liquid samples can be rapidly forced through filters of different pore sizes, sample debris can clog filters and retain bacteria. Removal of bacteria from filters following filtration can also be difficult. The commercially available Iso-Grid (Neogen Corp., Lansing, MI) is a dual filtration method developed for food products (227). Prefiltered food is passed through a 0.45-μm filter, which is then placed on an agar plate. Bacteria captured on the filter are grown and detected using selective and differential media. Iso-Grid filtration for *E. coli* O157:H7 has been approved by AOAC International, AOAC Official Method 997.11 (14, 88).

FTA filters (Whatman, Springfield, KY) have been developed for rapid isolation of nucleic acids from environmental, clinical, or food samples. Samples are added directly to the filter. The filter can then be washed and the nucleic acid remains bound to the filter. The filter is then ready for processing or long term storage at room temperature. FTA filters have been used for the detection of *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus megaterium* spores using nested PCR. The reported sensitivity of this method is 53 spores in the first round of PCR, and 5 spores after the second nested PCR round (169). The preparation of template DNA from biothreat agents such as *Bacillus anthracis* spores using FTA filters should be similar although samples such as powder have not yet been tested.

Dielectrophoresis. Dielectrophoresis takes advantage of the intrinsic dielectric properties of bioparticles to enable separation of particles in nonuniform electric fields. In dielectrophoresis, a nonuniform electric field consisting of positive and negative dielectrophoretic forces is generated by microelectrodes in a small chamber. Different bacterial cells and other components in the sample can be separated based on each particle's effective conductivity. Particles are released from regions near the electrodes when the dielectrophoretic response of each particle changes from attraction to repulsion from such regions. Different species of bacteria have different cell wall structures and compositions, and these differences give rise to large differences in particle conductivities. Changes in the physiological state of the cell will also affect particle conductivities. Dielectrophoresis has been incorporated into a microfabricated bioelectronic chip and used to separate *E. coli* from the sample (54, 315). Dielectrophoresis has also been used to clean target cells by removing inhibitors (228) and to isolate parasitized cells (98). Insulator-based dielectrophoresis has been used to isolate and trap *E. coli* and vegetative cells of *Bacillus* species into two distinct locations (170).

Immunomagnetic separation. Antibodies also can be used to purify and concentrate target biothreat agents. Magnetic particles (beads) with antibodies immobilized on their surfaces can bind to target cells, toxins, or other molecules found in samples. The magnetized particles are then collected using a magnetic field. Sample debris and nontarget organisms and molecules are removed by washing. The particles are released when the magnetic field is removed. The remaining solution contains mainly concentrated cells or molecules of interest that have bound to the antibody attached to the magnetic particle. This antibody-based method of cell separation is referred to as immunomagnetic separation. Immunomagnetic separation has been used to isolate and concentrate *Listeria monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 from stool and food samples (131, 148, 243, 267, 291). *Cryptosporidium* spp. have been successfully isolated from water samples using immunomagnetic separation (41), and the method is a component of U.S. Environmental Protection Agency Method 1622 and Method 1623 for the recovery and identification of *Cryptospo-*

ridium spp. and *Giardia* spp. in environmental waters (289, 290). Dynabeads (Dynal Inc., Oslo, Norway) supplies immunomagnetic separation beads that are functionalized for covalent linkage to antibodies or peptides that target biothreat agents. Beads with protein A or G linkage for noncovalent attachment of antibodies to the beads are also available.

Immunomagnetic separation has been used in a field-deployable automated electomagnetic flow cell fluidics system (Biodetection Enabling Analyte Delivery System [BEADS]) to separate and concentrate pathogenic bacterial cells as well as nucleic acid. The BEADS system engineered by Pacific Northwest National Laboratory (Richland, WA) was designed specifically for processing environmental and clinical samples prior to biodetection. Porous Ni foam was used to enhance the magnetic field gradient within the flow path so that the immunomagnetic separation particles could be immobilized throughout the fluid rather than at the tubing wall (50–52). *E. coli* O157:H7 was recovered at 32% efficiency directly from poultry carcass rinse without cell growth and enrichment using the BEADS system. Commercially available antibodies targeted against the O and K antigens were used for capturing the *E. coli* onto beads, and the recovered beads were used for direct PCR amplification and microarray detection (50). As long as antibodies or other affinity-based molecules are available for attachment to the beads, immunomagnetic separation can be a versatile technique potentially available for the purification of target biothreat agents and their products from heterogeneous sample matrices.

Nucleic acid extraction. The efficiency of extraction and purification of nucleic acid (DNA or RNA) from cells or spores of potential biothreat agents influences the sensitivity, reproducibility, and accuracy of any nucleic acid detection method. Substances in complex sample matrices can inhibit hybridization and enzymatic reactions, degrade the nucleic acid, and reduce the efficiency of cell or spore lysis. In addition, spores must be disrupted (e.g., through sonication) prior to nucleic acid extraction. In large samples, the nucleic acid must also be concentrated, often over 1,000–fold, into a smaller volume appropriate for nucleic acid analysis. There are many kits commercially available for purifying nucleic acid (56). The following are a few examples of rapid nucleic acid purification methods that have been developed to purify and concentrate nucleic acid from complex sample matrices.

The Cepheid GeneXpert and Biothreat agent detection system (Cepheid, Sunnyvale, CA) integrates sample preparation, PCR, and detection into a disposable cartridge. An instrument automatically processes the cartridge, allowing sample preparation in fewer than 5 minutes and detection (four-color real-time PCR) in 25 min. No special skills are required to use the system. Large volumes (100 μl to 5 ml) of raw sample can be handled, and up to four targets can be detected simultaneously per cartridge. The system automatically sonicates and purifies the sample; PCR reagent is then added to the extracted nucleic acid, and the mixture is dispensed into a PCR tube (199). Swab extracts, serum, cerebrospinal fluid, urine, bone marrow, sputum, tissue, and whole blood samples have been tested with this system (21, 22, 199). *Bacillus anthracis* (Ames strain) spores have also been detected using the system (199).

Extraction of many types of samples for nucleic acid purification has also been automated using the MagNaPure compact or LC instrument (Roche Applied Science, Indianapolis, IN). Eight or 32 samples, respectively, can be purified in 1 to 3 hours. The purification process involves the binding of nucleic acid to surfaces of magnetic glass particles, extensive washing, and then elution of nucleic acid. The MagNaPure instrument has been used successfully to purify nucleic acid from stool (254, 312), serum (104), whole blood (91, 151, 201), urine and swab specimens (63), atherosclerotic tissue (210), sputum (237), cerebrospinal fluid, synovial fluid, and ticks (90), and powders (190).

Sample Matrix Processing

Sample matrices can be of different compositions and, therefore, may require different methods for processing. The following are examples of sample preparation methods and the impediments encountered for specific sample types.

Food. The vast variety of food types makes it difficult to develop a universal sample preparation method for food. Many current methods rely on the time-consuming enrichment culture for the growth of the target microorganisms in the sample. More rapid methods such as immunomagnetic separation require specialized equipment and/or skilled labor. Obstacles to preparation of food samples for testing using rapid detection protocols include the requirement that food volumes need to be large (25 g or ml) and homogenous. The presence of food components such as lipids, polysaccharides, acid, and salts will inhibit enzymatic reactions required for certain rapid detection approaches such as PCR. Low concentrations of the target pathogen within the large sample will also decrease sensitivity of detection if small volumes are required for rapid detection analysis.

In conventional methods, solid food is diluted in a buffer solution and is blended or homogenized to obtain a homogenous mixture. The homogenous mixture is then further purified or cultured prior to testing. A homogenous mixture must still be prepared even when a rapid detection technique is used. The target microorganism can then be concentrated from the mixture by methods discussed previously. Unfortunately, the most effective technique for obtaining large numbers of target organisms in food is growth of the organisms by culture enrichment. Rapid effective separation and concentration of target pathogens remain a barrier to the rapid detection of microorganisms in foods.

Water. Microorganisms and their by-products in drinking water and recreational waters are usually too dilute for direct measurements. Water samples generally must be concentrated to obtain the number of target microorganisms necessary for microbiological analysis. Detection of microorganisms or their toxins from water samples is difficult because the recovery efficiency is poor and/or substances that are inhibitory to the detection technique are present. Target microorganisms must be isolated and concentrated by several orders of magnitude from the water samples. Concentration is most often performed using ultrafiltration in which parasites, bacteria, viruses, and potentially some high-molecular-weight biotoxins are retained on the filter (76, 205, 289, 290). The material that has accumulated on the filter is removed, collected, and analyzed. Potential interferents such as chlorine, salts, humic acids, and indigenous bacteria may also accumulate on the filter

and inhibit rapid detection. Target microbial pathogens have also been recovered from the water sample using immunomagnetic separation (87, 192).

Human specimens. (i) Blood. Blood contains heme, erythrocytes, and other cells and biologically active components that can interfere with antibody- and nucleic acid-based assays. Removal of these components and labeling of the target pathogen in such a complex mixture are major hurdles that must be overcome when processing blood samples for rapid detection. Instead of removing the sample matrix, Spectral Diagnostics, Inc. (Canada), took advantage of the components in blood by developing a rapid whole-blood elimination test for gram-negative bacterial infection for patients in the intensive care unit. The U.S. Food and Drug Administration has approved the test to identify patients at risk for developing severe sepsis on admission to intensive care units. The test is performed directly on whole blood; separation of the target bacteria from the blood is not necessary. Endotoxin, if present, reacts with anti-endotoxin antibody in the test. The endotoxin-antibody binding reaction primes the patient's neutrophils, resulting in an enhanced respiratory burst in the presence of zymosan. Zymosan is an insoluble preparation of yeast cells and has been shown to activate macrophages via toll-like receptor 2. The release of oxygen radicals results in chemiluminescence in the presence of luminol. The magnitude of the priming influence is proportional to the concentration of the endotoxin/antiendotoxin antibody complex (246).

