Chapter 18

LABORATORY IDENTIFICATION OF BIOLOGICAL THREATS

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INTRODUCTION

The ability of military laboratories to identify and confirm the presence of biological threats has significantly improved over the past decade. Identification approaches have advanced from classical identification methods performed in only a few reference laboratories to complex integrated diagnostic systems that are maturing as part of the Joint Biological Agent Identification and Diagnostic System (JBAIDS) for field laboratories. During the Persian Gulf War (1990–1991), deployed field laboratories and environmental surveillance units depended significantly on immunoassay methods with limited sensitivity and specificity. Because of intensive efforts by scientists at military reference centers, such as the US Army Medical Research Institute of Infectious Diseases (USAMRIID), the Naval Medical Research Center, the Armed Forces Institute of Pathology, and the US Air Force Institute for Operational Health, researchers are better prepared to identify and confirm the presence of the highest priority biological threats to human health (Exhibit 18-1).[1,2] However, the biological threat is more complicated than ever before. Future diagnostic and identification systems will depend on an integrated set of technologies, including new immunodiagnostic assays and rapid gene analysis methods to detect a broad spectrum of possible biological markers for diagnosing biological threats (see Exhibit 18-1).[2] The combination of several diagnostic approaches will improve reliability and confidence in laboratory results, which may shape medical treatment or response after an attack. Military and civilian clinical laboratories are now linked into a laboratory response network (LRN) for bioterrorism sponsored by the Centers for Disease Control and Prevention (CDC).[3] Together, these efforts have improved the national preparedness, but continuing research and development are needed to improve the speed, reliability, robustness, and user friendliness of the new diagnostic technologies. This chapter will review the agent identification approaches and state-of-the art diagnostic technologies available to protect and sustain the health of soldiers and other military personnel.

THE LABORATORY RESPONSE

Role of the Military Clinical and Field Laboratories

Military clinical and field laboratories play a critical role in the early recognition of biological threats. For the purposes of this chapter, a biological threat is any infectious disease entity or biological toxin intentionally delivered by opposing forces to deter, delay, or defeat US or allied military forces in the accomplishment of the mission. Biological agents can also be used in bioterrorism scenarios to create terror or panic in civilian and military populations to achieve political, religious, or strategic goals. Although the principal function of military clinical and field laboratories is to confirm the clinical diagnosis of the medical officer, laboratory staff also provide subject matter expertise in theaters of operation on the handling and identification of hazardous microorganisms and biological toxins. Because these laboratories have a global view of disease in the theater, they play an important sentinel role by recognizing unique patterns of disease. Military field laboratory personnel may also evaluate environmental samples and veterinary medicine specimens as part of a comprehensive environmental or preventive medicine surveillance system in a theater of operations.

Military Field Laboratories

If a complete medical treatment facility is part of a deployment, its intrinsic medical laboratory assets can be used. However, a medical laboratory may not be available for short duration operations in which the health service element is task organized for a specific mission. In this case, medical laboratory support should be provided by a facility outside the area of operations.[4] Army medical treatment facilities in a theater of operations have limited microbiology capabilities unless supplemented with a microbiology augmentation set (M403), which is fielded with an infectious disease physician, a clinical microbiologist, and a laboratory technician. The M403 set contains all of the necessary equipment and reagents to identify commonly encountered pathogenic bacteria and parasites, evaluate bacterial isolates for antibiotic sensitivity, and screen for some viral infections. Although this medical set does not contain an authoritative capability for definitively identifying biological warfare agents, it supports ruling out common infections. Specimens requiring more comprehensive analysis capabilities are forwarded to the nearest reference or confirmatory laboratory. After the Persian Gulf War, all of the military services recognized a need to develop additional deployable laboratory assets to support biological threat identification and preventive medicine efforts (described below).