An integrated miniaturized biochip device that automatically integrates sample preparation with PCR and DNA microarray detection has been reported by Liu et al. (186). This automated device mixes the blood sample with immunomagnetic separation beads in a sample storage chamber using air bubbles in a phenomenon termed microstreaming. The target pathogen bound to the immunomagnetic separation beads is then pumped to a PCR chamber, where the pathogen is concentrated using the magnetic element of the device and separated from whole blood using a washing step. The sample is then ready for direct addition of PCR reagents to the PCR chamber (186).

Blood is slightly acidic, thereby inhibiting antibody binding and potentially other enzymatic reactions. *Taq* DNA polymerase, the enzyme used to amplify DNA in PCR, is totally inhibited by as little as 0.004% (vol/vol) human blood (3); this inhibition is due to the heme in the blood (4). In addition, in many situations, the target microorganism must first be concentrated from large volumes of blood prior to DNA extraction and analysis. A procedure to rapidly process large-volume (6 ml) blood samples using a fibrin lysis cocktail and the Roche LC DNA isolation kit III (Roche Applied Science, Indianapolis, IN) has been reported (M. S. Ewert, personal communication). DNA preparations from blood processed using this procedure were analyzed by real-time PCR to detect low concentrations (<44 CFU/6 ml) of *Yersinia pestis*, *Brucella suis*, and *Bacillus anthracis*.

Dielectrophoresis can be used to discriminate and separate *Plasmodium falciparum*-infected cells from blood (98, 99). The plasma membranes of the infected erythrocytes have an increased ionic permeability, so that infected cells lose internal ions when suspended in a low-conductivity medium (98).

(ii) Urine. It is unlikely that biothreat agents would need to be detected in the course of a urinary tract infection; however, urine contains yet-unidentified substances that are inhibitory to PCR (35, 40, 282, 297), and many components such as bacterial contamination commonly found in urine can interfere with detection results. For example, Western blots of urine samples used to detect the presence of protease-resistant prion proteins could be misinterpreted. Bands with a molecular mass similar to prion proteins can be detected from these Western blots (96).

There are several rapid methods available for processing urine in order to detect the total number of bacteria present. These methods include the FiltraCheck-UTI (Applied Polytchnology, Inc., Houston, TX), the UTIscreen (Los Alamos Diagnostics, Los Alamos, NM), and the Flash Track (Gen-Probe, San Diego, CA). These tests, along with the conventional cytospin Gram stain smear method, are used to process urine when large numbers of bacteria ($\geq 10^5$ CFU/ml) are present (189, 244). Studies that have investigated sample preparation methods for urine PCR have found that centrifugation of the specimen and DNA extraction of the pellet were critical factors to reducing PCR inhibition by urine constituents (26, 35, 297). A urine sample preparation method for PCR has been developed for *Borrelia bugdorferi* infection using seven different extraction methods (26). Only one of these methods, the DNAzol extraction method (Molecular Research Center, Inc., Cincinnati, OH), yielded positive results with 10 cells per PCR mixture. This method included a preextraction step, in which the sample was centrifuged at $36,000 \times g$ for 30 min and ethanol precipitated after the addition of DNAzol (26).

(iii) Stool specimens. Stool specimens contain many substances, such as bile salts and complex polysaccharides, that will inhibit PCR and other enzymatic reactions. In addition, there will be large numbers of background bacteria that will interfere with immunoassays and other affinity-based detection techniques. Immunomagnetic separation and the MagNaPure system, previously described in this article, are techniques that can be used to isolate and concentrate bacteria and nucleic acid from stool specimens. The QIAamp DNA stool purification kit (QIAGEN, Inc., Valencia, CA), intended for *Taq* DNA polymerase inhibitor removal, has been tested for detection of Shiga-toxin-producing *E. coli* by PCR directly from cattle fecal samples (106). The *stx* gene, encoding Shiga toxin, a defining characteristic of enterohemorrhagic *E. coli* strains, was amplified in the PCR, and the results were equivalent to culture, suggesting successful removal of PCR-interfering inhibitors from the feces.

Detection of *Helicobacter pylori* is usually performed on gastric biopsy samples because noninvasive detection directly from feces has low sensitivity. Immunomagnetic separation was used with magnetic beads coated with anti-*Helicobacter pylori* antibody to concentrate the bacteria from the feces of patients with *Helicobacter pylori*-positive gastric biopsies. The sensitivity of PCR detection was improved with immunomagnetic separation compared with direct detection from feces. However, only 61% of the fecal samples tested positive for *Helicobacter pylori*, suggesting that gastric biopsies are still more effective for this diagnosis (303).

(iv) Nasal and throat swab specimens. Luna et al. (190) have developed a method to safely extract DNA from powder sam-

ples suspected of containing *Bacillus anthracis* spores (described later in this review). This method has also been used to safely extract DNA from nasal swabs that contain *Bacillus anthracis* spores.

Vaccinia virus is commonly used as a surrogate for measuring the effectiveness of detection tests for variola, the smallpox virus. Donaldson et al. (81) have described a procedure in which throat swab specimens spiked with vaccinia virus were resuspended in buffer and directly detected using an evanescent wave fiber optic biosensor. Vaccinia virus-specific antibodies attached to the biosensor's fiber optic waveguide were able to separate and capture virus particles from other cells and debris in the specimens.

Powders and soil. During the most recent threat of *Bacillus anthracis* spores (47), powders were one of the most common nonclinical specimens submitted to designated laboratories (http://www.bt.cdc.gov/lrn/factsheet.asp). A rapid method was developed by Luna et al. (190) to render powder and other environmental samples harmless for safe extraction and identification of DNA. DNA was safely extracted from ≤10 *Bacillus anthracis* spores and detected using PCR. Samples were prepared for PCR by germinating spores by heat shock (80°C for 2 minutes), followed by sonication (30 minutes) and autoclaving (121°C, 20 minutes). The DNA was then purified using the MagNaPure instrument as previously described in this review.

An evanescent wave fiber optic biosensor method has also been developed to directly test powders for *Bacillus anthracis* spores (281). Talc-based baby powder, corn starch-based baby powder, confectioner's sugar, baking soda, and *Bacillus thuringiensis*-based pesticide spiked with *Bacillus anthracis* spores were successfully processed by this method. Powders were resuspended in buffer and tested directly by the biosensor. Use of unspiked talc-based baby powder as a reference baseline eliminated 100% of potential false-positive readings from powder samples.

Numerous kits have been developed to expedite extraction of total DNA from natural microbial communities within soils and sediments. Three commercially available kits for DNA purification were compared by extracting target DNA from *E. coli* DH5α that had been seeded into freshwater lake and river sediments. DNA recovery was higher for the two kits (UltraClean Soil DNA Kit, MoBio Inc., Solana, CA, and FastDNA SPIN Kit, Bio 101, Carlsbad, CA) that used mechanical lysis through bead beating. The third kit (SoilMaster DNA Extraction Kit, EpiCentre, Madison, WI) lysed cells with hot detergent and recovered less but a higher quality of DNA (208).

Aerosols. Particles of two major size distribution groups are present in infectious aerosols: particles >5 μm in diameter and particles 1 to 5 μm in diameter. Particles 1 to 5 μm in diameter behave like gases (61). Larger particles (>5 μm in diameter) settle from the atmosphere and bind to surfaces (82). The smaller 1- to 5-μm particles can be collected by processing large volumes of air and passing the air through a filter or impinging the particles from the collected air into a liquid or semisolid sample (271).

Impaction samplers collect culturable air-borne bacteria and fungi by depositing air-borne particles onto a semisolid agar surface. The semisolid surface then must be cultured in order to detect the presence of collected microorganisms. Although these samplers are capable of collecting small numbers of bacteria (approximately 20 CFU), their usefulness is limited at higher levels of bacteria (>10^4 CFU) and by sampling time (43). Longer sampling times (>5 min) dry the semisolid collection surface, thereby reducing collection efficiency. Impingement samplers that collect air-borne cells into a liquid can collect particles over longer periods of time and are not limited by the types of analytical methods that can be used for detection of the collected microbes.

Collected air can also be passed through a porous filter. Filtration may desiccate vegetative cells, but the DNA from the cells would be preserved for PCR analysis. Evidence of only the DNA, however, may not be an ideal way to detect a potential biothreat agent because the presence of intact viable cells cannot be confirmed. Filtration collectors currently are used in more than 30 major cities in the United States in the BioWatch biosurveillance program. Filters from these collectors are periodically removed and processed manually for the presence or absence of biothreat agents by participating laboratories. The Department of Homeland Security and the Centers for Disease Control and Prevention's Laboratory Response Network provide early detection of biothreat agents (37).

The Lawrence Livermore National Laboratory (Livermore, CA) has developed a high air volume to low liquid volume aerosol sampler that concentrates air-borne materials from large volumes (2,300 liters/min) of air into a 4-ml liquid sample for subsequent automatic analysis. This sampler, part of the Advanced Pathogen Detection System, has been field tested for viable biothreat agents *Bacillus anthracis* and *Yersinia pestis* in a biosafety level 3 facility (197).

Northrop Grumman (Arlington, VA) has incorporated the existing GeneXpert technology into its high-volume Biohazard Detection System for screening mail at U.S. Postal Service facilities. During mail operations, the Biohazard Detection System collects air samples directly above the cancellation equipment and concentrates air samples for 1 hour by absorbing and concentrating air-borne particles into a sterile water base. The fully automated system is a Cepheid GeneXpert module that identifies the presence of *Bacillus anthracis* spores from air samples. In the fall of 2003, after extensive testing, Northrop Grumman was awarded a production contract to install and manage these systems at U.S. Postal Service sorting centers nationwide (141).

Testing for potential biothreat agents in air is possible only at facilities that contain approved air chambers, such as the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) and U.S. Army Dugway Proving Ground, Utah. The Harry Reid Center at the University of Nevada (Las Vegas, NV) contains aerosol chambers and wind tunnels capable of handling items contaminated with chemical, biological, or radiological materials (or surrogates) of interest. Commercial and academic facilities cannot test select agents unless registered with the Centers for Disease Control.

Surfaces. The Brentwood Mail Processing and Distribution Center in Washington, D.C., was extensively contaminated with *Bacillus anthracis* spores in 2001 after two letters containing spores were processed at the facility (47). The level of *Bacillus anthracis* spores obtained by surface wet or dry-swab, wipe, and HEPA vacuum sock sampling methods was mea-

sured at the Brentwood facility. The numbers of spores detected using the wipe and HEPA vacuum methods were similar, but the wet-swab and dry-swab methods did not detect spores >33% and 66% of the time, respectively. No single sampling method was found to completely remove spores from sampled surfaces (250). Spores from the *Bacillus anthracis* surrogate *Bacillus atrophaeus* sprayed onto different surfaces were used to study the effectiveness of sampling using a swipe (Speci-songe, Nasco, Fort Atkinson, WI), heavy wipe (Handy Wipes Heavy Wipe, First Brands Corp., Danbury, CO), or swab sample processing kit (ASD, Ft. Lauderdale, FL). The three sampling methods performed equally well, but no method completely recovered all spores (42). Different swab materials were also found to affect the recovery of *Bacillus anthracis* spores from steel surfaces. Premoistened macrofoam and cotton swabs that had been mixed 2 minutes recovered the greatest percentage (>40%) of spores (247).

The Microbial-Vac (M-Vac, Microbial-Vac Systems, Inc., Jerome, ID) uses vacuum and centrifugation to sample and concentrate microorganisms from fruit and vegetable, carcass, and equipment surfaces. A study compared recovery of *Salmonella enterica* from cantaloupe rind surfaces using M-Vac and by resuspending rinds in peptone water containing Tween 20 and massaging the rinds in solution by hand or by stomacher (31). M-Vac was the only method that could recover *S. enterica* (6% recovery) from the rind surface when the surface was seeded with a low quantity of the bacterium (2.0×10^2 CFU/100 cm^2).

COMMERCIAL DETECTION AND IDENTIFICATION OF BIOLOGICAL THREAT AGENTS

Numerous methods for detection and identification of human pathogens have been used for more than a century and thus are well established and well understood (209). These methods rely on cultivation and biochemical assays which can take from 24 h to 1 month to perform in the hands of a well-trained clinical technician. While these methods are reliable, they generally cannot be used in the field or are not capable of providing real-time or nearly real-time detection and identification of biothreat agents in a bioterrorism event. With recent bioterrorism incidences, there is a critical need to develop more rapid, accurate methods to detect and identify biothreat agents.

Much research and money have been invested in commercial development of technologies that can rapidly and accurately detect and identify biothreat agents both in the field and in the clinical laboratory. The following sections cover only those technologies that are currently available in an "off-the-shelf" format. Those products still in development or in beta-testing stages are discussed in a later section of this review. While an attempt has been made to broadly and thoroughly cover each area of detection methodology, the following discussion is by no means an exhaustive accounting of products available on the market. Many products are too new to have been tested and reported upon in the scientific literature; others are proprietarily owned or licensed to the U.S. Government for defense purposes and, thus, are excluded from this review. Detection assay types discussed include those based on biochemistry, antibodies, and nucleic acids.

TABLE 1. Examples of biothreat agents and identification tests for Laboratory Response Network level A laboratories

Screening test or medium	Suitability of test for bacterium			
	B. anthracis	*Y. pestis*	*F. tularensis*	*Brucella* spp.
Gram stain	+	+	+	+
Catalase test	+	+	+	+
Oxidase test		+	+	+
Urease test		+	+	+
India ink	+			
Wright-Giemsa stain		+		
Beta-lactamase			+	
XV or *S. aureus*[a]			+	+
Motility medium	+			
5% sheep blood agar	+	+	+	+
Chocolate agar	+		+	+
MacConkey agar	+	+	+	+
Phenyl ethyl alcohol agar	+			
Tryptic soy broth	+			
Thioglycollate broth	+		+	
Brain heart infusion agar		+		
Eosin methylene blue agar[b]		+	+	
Thayer-Martin agar[c]			+	+

[a] XV factors or *S. aureus* for satellite growth.
[b] Alternative to MacConkey agar.
[c] Alternative to chocolate agar.

Manual Biochemical Tests

Local clinics and hospital laboratories are the first facilities likely to encounter biothreat agents as infected or exposed individuals seek medical assistance (263). Recognition of unusual symptoms or culture test results is of utmost importance, while at the same time unnecessary alarm at false positives should be minimized. Widely available microbial identification systems used on a routine basis by clinical laboratories are not designed or optimized for detection of biothreat agents. Thus, screening hierarchies have been established using standard microbiology biochemical tests to reduce the potential for false alarms and rule out biothreat agents as quickly as possible at the clinical laboratory level. For example, testing and presumptive agent identification protocols for *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and *Brucella* spp. can be obtained online at http://www.bt.cdc.gov/labissues/index.asp#testing. These protocols, which outline the steps to determine if a suspicious culture can be determined not to be of significance or must be further examined by additional tests, are summarized in Table 1.

Automated Biochemical Tests

Most commercial biochemistry-based clinical microbe identification systems are not developed specifically for identification of biothreat agents. However, some commercial systems that utilize pattern recognition databases or libraries have recently begun to offer biothreat update packages. The patterns generated by these types of systems typically are based either on the bacterium's ability to metabolize specified compounds or on gas-liquid chromatography of cellular components.

Substrate utilization patterns. Several automated identification systems such as VITEK (bioMérieux, Hazelwood, MO)

and MicroLog (BiOLOG, Hayward CA) utilize pattern recognition or "fingerprinting" systems based on metabolized substrates and carbon sources or susceptibility to antimicrobial agents. A turbidometrically controlled aliquot of a pure bacterial suspension is added to analysis or identification cards or plates. These cards or plates contain numerous wells with different types of biochemical substrates (usually proprietary) and can be purchased in many different configurations depending on the expected needs of the laboratory. The inoculated cards or plates are then incubated for a set amount of time and read using an automated system. A similar system offered by bioMérieux is the API Series, which consists of biochemical tests in a strip format that, after inoculation and incubation, are processed and read manually. These systems have been used to identify biothreat agents such as *Bacillus anthracis* (20), *Yersinia* spp. (16, 183), *Vibrio cholerae* (176), and other pathogens (219). BiOLOG has recently introduced a "dangerous pathogen" supplement (DP Database) to its MicroLog system, although no published evaluations of the system are available currently.

Fatty acid profile. The Microbial Identification System (MIDI Inc., Newark, DE) converts cellular fatty acids from pure cultures of bacteria to fatty acid methyl esters and uses a gas chromatograph for separation and identification. Samples are harvested, saponified, and methylated and then extracted and washed. The resulting organic phase is used for identification. Pattern recognition software is used to identify isolates from the chromatograph reading. This method has been used to identify and differentiate *Bacillus* spores (266), *Burkholderia* spp. (136, 268), *Francisella* spp. (27, 57), and *Yersinia* spp. (172). MIDI Inc. recently introduced the Sherlock Bioterrorism Library that can be added to its identification system to specifically target biothreat agents and challenge organisms. The MIDI Sherlock system containing the MIDI BIOTER database version 2.0 has been been awarded AOAC Official Methods of Analysis status for confirmatory identification of *Bacillus anthracis* (13).

Each of these biochemical methods is inherently time-consuming as each requires pure isolates for the tests to be accurate. In addition, well-trained, experienced technicians are required to accurately identify and subsequently handle further testing that may be necessary. While automated systems are initially expensive, once a pure isolate has been obtained, the benefits of such systems include the ability to perform multiple biochemical tests simultaneously, the speed of obtaining results after minimal incubation times, and minimal advanced technician interaction. These systems generally are more convenient (i.e., plug in sample, obtain print out) than manual procedures but typically cannot be used in the field and their usefulness may limited by the extent of their databases, which must be updated on a regular basis.

Immunological Detection Devices

Since its inception, the immunoassay has increasingly been used and developed for detection of infectious diseases, drugs, toxins, and contaminants in the medical, pharmaceutical, and food industries. In addition, immunological detection has been successfully employed for detection of biothreat agents such as bacterial cells, spores, viruses, and toxins based on the concept that any compound capable of triggering an immune response can be targeted as an antigen. The various types of immunoassays used for biothreat detection have been reviewed previously (12, 137, 230, 231).

Andreotti et al. (12) summarized the basic principles as follows. "Immunoassays rely upon four basic components regardless of the application and underlying technology: (i) the antigen to be detected; (ii) the antibody or antiserum used for detection; (iii) the method to separate bound antigen and antibody complexes from unbound reactants; and (iv) the detection method. The efficacy of any given immunoassay is dependent on two major factors: the efficiency of antigen-antibody complex formation and the ability to detect these complexes."

Typically, immunoassays generally test for only one analyte per assay. This limitation means that multiple simultaneous or sequential assays must be performed to detect more than one analyte in a sample or specimen. Advances in assay design and in matrix format have resulted in development of multiplex assays that can be performed on multiple samples simultaneously by automated systems. However, the specificity of immunoassays is limited by antibody quality, and sensitivity (detection limits $\sim 10^5$ CFU) is typically lower than with PCR and other DNA-based assays. As improvements are made in antibody quality (e.g., production of antibodies from recombinant libraries) and in assay parameters, it may be possible to increase immunoassay sensitivity and specificity.

Many different immunoassay formats are currently commercially available for a wide variety of detection needs (Table 2). Many formats are similar to, or derived from, the classic sandwich assay based on the enzyme-linked immunosorbent assay (ELISA) design (209).