Army

Army medical laboratories (AMLs) are modular, task-organized, and corps-level assets providing

EXHIBIT 18-1

REGULATED BIOLOGICAL SELECT AGENTS AND TOXINS

US DEPARTMENT OF HEALTH AND HUMAN SERVICES SELECT AGENTS AND TOXINS

Abrin
Cercopithecine herpesvirus 1 (Herpes B virus)
Coccidioides posadasii
Conotoxins
Crimean-Congo hemorrhagic fever virus
Diacetoxyscirpenol
Ebola virus
Lassa fever virus
Marburg virus
Monkeypox virus
Reconstructed replication competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (Reconstructed 1918 Influenza virus)
Ricin
Rickettsia prowazekii
Rickettsia rickettsii
Saxitoxin
Shiga-like ribosome inactivating proteins
South American Haemorrhagic Fever viruses
- Flexal
- Guanarito
- Junin
- Machupo
- Sabia

Tetrodotoxin
Tick-borne encephalitis complex (flavi) viruses
- Central European Tick-borne encephalitis
- Far Eastern Tick-borne encephalitis
- Kyasanur forest disease
- Omsk hemorrhagic fever
- Russian Spring and Summer encephalitis

Variola major virus (Smallpox virus) and Variola minor virus (Alastrim)
Yersinia pestis

OVERLAP SELECT AGENTS AND TOXINS

Bacillus anthracis
Botulinum neurotoxins
Botulinum neurotoxin producing species of *Clostridium*
Brucella abortus
Brucella melitensis
Brucella suis
Burkholderia mallei (formerly *Pseudomonas mallei*)
Burkholderia pseudomallei (formerly *Pseudomonas pseudomallei*)
Clostridium perfringens epsilon toxin
Coccidioides immitis
Coxiella burnetii
Eastern equine encephalitis virus
Francisella tularensis
Hendra virus
Nipah virus
Rift Valley fever virus
Shigatoxin
Staphylococcal enterotoxins
T-2 toxin
Venezuelan equine encephalitis virus

US DEPARTMENT OF AGRICULTURE SELECT AGENTS AND TOXINS

African horse sickness virus
African swine fever virus
Akabane virus
Avian influenza virus (highly pathogenic)
Bluetongue virus (Exotic)
Bovine spongiform encephalopathy agent
Camel pox virus
Classical swine fever virus
Cowdria ruminantium (Heartwater)
Foot-and-mouth disease virus
Goat pox virus
Japanese encephalitis virus
Lumpy skin disease virus
Malignant catarrhal fever virus (Alcelaphine herpesvirus type 1)
Menangle virus
Mycoplasma capricolum / M.F38 / *M mycoides Capri* (contagious caprine pleuropneumonia)
Mycoplasma mycoides mycoides (contagious bovine pleuropneumonia)
Newcastle disease virus (velogenic)
Peste des petits ruminants virus
Rinderpest virus
Sheep pox virus
Swine vesicular disease virus
Vesicular stomatitis virus (Exotic)

US DEPARTMENT OF AGRICULTURE PLANT PROTECTION AND QUARANTINE (PPQ) SELECT AGENTS AND TOXINS

Candidatus Liberobacter africanus
Candidatus Liberobacter asiaticus
Peronosclerospora philippinensis
Ralstonia solanacearum race 3, biovar 2
Schlerophthora rayssiae var *zeae*
Synchytrium endobioticum
Xanthomonas oryzae pv. *oryzicola*
Xylella fastidiosa (citrus variegated chlorosis strain)

Reproduced from: US Department of Health and Human Services and US Department of Agriculture Select Agents and Toxins, 7 CFR Part 331, 9 CFR Part 121, and 42 CFR Part 73. Available at: http://www.cdc.gov/od/sap/docs/salist.pdf. Accessed February 23, 2006.

comprehensive preventive medicine laboratory support to theater commanders. AMLs are capable of testing environmental and clinical specimens for a broad range of biological, chemical, and radiological hazards. For biological agents, the laboratory uses a variety of rapid analytical methods, such as real-time PCR, electrochemiluminescence (ECL), enzyme-linked immunosorbent assay (ELISA), and more definitive analyses involving bacterial culture, fatty acid profiling, and necropsy and immunohistochemistry.[2] AMLs have significant "reach back" capability to reference laboratories in the continental United States (CONUS) for support. The largest of the service laboratories, AMLs can identify "typical" infectious diseases including endemic disease threats and they contain redundant equipment for long-term or split-base operations. The laboratory contains all of the necessary vehicles and equipment to move and maintain itself in the field.