Commercial versions of the sandwich method have expanded to include newer innovations of biotechnology, principally in detection capabilities. Assays can be performed with a variety of substrates and labels (fluorescent, chemiluminescent, and electrochemiluminescent), as well as on multiple platform types (biosensors, flow cytometry, microarray, and lateral flow diffusion devices). Many systems available today use different combinations of these advances. However, there are two main categories of immunoassays used with a few adaptations, mainly variations of solid-support models and lateral flow diffusion apparatuses, also known as "smart ticket" technology or hand-held assay devices.

Solid-support platforms. (i) Luminex xMAP. Luminex xMAP (Luminex Corp, Austin, TX) technology uses the basic "sandwich" assay format, but the capture antibody is coated onto the surface of a polystyrene bead rather than in a microwell plate. These beads are processed through the assay and separated for analysis via flow cytometry (298). The beads (microspheres) are spectrally unique and color coded into different sets that can be differentiated by the Luminex100 analyzer. As each type of bead can be labeled with different antibodies, this labeling enables multiple analyses to be simultaneously performed in the same well. The beads are maintained in solution throughout the assay, thereby permitting liquid phase binding of target and separation spectrally by a dual laser detection system. The laser detects excitation of internal bead dyes and reporter dyes of any captured targets. The signal intensity is proportional to the amount of target

TABLE 2. Commercially available biothreat detection systems[a]

Type	Test format	Test name	Manufacturer	Targets[b]	References[c]
Biochemical	FAME-GC	Sherlock Bioterrorism Library	MIDI, Inc.	*B. anthracis, Y. pestis, Brucella* spp., *F. tularensis, B. mallei, B. pseudomallei*	27, 57, 136, 152, 172, 266, 268
	Substrate utilization	MicroLog Dangerous Pathogen Database	BiOLOG	*B. anthracis, Y. pestis, Brucella* spp., *F. tularensis, B. mallei, B. pseudomallei*	20, 219
		Vitek	bioMérieux	*Y. pestis, V. cholerae, C. botulinum*	176, 183, 219
		API Series	bioMérieux	*B. anthracis, C. botulinum, Y. pestis, Salmonella* spp.	16, 188, 258, 309
Antibody	Microarray	NanoChip	Nanogen	Pathogens, SEB, *V. cholerae* toxin B	89
	ELISA	xMap Technology	Luminex	Pathogens	29, 30, 123, 196
		BV Technology	BioVeris Corp.	*B. anthracis, E. coli* O157:H7, *Listeria* spp., *Salmonella* spp., *C. botulinum* toxins A, B, E, & F, ricin, SEB	100, 154, 260, 318, 319
		QuickELISA *B. anthracis*-PA kit	Immunetics Inc.	*B. anthracis*	NA
		RAPTOR	Research International	*B. anthracis*, *F. tularensis*, *Y. pestis, Brucella* spp., *V. cholerae*, ricin, SEB, *E. coli* O157:H7, *Salmonella* spp.	9–11, 44, 180
		Bio-Detector	Smiths	Assorted organisms	NA
	Smart Tickets	Instant Check	EY Laboratories	Ricin	NA
		BioThreat Alert	Tetracore	*B. anthracis, F. tularensis, Y. pestis, C. botulinum, Brucella* spp., ricin, SEB	157
		Redline Alert	Tetracore	*B. anthracis*	NA
		SMART-II	New Horizons Diagnostic, Inc.	*B. anthracis*, *F. tularensis*, *Y. pestis*, *C. botulinum*, *V. cholerae*, ricin, SEB, *E. coli* O157:H7, *Salmonella* spp.	157
		Biowarfare Agent Detection Device (BADD)	Osborne Scientific	*B. anthracis*, *C. botulinum*, ricin	157
		RAMP	Response Biomedical Corp.	*B. anthracis*, *C. botulinum*, *Y. pestis*, ricin, smallpox	NA
		HHA & HHMA	ANP Tech	*B. anthracis*, *C. botulinum*, *Y. pestis*, smallpox	NA
	TRF	DELFIA	Perkin-Elmer	Pathogens	229, 320
DNA	Q-PCR	GeneXpert/Smart Cycler	Cepheid	*B. anthracis*	126, 134, 167, 193
		RAPID	Idaho Technology	*B. anthracis, F. tularensis, Y. pestis, C. botulinum, E. coli* O157:H7, *Salmonella* spp., *Brucella* spp., *Listeria* spp.	121, 296
		LightCycler detection kit	Roche	*B. anthracis*	23, 126, 134, 167, 193
		BioSeeq	Smiths Detection	Pathogens	83
		B. anthracis PCR kit	Takara Mirus Bio	*B. anthracis*	193
		O157:H7; StxI; StxII	Applied Biosystems Inc.	*E. coli* O157:H7	64
		RealArt PCR kits	Artus	*B. anthracis, Salmonella* spp. dengue virus orthopox virus, other viruses	NA
		PathAlert detection system	Invitrogen	*B. anthracis*, *Y. pestis*, *F. tularensis*, smallpox	NA
		Certified Lux primer set	Invitrogen	*B. anthracis*, *Y. pestis*, *F. tularensis*, smallpox, *C. botulinum*	NA
Other	Mass spectrometry	TEEMmate	JEOL	Spores	28
	Bioluminescence	Profile-1	New Horizon Diagnostic, Inc.	Pathogens plus spores	62, 174, 273

[a] Abbreviations: FAME, fatty acid methylester; GC, gas chromatography; SEB, staphylococcal enterotoxin B; NA, not available to public; HHA, handheld assay; HHMA, handheld microarray assay.
[b] Examples of targets detected, not a complete accounting.
[c] Peer-reviewed publications accessible to the public.

analyte in the sample. The xMAP technology has been incorporated into continuous environmental monitoring systems (123, 196) and for detection of exposure to biothreat agents in humans (29, 30).

(ii) BV technology. The BV M-Series instrument (BioVeris Corp., Gaithersburg, MD) (formerly ORIGIN-Igen Corp.) uses electrochemiluminescence to detect reporter molecules used in the sandwich assay. BV assays use paramagnetic beads as the support structure for the capture antibodies. After the target is captured, the reporter antibodies labeled with BV-TAG, $Ru(bpy)_3^{2+}$, are passed through the system and any target analyte captured on the beads is labeled. The beads are passed via a flow cell over a magnet on the surface of an electrode. The beads are captured by the magnet and are thus separated from unbound label and remaining matrix components. An electrical potential is applied to the electrode that excites the BV-TAGs, which then emit light that can be detected and processed. This technology has been mainly used in clinical settings, but is being applied to detection of biothreat agents such as *E. coli* O157 (260, 318, 319), *Bacillus* spores (100, 318), *Yersinia* spp. (318), *S. enterica serovar Typhimurium* (319), and toxins and toxoids (100). Numerous biothreat agent assays have been developed and many are available as kits (Table 2).

(iii) Bio-Detector. The Bio-Detector (Smiths Detection, Edgewood, MD) uses ELISA principles on a tape format in a portable rugged housing. Liquid samples are injected and separated for detection of different biothreat agents. During the reaction, each subsample is mixed with biotin-labeled and fluorescein-labeled antibodies as well as with streptavidin, which attaches to the biotin label. After the labeled antibodies have attached to the targets within the subsample, they are filtered and captured by biotin-coated biotape in different locations for each subsample. The streptavidin acts as a bridge between the biotin-tagged targets and the biotin-coated tape. A solution of antifluorescein antibody, conjugated to the enzyme urease, is filtered through the tape and tags the bound target. The tape is then positioned over the sensor, where it is covered in a solution of urea. If the tape has trapped a targeted agent, the urease reacts with the urea and causes a change in pH. The rate of change is proportional to the amount of target present. The sensor processes the signal and determines the presence and quantity of target agent present.

(iv) DELFIA. The DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) (Perkin-Elmer Life Sciences, Akron, OH) system is one example of a format based on time-resolved fluorescence. This technique relies on lanthanide chelate labels that have long fluorescence decay times, which allow measurements of fluorescence without interference from background signals. Typically, this chemistry is incorporated into standard ELISA microplate assay format (detection antibodies are labeled with the lanthanide) and, after the reaction has occurred, the label is disassociated from the detection antibodies using a low-pH enhancement solution. The free molecules rapidly form new stable highly fluorescent chelates, which can be read by the system (12, 230). The DELFIA system has been used to detect *Francisella tularensis*, *Clostridium botulinum* toxin, staphylococcal enterotoxin B and *E. coli* O157:H7 (229, 320).

Lateral flow platforms. Lateral flow devices have been principally developed for rapid field assay formats but are quickly becoming incorporated in clinical laboratory settings. These tests are usually single-use, disposable cartridge tests in the form of either immunochromatographic line assays or enzyme immunoassays generating detectable colored end products which give a yes or no answer. Basically, these assays use antibodies mounted on a paper strip or membrane as the capture antibody and use capillary flow to move colloidal gold or colored microparticle-labeled antigen detection antibody complexes in the fluid phase toward the capture antibody. A positive result is obtained from the capture of the labeled antigen-antibody complex with a second immobilized antispecies antibody (typically an anti-immunoglobulin G for the detection antibody host) and the formation of a line or pattern in the appropriate result window (209). Also incorporated are control reagents which result in either in a contrasting design or pattern appearance in a separate window for a negative result depending on the format. The negative result (control) is also an indicator that the test worked correctly and the sample was passed through the testing field. These devices are commonly referred to as dipstick tests, and the public is most familiar with their use as off-the-shelf pregnancy test kits.

Although lateral flow device assays are easier to perform and more rapid than classic instrument-based immunoassays, they typically are not as sensitive and can have the potential for higher rates of false positives. However, such devices can be useful in rapid preliminary screening of samples for a biothreat agent, and any positives should be followed by confirmatory tests such as PCR.

Lateral flow devices have been developed by many companies for several biothreat agents, including *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Clostridium botulinum*, and several toxins such as ricin and staphylococcal enterotoxin B (Table 2). Although lateral flow devices have been available for use in the field for some time, there is little information available about their performance other than company literature, promotional items, and news reviews. With the exception of one paper by King et al. (157), no peer-reviewed published data are available on these kits. However, the U.S. Food and Drug Administration recently approved two test kits (Redline Alert, Tetracore Inc., Gaithersburg, MD; and RAMP, Response Biomedical Corp., Burnaby, British Columbia, Canada) for use with pure culture isolates to test for *Bacillus anthracis* in the clinical setting (Redline: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfRL/listing.cfm?&ID-90045; RAMP: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfRL/listing.cfm?&ID=79402) In addition, the RAMP system was also awarded AOAC Official Methods status (13, 111). These approvals may lead to more thorough and descriptive testing and reporting of these types of systems.

Nucleic Acid Detection via Quantitative PCR

Quantitative real-time PCR (Q-PCR) combines PCR amplification with simultaneous detection of amplified products based on changes in reporter fluorescence proportional to the increase in product (118, 187). There are two formats of Q-PCR, nonspecific detection and specific detection. Nonspecific detection uses DNA-intercalating dyes that fluoresce when

bound to DNA (e.g., SYBR green) (Molecular Probes Inc., Eugene, OR). As the DNA is amplified, the dye is intercalated into the product. After amplification, a melting curve is performed and, as the DNA dissociates, the fluorescent signal decreases proportionally. This format is useful for optimizing PCR conditions and checking specificity of primer designs and is cost efficient compared to specific detection formats. For specific detection, the change in fluorescence relies on the use of dual-labeled fluorogenic probes containing both a reporter fluorescent dye and a quencher dye. An increase in fluorescence indicates that the probe has hybridized to the target DNA and the quencher dye is no longer able to mask the signal of the fluorescent dye. Q-PCR is used for a variety of applications from quantitative presence/absence tests and rapid confirmation tests to monitoring gene expression (the most common use).

The main Q-PCR format used for biothreat agents is specific target detection, and a wide variety of primer and probe combinations are available from many companies in a multitude of configurations. Many of these specific target configurations rely on mechanistic variants in the primer/probe construction and combinations which can include TaqMan probes (double-dye probes) (127), locked nucleic acid probes (161, 218), black hole quenchers (146), molecular beacons (287), lux primers (213), and scorpion primers (306). Each type has a slightly different method of separating the fluorophore from the quencher for reporting the amplification process, and all are available commercially and can be customized.

As the fluorescent reporter dyes attached to these primer and probe combinations can have different excitation/emission spectra, Q-PCR can be utilized to detect several targets simultaneously using different reporter dyes for different targets. Some of the more common reporter dyes include 6-carboxyfluorescein, 6-carboxytetramethylrhodamine, Cy5, Cy3, Rox, Texas Red, rhodamine, fluorescein, and Oregon Green. Quenchers have the ability to quench throughout their absorption spectrum, but optimally have their maximum absorption matched closely with the reporter dye emission for the best performance.

Typically the Q-PCR thermocyclers incorporate intricate software to monitor the progression of the reactions. At the point when the product signal is detected above the background (C_t value) and in the exponential phase, the software can quantitate and compare the signal to standards. The more abundant the target, the faster it is detected above background and the lower the C_t value. This C_t value can be either qualitative or quantitative, depending on how the standards are set up and based on the needs of the user. As technology progresses, these instruments are becoming smaller, faster and more sensitive, thus making them highly appealing as rapid detection methods for biothreat agents.

However, accurate characterization or identification of bacteria by Q-PCR is limited by the same bias and variations that are inherent in many nucleic acid techniques. The main concerns are biased nucleic acid extraction (e.g., efficiency of extraction or cell lysis if using whole-cell methods), degradation of nucleic acids by nucleases, probe and primer reactivity (i.e., sensitivity, specificity, accessibility, and quantitation), and inherent PCR bias (e.g., variances in polymerase, buffer, and thermocycler performances). The ability to either extract the DNA or rupture the cells or spores for accessibility significantly influences the sensitivity, reproducibility and accuracy of any PCR biothreat agent detection method. Additionally, the presence of inhibitors such as humic acids or chelating agents can interfere with target sites of the probes and primers, thereby resulting in false negatives. Care must be taken to remove these inhibitory compounds prior to analysis.

In spite of the limitations, PCR-based analysis can be highly specific and sensitive for the target of interest if the number of cells present is at or above the detection limits of the particular assay (typically 10 to 100 cells). Use of Q-PCR to obtain rapid quantitative estimates for biothreat agent presence is an invaluable asset. The new advances in size reduction and speed of thermocycling enable these units to be used both as portable and as laboratory-based platforms.

Q-PCR thermocycler platforms. While many Q-PCR technologies are available, a few have had more focused development towards pathogen and biothreat agent detection. Most of these systems are sophisticated molecular-based platforms that perform best in a laboratory setting such as hospitals, monitoring offices, and research laboratories. The GeneXpert Q-PCR DNA detection system (Cepheid, Sunnyvale, CA) incorporates the standard Q-PCR features of the Smart Cycler system (Cepheid) with disposable automated testing cartridges that can process "dirty" samples and perform amplifications followed by product detection (22). This automated process removes the human variable and speeds the processing time by eliminating the need to extract DNA from samples prior to analysis. A GeneXpert test cartridge specific for *Bacillus anthracis* is available, and other targets are in development.

The LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) also has assays specifically developed for biothreat agents and a LightCycler-*Bacillus anthracis* detection kit (Roche Applied Science) is commercially available (23, 288). The LightCycler system utilizes a disposable glass capillary system into which extracted DNA is placed and analyzed, instead of a cartridge system (310). Applied Biosystems (ABI, Foster City, CA) offers several 96-well plate format Q-PCR systems, including the 7300 and the 7500 real-time PCR systems and the 7000 and the 7900 ABI Prism sequence detection systems. ABI also sells TaqMan-based detection kits for pathogens (e.g., Taqman *E. coli* O157:H7 and *E. coli* Stx1 and Stx2) (64). Numerous Q-PCR assays have been developed and validated for several biothreat agents (e.g., *Bacillus anthracis* and variola virus), and many have had their conditions optimized for use on these different platform types (126, 134, 139, 167).

While these Q-PCR instruments are useful in a laboratory environment, recent bioterrorism events have underscored the need for fieldable Q-PCR platforms. The Ruggedized Advanced Pathogen Identification Device (RAPID) (Idaho Technology, Salt Lake City, UT) is an automated, portable Q-PCR platform that integrates the LightCycler technology in a compact unit. This instrument was designed for use by military field hospitals and first responders and in other rugged environments that require a portable impact-resistant platform. Many biothreat agent and pathogen detection kits are available in freeze-dried format for the RAPID (121, 296). Another portable instrument is BioSeeq (Smiths Detection, Edgewood, MD), a commercial version of the handheld advanced nucleic acid analyzer by Lawrence Livermore National Laboratory.

This unit has a small, rugged design with features that make it possible to operate with the heavy gloves of a hazmat suit and was designed for military field use. BioSeeq uses either a prepared PCR cartridge and preset protocols to analyze samples in the field or individual samples with programmable protocols in a laboratory mode for customized assay designs (83).

PCR reagent kits for biothreat agents. There are innumerable sets of probes and primers in the literature databases that can be used with any of a number of pathogens or biothreat agents. Recently, however, several companies have begun to offer PCR kits in various formats for detection of biothreat agents (Table 2). These kits eliminate the need for extensive primer/probe design and facilitate rapid detection and monitoring programs. The kits often come with prepared controls and only lack the sample DNA to be tested. The removal of inadvertent investigator influence (e.g., pipeting or calculation errors and cross-contamination issues) on these systems results in simplified processing and reproducible data. One kit, PathAlert (Invitrogen Corp., Frederick, MD) (Table 2), can be coupled with the Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE) for an automated multiplex assay in a lab-on-a-chip format. PCR products produced with the Q-PCR kit are passed through the Bioanalyzer and identified.

Bioluminescence Detection

Bioluminescence has been used widely in clinical, food, and environmental settings for monitoring incidences of bacterial contamination. The most common example of bioluminescence is the luciferin-luciferase reaction in the presence of ATP. The basic principle of ATP bioluminescence monitoring is that the amount of ATP in a sample correlates proportionally to the biomass. Since ATP is found in all living cells, steps must be taken during sample processing to eliminate ATP contamination from nontarget sources (e.g., plant cells, mammalian cells, and nontarget bacteria). Depending on the selected target, different strengths of detergents are used to lyse nontarget cells (e.g., mold, pollen, somatic cells) in the sample, so that only the intended cells are monitored (62).

The Profile-1 hand-held system (New Horizon Diagnostics Inc., Columbia, MD) uses a microluminometer to read sample bioluminescence and a Filtravette (New Horizon Diagnostics Inc.) sample processing unit to remove nonbacterial ATP sources. This combined system has been used to examine contamination on meat carcasses (62), test biological aerosols (273) and detect *Bacillus* species spores in powders (174). The main limitation to this method is that it is nonspecific for biothreat agents. ATP monitoring can report the presence and relative abundance of bacteria or germinating spores in a sample but cannot identify the type of bacteria. Processing samples via this method only indicates if ATP-producing microorganisms are present; further processing of the sample by other methods is needed to determine whether or not a serious threat is present. While this type of test may be well suited to the food industry, where the presence of any bacterial contamination may be important, its usefulness for biothreat agent detection is limited.

DEVELOPING TECHNOLOGIES

Developing sensor technologies for bioterrorism defense can be divided into a few broad groups according to the way they recognize and respond to a biological threat agent. For the purposes of this review, these groups are defined as biochemical, immunological, nucleic acid, cell/tissue, and chemical/physical technologies. Each of these recognition systems must be paired with a transducer that can transform the response into an analyzable signal. Detection platforms that combine a biological recognition system and a physical transducer are termed biosensors. There are a variety of transducers that can be paired with a given biological recognition system to produce an analyzable signal, including electrochemical, optical, mass, thermal, and high frequency (2, 67). Hence, this review will focus on only some of the most active areas of research and advanced biosensor development efforts reported in the literature. Additional information on biosensor development and potential applications can be found in other review articles (67, 103, 113, 137, 138).

Biochemical Detection

Biochemical systems for recognition of biological targets include measurements of products and enzymatic activity associated with microbial metabolism. These methods generally are not as specific as antibody- or nucleic acid-based methods because the targeted product or enzyme may also be present in other organisms.

One example of a technology that detects the metabolic products of organisms is electronic nose devices. This technology uses a transducer, such as a cantilever, acoustic wave, or conducting polymer, which has been coated with a chemical that reacts with specific volatile organic compounds or gases to create a sensing element (67). An array of sensors, each specific for different vapors or gases, can be constructed and used to detect multiple analytes. The technology has been used by researchers to detect and identify volatile organic compounds produced by specific bacteria or fungi (1, 128, 149, 150, 191, 224–226, 252, 286). Electronic nose devices have been used for detection of microbes in food (150, 191) and infections in humans (225, 226). This technology requires complex pattern recognition software to interpret the results. It can be rapid and sensitive but is not very specific because the compounds produced by microorganisms can fluctuate with energy and/or carbon source and with environmental conditions. Furthermore, different organisms can produce similar volatile products.

Conducting polymers may also be used to detect biologically produced chemicals other than volatile organic compounds and could be used to detect products of microbial metabolism such as toxins. Conducting polymers are organic polymers that can conduct electricity. These polymers can be doped with enzymes, antibodies, or other biomolecules that allow them to capture specific types of target biological compounds. Much of the work in this area has concentrated on detection of biological compounds, such as glucose, urea, and cholesterol, making them useful as biochemical detectors. A recent review by Gerard et al. (103) provides detailed information on the nature of

these polymers and their potential applications in health, food, and environmental monitoring.

Biosensors that detect the presence of a bacterial toxin based on inhibition of enzyme activity have been reported in the literature. A portable sensor for anatoxin-a, a toxin released by certain species of cyanobacteria, detected inhibition of acetylcholinesterase activity using an electrochemical transducer (299). The sensor performed well; however, it is nonspecific since several other compounds, including common organophosphate and carbamate pesticides, also inhibit acetylcholinesterase activity (74, 256). In an attempt to compensate for the lack of specificity, mutations were induced in acetylcholinesterase and mutant enzymes were screened for both increased sensitivity and specificity to anatoxin-a (74).

β-Galactosidase activity with amperometric detection was used to quantify both *E. coli* and *Klebsiella pneumoniae*, members of the fecal coliform group of bacteria, in sterile water (202). Specific detection of *E. coli* was also accomplished by selective capture of the bacteria on an electrode functionalized with antibodies specific for the bacteria. An alternative method increased specificity by using phage specific for *E. coli* to lyse the bacteria followed by amperometric detection of β-galactosidase activity (214).

Immunological Detection

Antibody-based and similar affinity probes are used in many of the technologies currently under development. Technologies based on this type of detection utilize the specificity of the immune system to target agents of interest. Shape recognition technologies can be used to detect a range of potential threat agents, including viruses, bacteria, toxins, and bioregulators. A substantial amount of research is concentrated on improving antibody sensitivity and specificity through the generation of recombinant antibodies, antibody fragments, and phage probes. Other types of shape recognition probes are also under investigation, including receptors, aptamers, and peptide ligands. A primary advantage to using affinity probes is the potential to recover live organisms for culture and further study (165, 280). Several reviews have been published that discuss the use of antibodies and other affinity probes and their applications (36, 125, 137, 166, 232).

Antibodies and fragments. Conventional antibodies are the predominant affinity probe used in shape recognition-based technologies. However, other forms of antibodies, including mono- and divalent antibody fragments such as Fab′ and $F(ab')_2$ respectively, and single-chain variable regions have also been explored to determine any advantages they might offer in sensitivity, specificity, or durability compared to antibodies (84, 163, 177, 232, 238, 323). Such fragments may also be modified using recombinant technology in an attempt to improve the binding kinetics of the antibody fragments, thus making them more favorable as probes (232). Phage display libraries have improved the development of affinity probes by permitting biopanning of libraries containing thousands of possible peptides for the ones that bind best to a specific antigen. Identified antibodies or fragments can then be chemically synthesized or produced in large quantities in a recombinant host (137, 233), or the phage expressing the antibody or fragment may also be used as the probe (233). Using this methodology, probes have been developed that bind to bacteria, viruses, and toxins, e.g., *Clostridium difficile* toxin B (73), *Brucella melitensis* (116), vaccinia virus (253), and botulinum toxin (84). The final products may be isolated or remain attached to the phage (73, 116, 233).

Aptamers and peptide ligands. Aptamers and peptide ligands are other alternatives to antibodies. Aptamers are small DNA or RNA ligands that recognize a target by shape, not by sequence, and that are generated using combinatorial methods. Aptamers have been used to detect the toxin ricin in a bead-based biochip sensor (159) and cholera toxin, staphylococcal enterotoxin B, and *Bacillus anthracis* spores in an electrochemiluminescence assay (38, 39). Two variations of aptamers include ribozymes, autocatalytic RNA aptamers that can be engineered to generate a signal after target capture (119), and photoaptamers, DNA aptamers that have been modified to bond covalently to a bound target when exposed to UV light (262). A review by Breaker (33) provides more detailed information on the characteristics and potential applications of aptamers and similar nucleic acid affinity probes.

Short recombinant peptide sequences have also been tested as capture and detection elements in biosensors. As with phage display-generated antibodies and fragments, these peptide ligands may be chemically synthesized or remain as a phage probe. Sequences that bind specifically to ricin (153), *Bacillus anthracis* and other *Bacillus* species spores (34, 284, 307), protein A of *Staphylococcus aureus* (195), and staphylococcal enterotoxin B (108) have been developed and tested. In addition, hemin tethered to glass slides has been used to capture and detect various types of bacteria using intrinsic NADH/NADPH fluorescence (156, 195).

Flow cytometry. Biosensors that exploit shape-based recognition to capture and detect specific biothreat agents are being developed. One example is the flow cytometer that serves as the detector for the Autonomous Pathogen Detection System (APDS) developed at Lawrence Livermore National Laboratory. This flow cytometer is based on proprietary Luminex technology, in which Luminex color-coded beads are conjugated to antibodies that bind to specific target agents (196, 197). Each differently coded bead is labeled with a target-specific antibody, thereby providing extensive multiplex capabilities. The APDS has been used to detect the threat agent simulants *Bacillus globigii*, *Erwinia herbicola*, MS2, and ovalbumin singly and in mixtures (196) and to simultaneously detect of *Bacillus anthracis* and *Yersinia pestis* in air (197). Researchers have recently reported a successful integration of PCR with the APDS to produce a completely automated system that will automatically perform confirmatory PCR on any sample that produces a positive immunoassay result (124). During aerosol chamber tests with *Bacillus anthracis*, *Yersinia pestis*, *Bacillus globigii*, and botulinum toxoid, all agents were detected using the automated immunoassay procedure, and the presence of the three bacteria was confirmed using the automated PCR process.

Biochip arrays. Several methods of detecting targets using biochip technology are under investigation. One approach uses dielectrophoresis to concentrate target agents for identification and then detects them using an electric-field-driven immunoassay (89, 132, 315). Bioparticle separation and enrichment using dielectrophoresis was discussed previously under

Sample Matrix Processing. Dielectrophoresis and electric fields are used to direct the assay components and targets to the proper position on the array. The resulting fluorescence is visualized microscopically. The method has been tested with *E. coli* O157:H7 and *Bacillus globigii* spores (132, 315) and staphylococcal enterotoxin B and cholera toxin B (89, 315).

A second method uses a sensor array constructed on a complementary metal oxide semiconductor integrated circuit to detect targets on a microarray (207). The optical detector can interrogate all spots on the array simultaneously or individually. A system based on this technology has been designed for detection of *Bacillus globigii* spores in air by integrating a portable air sampler with an on-chip ELISA (274).

A complementary metal oxide semiconductor platform is also used in an immunoassay microarray that self-assembles oligonucleotide-labeled antibodies onto the chip through matching with their complementary oligonucleotides on the array (77). Detector antibodies labeled with horseradish peroxidase provide amplified electrochemical detection. The system has been tested with ricin, M13 phage, and *Bacillus globigii* spores.

A nanoscale detector comprised of porous silicon has been developed that can rapidly distinguish gram-positive and gram-negative microorganisms (48). Microcavities in the silicon were functionalized with an antibody alternative, a synthetic organic receptor specific for lipid A. Binding of gram-negative bacteria produced a photoluminescence red shift. No shift was observed with gram-positive bacteria.

Microspheres arranged in cavities micromachined into a silicon wafer constitute another version of a biochip. The microspheres are functionalized with antibodies or aptamers directed to specific targets. This "taste chip" technology has been tested for its ability to detect several proteins, including ricin (109, 159).

Surface plasmon resonance-based biosensors. Biosensors based on surface plasmon resonance are also being studied for use as detectors of biothreat agents. These sensors directly detect target analytes by measuring the refractive index changes that occur when a target binds to the surface of a metal-coated (generally gold or silver) surface (200, 203). Commercial instruments are large, but a small unit for field applications has been developed (Spreeta, Texas Instruments, Attleboro, MA) and is being tested for detection of staphylococcal enterotoxin B (211, 313). The miniature gold-coated sensor uses a gold-binding peptide to functionalize the surface for a specific target and tracks the angle of reflectance at a fixed wavelength to produce a signal. It is temperature controlled and has two channels, with one channel serving as a control channel. Another miniature surface plasmon resonance device uses a side-polished, single-mode optical fiber to monitor spectral changes at a fixed angle of incidence (261). This device was used to directly detect staphylococcal enterotoxin B in milk. Sensitivity was increased when a secondary antibody was used to amplify the signal (129).

Evanescent-wave biosensors. Evanescent-wave excitation of fluorophore-labeled antibodies attached to targets captured on a waveguide surface is another method of using biosensors for detection (200, 203). Several of these devices are being developed and tested for detection of biothreat agents. The RAPTOR (Research International, Monroe, WA) is a rugged biosensor that was developed by the Naval Research Laboratory (Washington, DC) (9–11, 158, 180, 212). For this format, the capture antibodies are coated onto polystyrene fiber optic waveguides encased in a coupon that can be prepared in advance. Samples are passed over the waveguides, and target analytes are captured by the antibodies. Fluorophore-labeled reporter antibodies are then passed through the coupon and bind to the captured target analytes on the waveguides. Fluorescent reporter molecules within 100 to 1,000 nm of the waveguide surface are excited by the evanescent field of the laser, and a portion of the emission energy recouples into the fiber and is quantified by the photodiode. Increases in fluorescence are proportional in rate and magnitude to the target analyte concentration and are recorded as pAmp signals.

The RAPTOR biosensor and its predecessor, the Analyte 2000 (Research International), have been used to develop biothreat agent assays in many different complex matrices. These include foods (69–71, 102, 164, 180, 181), river and spent sprout irrigation waters containing potentially interfering organic compounds and microbes (164, 212), powders (281), and clinical specimens (44, 81, 278). Furthermore, methods have been developed to recover live bacteria from the waveguides used in RAPTOR/Analyte 2000 assays to confirm viability and perform PCR and other confirmatory tests (165, 280).

The Naval Research Laboratory has also developed an array biosensor and an integrating waveguide biosensor. In the array biosensor, target analytes are captured by antibodies on a patterned glass slide. Fluorescent reporter antibodies are added and, after evanescent wave excitation by a laser, images are recorded by a charge-coupled device camera (68, 179, 276, 277). The array biosensor has been tested with target analytes in different complex matrices, including toxins in clinical fluids, environmental samples and food matrices (179), and bacteria in food (251, 277). The assay process has been automated with development of a small fluidics module that can be coupled to the array detector (79). The integrating waveguide biosensor uses a glass capillary tube as the waveguide and has been used to detect staphylococcal enterotoxin B at pg/ml levels (178).

Cantilever and acoustic wave. Cantilever technology uses changes in mass to detect target agents trapped on the device surface. Target specificity is achieved by adsorbing or attaching some type of capture molecule to the surface substrate. As target is captured, the added mass results in a frequency shift from the normal oscillation of the cantilever. An additional shift in resonance frequency is observed upon capture of the target (113). A cantilever biosensor was constructed and used to detect *E. coli* O157:H7 following immersion of the cantilevers in suspensions containing 10^6 to 10^9 cells/ml (135). No frequency shifts were observed when buffer alone or buffer containing *Salmonella enterica serovar Typhimurium* was incubated with the cantilevers.

A magnetoelastic cantilever immunosensor was developed that used a magnetic field to induce oscillation of the sensor (248, 249). The sensor surface was coated with antibodies to permit specific capture of the desired target agent. Following agent capture, alkaline phosphatase-labeled antibodies to the target were added to amplify the signal by increasing the total mass on the sensor. The sensor was tested with both *E. coli* O157:H7 and staphylococcal enterotoxin B with reported sen-

sitivities of 10^2 cells/ml and 0.5 ng/ml, respectively.

Impedance analysis of an oscillating quartz crystal was used as the basis of a piezoelectric biosensor that was tested with *Salmonella enterica serovar Typhimurium* (155). Antibodies coated onto paramagnetic microspheres provided specificity and increased the response of the sensor. The detection limit was reported as 10^3 cells/ml.

A device based on surface acoustic wave technology is under development and has been tested using spores of *Bacillus thuringiensis* as a surrogate for *Bacillus anthracis* spores (32). The use of shear horizontal waves permitted acoustic measurements in a liquid environment and preparation of the surface with a monoclonal antibody provided specific capture of the target. Detection of *Bacillus thuringiensis* spores at or below *Bacillus anthracis* spore levels for human infection via inhalation was reported.

Quantum dots and upconverting phosphors. Quantum dots are inorganic fluorescent nanocrystals that are color-tunable by varying the size and composition of the crystal core. They have a wide absorption spectrum and a narrow emission peak, which makes them ideal for multiplexing. In addition, quantum dots are highly photostable and have a high quantum yield. These novel reporters and their potential use as labels in biological assays are discussed in more detail by Chan et al. (49) and Riegler and Nann (242). Quantum dots have been used as tags to replace more traditional fluorescent dyes such as fluorescein and rhodamine. Assays to detect *E. coli* O157:H7 (275), *Cryptosporidium* and *Giardia* spp. (175, 324), and a multiplexed assay to detect cholera toxin, ricin, shiga toxin 1 (called shiga-like toxin 1 or SLT-1 by the authors), and staphylococcal enterotoxin B (107) have been developed using quantum dot-labeled antibodies.

Upconverting phosphor technology provides another labeling replacement for fluorescent dyes and utilizes unique submicron-sized ceramic particles containing a rare earth element (114, 216). These unusual labels absorb more than one photon of low energy (in the infrared) to achieve a higher energy state that is emitted as phosphorescence when the crystal returns to its ground state. Since this process does not occur naturally, there should be no background when these reporters are used as labels. Hampl et al. (114) attached upconverting phosphors to antibodies and used them in both lateral flow and plate assay formats to detect human chorionic gonadotropin and in a duplex assay to detect both human chorionic gonadotropin and ovalbumin simultaneously. Niedbala et al. (216) used upconverting phosphor technology in a lateral flow sandwich assay format. These researchers were able to detect 10^3 CFU/ml *E. coli* O157:H7 spiked into an enrichment medium containing 10^9 organisms/ml grown from a ground beef inoculum. This enriched medium served as a negative control background for the assays. Upconverting phosphor technology has also been tested in nucleic acid-based assays (60, 294, 325).

Nucleic Acid Detection

Nucleic acid-based detection uses the specificity of base pair matching to detect and identify biothreat agents. Any biothreat agent that contains DNA or RNA can be detected using nucleic acid-based detection methods; however, a limitation of these types of sensors is that they are not able to detect protein threat agents such as toxins and bioregulators. Many of the nucleic acid approaches for detection of biothreat agents are described in recent review articles (137, 139, 182, 269). Some of these development efforts focus on improving the speed, portability and simplicity of PCR while maintaining the sensitivity and specificity. Additional approaches also investigate methods to detect nucleic acids from target agents of interest using isothermal amplification or directly from samples without using an amplification step.

Amplification methods. A hand-held real-time thermal cycler has been developed that can rapidly analyze up to four prepared samples (122). This platform has been tested successfully with *Bacillus anthracis* cultured from swab samples taken after a recent bioterrorism incident and *E. coli* cultured from streamwater samples. SYBR green dye was used as the label and positive results were observed in 13 to 32 minutes. The appearance of background fluorescence was noted as a problem in the *E. coli* assays. *Erwinia herbicola* from an overnight culture was detected in approximately 7 minutes using a TaqMan probe and 500 cells as a template.

NASBA relies on the isothermal amplification of single-stranded RNA for detection of target organisms. In this method, a primer binds to the target RNA sequence and a reverse transcriptase produces a cDNA strand. RNase digests the template RNA and a second primer binds to the cDNA, which the reverse transcriptase uses to form a double-stranded cDNA. T7 RNA polymerase is then used to produce RNA transcripts via an amplification process. The uses, advantages, and limitations of NASBA have been discussed in reviews of the technique (58, 66). Assays have been developed and tested for several pathogenic microorganisms, including viruses (46, 120, 144, 145, 168, 204, 321), bacteria (18, 19, 59, 245), fungi (317), and protozoans (255). Successful detection of microorganisms in both environmental (18, 145, 168, 245) and clinical (120, 168, 204, 255, 317) samples has been reported. Data from these investigations indicate that NASBA is a sensitive, specific, and rapid analysis method. The method may also be useful for detecting viable organisms when mRNA is used as the template (58).

Loop-mediated isothermal amplification is a method of isothermally amplifying DNA using a novel strand displacement approach (217). The technique uses DNA polymerase and four specially designed primers that are specific for sequences on the sense and antisense strands of the target DNA. Loop-mediated isothermal amplification-based assays have been used to detect several viruses (222, 234, 235, 279) and bacteria (86, 130, 140, 194, 257, 265) and at least one fungus (85). Successful detection has been reported in tests involving several clinical samples (85, 140, 234, 235, 257, 279).

Microarrays. A method of identifying bacteria using rRNA was tested using *E. coli* in a mix containing *Bordetella bronchiseptica* (101). After single-stranded DNA capture of rRNA from the bacteria on a self-assembled monolayer, the bacteria were tagged with another single-stranded DNA probe labeled with fluorescein. An antifluorescein antibody labeled with peroxidase was then used to amplify the signal, followed by amperometric detection of peroxidase activity. The authors reported detection at approximately 10^3 *E. coli* cells with this method.

Many of the biochip technologies discussed under Immuno-

logical Detection have also been tested for detection of nucleic acids. The electric-field-driven assay described for immunological detection has also been used to detect bacterial DNA. Bacteria concentrated by dielectrophoresis are lysed and the DNA is denatured in a heated denaturation module. The released DNA is amplified using strand displacement amplification (304, 315) and is analyzed using an on-chip electric-field-driven hybridization assay. This electric-field-driven assay system has been used to amplify and discriminate six gene sequences from six different bacteria (304).

Another antibody-based detection chip technology that has been adapted for detection of DNA uses capillary array electrophoresis and laser-induced fluorescence to detect PCR-amplified DNA (264). DNA from an enterotoxigenic strain of *E. coli* was used to test chip performance. The results indicated that the method was comparable to gel analysis for identifying specific amplicons and offered the advantages of greater speed and multiplexing capabilities on a microarray platform.

The "taste chip" technology used in antibody-based biochips has also been used to rapidly distinguish a mixture of similar 18-base DNA oligonucleotides (6). Microbeads labeled with DNA probes were placed in micromachined cavities on a silicon chip and samples containing target DNA were recirculated through the cavities. Rapid identification of single nucleotide mismatches and a limit of detection of about 10^{-13} M using this method were reported.

A nucleic acid-based biochip for detection of cells has been developed and tested with *E. coli* K-12 cells (186). This biochip incorporates sample capture, preparation, PCR, hybridization, and electrochemical detection on a totally self-contained chip. The authors claimed positive recognition at 10^3 cells but with a very low signal-to-noise ratio.

Tissue- and Cell-Based Detection

Tissue- or cell-based detection systems use the intrinsic response of a specific cell type to a potentially toxic or infectious foreign substance to identify a biothreat agent. In these devices, the cells constituting the sensor produce an action potential signal that can be measured by an electrode or optical detector (162). The detector cells may originate from a specific unicellular organism or a tissue type, such as nerve or heart cells, and may be either primary or immortalized. Each has its advantages and disadvantages, which are reviewed by Kovacs (162). Potential applications of this approach for the detection of biothreat agents have been described (162, 270). In some instances, whole organisms may be used as the detector, but these are not generally useful in a clinical setting and will not be discussed in this review.

Biosensors based on cells from sources such as neurological and cardiac tissues are being explored for detection of harmful substances including biological threat agents (110, 162, 220, 270). One example of such a cell-based biosensor that has been tested for field use incorporates cardiomyocytes that have been genetically engineered to more selectively respond to specific functional activity (15, 65, 105). An automated biosensor based on neuronal cells has also been tested and was found to respond to the biological toxins tetrodotoxin and tityustoxin (220, 221). Neither of these biosensors has been explored for use with biological agents other than toxins. These cell-based biosensors generally are not as specific as those based on shape or sequence recognition, such as the antibody and nucleic acid sensors described above, but this characteristic can be an advantage when the threat agent is unknown.

Another type of cell-based biosensor, based on B lymphocytes, is also under development (239, 241). B lymphocytes are cells in the human body that display surface antibodies that act as pathogen receptors. Rider et al. engineered the B lymphocytes to express both aequorin, a jellyfish protein that emits light in response to calcium flux, and pathogen-specific surface antibodies (241). Although this type of cell-based biosensor provides increased specificity compared to other cell-based systems, it is subject to the same kind of antibody cross-reactivity problems as other antibody-based technologies. In addition, it faces the same problems with storage and maintenance as other cell-based systems.

Another cell type that has been investigated for detection of biological agents is chromatophores, the brightly colored cells found in the skin of cold-blooded animals (53, 198). Chromatophores are responsible for the pigmentation and camouflage exhibited by these animals and will change color when exposed to many classes of biologically active substances, such as pesticides, neurotransmitters, and bacterial toxins. The color change can be observed with a microscope or spectrophotometer. A biosensor incorporating chromatophores from fish has been developed and tested for its ability to detect harmful chemicals, biological toxins, toxin-producing pathogens, and other biologically active agents (53, 75, 259). One advantage of this biosensor is that the fish chromatophores do not grow and, therefore, the culture medium does not need to be replaced frequently.

Chemical and Physical Detection

Biosensors based on physical and chemical properties respond to specific characteristics intrinsic to the target analyte. Examples of such technologies include mass spectrometry, Raman spectrometry, and intrinsic fluorescence/luminescence. These methods do not require additional biological reagents; however, affinity probes may be used to aid in target capture and increase specificity.

A multiwavelength, UV/visible spectroscopic method of detecting pathogens that uses both the light-scattering and absorbing properties of vegetative cells and spores to generate a spectrum is under development (7, 8). It incorporates a relatively simple model to interpret the resulting spectrum. Data acquired to date indicate that vegetative cells of different species of bacteria may be distinguishable and that vegetative cells can be differentiated from spores. One problem with this method is that it currently requires extensive sample preparation to produce a pure suspension of target in a nonabsorbing medium. Furthermore, the method is nonspecific for most clinical applications.

There have been many attempts to develop a biosensor based on mass spectrometry (93, 171, 292). Mass spectrometry selectively identifies components of a sample by molecular weight analysis. The technique has been used to identify bacterial and viral proteins (72, 301, 316) and intact bacterial cells (28, 142, 147, 300, 302, 308) and to distinguish aerosolized spores of *Bacillus thuringiensis* and *Bacillus atrophaeus* (94).

Most of these approaches utilize matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). These systems are reagentless and rapid, require only a small sample volume, and potentially require little sample preparation. MALDI-TOF/mass spectrometry is theoretically capable of identifying all types of biological agents, including viruses, bacteria, fungi, and spores. Drawbacks to this approach include the requirement for a highly concentrated sample (10^5 to 10^7 cells/ml for whole-cell analysis), the need to develop complex spectral fingerprints for every target agent, and the possible lack of specificity in complex matrices or mixtures of targets. Flow field-flow fractionation is being investigated as one way of increasing specificity by separating particles in a complex matrix prior to analysis by MALDI-TOF/mass spectrometry (173, 240).

Electrospray ionization Fourier transform ion cyclotron resonance (EIS-FTICR) mass spectrometry has been used to analyze alleles from variable-number tandem repeats and single-nucleotide polymorphisms simultaneously (295). DNA was amplified and fragment size was kept to less than 200 base pairs to stay within the optimum size range for the mass spectrometry procedure. Analysis of variable-number tandem repeat and single-nucleotide polymorphism PCR products from *Bacillus anthracis* using EIS-FTICR-mass spectrometry produced results comparable to traditional gel electrophoresis.

Surface-enhanced Raman scattering (SERS) is under study as a means of identifying nucleic acids, pathogens, and toxins. As with mass spectrometry, the technique generally requires generation of fingerprints for each target analyte. Two methods of detecting the dipicolinic acid present in bacterial spores using SERS have been described (24, 322). Bell et al. improved the sensitivity of detection of dipicolinic acid by adding sodium sulfate to aggregate the silver colloid surface material and included thiosulfate as an internal standard to permit quantitation (24). Zhang et al. used a silver film over glass nanospheres to detect dipicolinic acid extracted from *Bacillus subtilis* spores, obtaining a sensitivity of approximately 10^3 spores in 20 μl (322). The sensitivity was determined to be approximately 10^4 spores in 20 μl for a field-portable detector that the authors also tested. SERS technology was incorporated into a biochip platform that used antibodies to specifically capture target analytes (112). The authors presented data indicating detection and identification of *Listeria* species, *Legionella* species, *Bacillus* species spores, and *Cryptosporidium parvum* and *Cryptosporidium meleagridis* using their biochip. The authors also reported differentiating between nonviable and viable organisms and identifying specific toxins in a mixture based on their SERS fingerprints.

The optical capture of individual particles using a near-infrared SERS method has been tested (5). Following capture, the authors used near-infrared SERS to discriminate between spores of two strains of *Bacillus stearothermophilus*. Near-infrared SERS has also been used to differentiate seven strains of *Escherichia coli* and six clinical isolates from urinary tract infections (143). SERS and a related technology, surface-enhanced resonance Raman scattering (SERRS), have been tested as assay reporters. In this approach, the target was either labeled directly with a SERS active substance (92, 223) or indirectly via gold or silver nanoparticles with attached SERS/SERRS-active compounds (45, 78, 80, 215, 314). SERS-active dyes were used to detect two oligonucleotides using a novel microfluidics platform (223) and spectra and detection limits for eight dye-labeled oligonucleotides were determined using SERRS (92). Using a sandwich hybridization technique on a biochip platform and a nanoparticle probe labeled with a SERS-active dye, Cao et al. (45) were able to differentiate between six different DNA strands and between two different RNA strands. Detection down to the femtomolar range was reported. Detection of rat and goat immunoglobulin G antibodies was reported using a sandwich assay that utilized antibody- and SERS-labeled colloidal gold nanoparticles as reporters (215). Detection of the hepatitis B virus surface antigen was also reported using an antibody- and SERS-labeled probe in a sandwich assay format (314). SERS/SERRS probes are a potential means of increasing the specificity of Raman scattering methods and eliminate the need to generate separate spectra for each target of interest.

CONCLUSION

The intentional release and dissemination of *Bacillus anthracis* spores by contaminated U.S. Postal Service mail in the fall of 2001 made public health officials acutely aware of the importance of rapidly and accurately detecting such events. Because bioterrorism is difficult to predict or prevent, reliable detection platforms are especially important to minimize dissemination of biothreat agents and to protect the public health.

The United States and other countries have committed large amounts of time, effort, and funds in recent years to develop reliable platforms to rapidly detect and identify biothreat agents. Although many different detection technologies have been introduced, few of these technologies have been extensively evaluated or reviewed under field conditions. Many challenges, including processing of complex sample matrices and detection of multiple types of agents and modified or previously uncharacterized agents in a sample, remain to be resolved.

This review has attempted to provide a survey of commercially available and developing technologies for biothreat agent detection. Only technologies that have been evaluated and published have been included. Many other technologies have not been included because insufficient published data were available to ascertain their accuracy and reliability. While an ideal platform has yet to be developed, many of the systems described in this review have proved invaluable in rapidly and accurately identifying biothreat agents. Although the risk of bioterrorism remains, detection technologies will continue to be improved to meet the challenges of this threat.

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