Navy

The Navy's forward deployable preventive medicine units (FDPMUs) are medium-sized mobile laboratories using multiple rapid techniques (polymerase chain reaction [PCR] and ELISA) for identifying biological warfare agents on the battlefield. The FDPMUs are also modular and have the ability to analyze samples containing chemical and radiological hazards. These laboratories specialize in identifying biological threat agents in concentrated environmental samples (high confidence), but they can also identify endemic infectious disease in clinically relevant samples.

Air Force

Air Force biological augmentation teams (AFBATs) use rapid analytical methods (such as real-time PCR) to screen environmental and clinical samples for threat agents. The teams are small (two persons), easily deployed, and designed to fall in on preexisting or planned facilities. The units are capable of providing early warning to commanders of the potential presence of biological threat agents.

The theater commander, in conjunction with the theater surgeon and nuclear, biological, and chemical officer, must decide which and how many of these laboratories are needed, based on factors such as the threat of a biological attack, the size of the theater, the number of detectors and sensitive sites in the theater, and the confidence level of results needed. A critical but little understood concept is that the rapid recognition of biological warfare threats must be fully integrated with preventive medicine activities and the response to endemic infectious diseases.

Laboratory Response Network

The response to future biological threats will require the entire military laboratory network. The logistical and technical burden of preparing for all possible health threats will be too great for the military clinical or field laboratories, which have limited space and weight restrictions. The most important role of these laboratories is to "listen to the hoof beats" of medical diagnosis, rule out the most common of threats, and alert the public health network about suspicious disease occurrences. The military LRN consists of the front-line medical treatment facility clinical laboratories or deployed AMLs backed by regional medical treatment facilities or military reference laboratories with access to more sophisticated diagnostic capabilities. The clinical laboratories in the regional medical centers or large medical activities are the gateways into the civilian LRN sponsored by the CDC. At the top of the military response pyramid are research laboratories, such as USAMRIID (Fort Detrick, Md) and the Naval Medical Research Center (Silver Spring, Md). Other laboratories, such as the Armed Forces Institute of Pathology (Washington, DC) and the US Air Force Institute for Operational Health (San Antonio, Texas) also provide reference laboratory services for endemic infectious diseases. Military research laboratories are best used to solve the most complex and difficult diagnostic problems, because usually they are not organized to perform high-throughput clinical sample processing and evaluation. Sentinel laboratories are generally supported by the network's designated confirmatory laboratories but may communicate directly with national laboratories when hemorrhagic fevers or orthopoxviruses (ie, smallpox virus) are suspected. The network of military laboratories with connections to federal and state civilian response systems provides unparalleled depth and resources to the biological threat response (Figure 18-1).

Biosafety and Biosecurity in the Military Clinical and Field Medical Laboratories

Biosafety Considerations

Specific guidelines for handling hazardous agents are contained in "Biosafety in Microbiological and Biomedical Laboratories" published by the US Department of Health and Human Services (DHHS).[5] By avoiding the creation of aerosols and using certain safety practices, most bacterial threats can be handled using standard microbiological practices at biosafety level (BSL) 2. BSL-2 conditions require that laboratory

personnel have specific training in handling pathogenic agents and are directed by competent scientists. Access to BSL-2 laboratories is restricted when work is being conducted and safety precautions are taken with contaminated sharp items. Procedures that may create infectious aerosols are conducted only in biological safety cabinets or other physical containment equipment. When samples must be processed on a bench top, laboratory personnel must use other primary barrier equipment, such as plexiglass shields, protective eyewear, lab coat and gloves, and work in low-traffic areas with minimum air movement. BSL-3 conditions, which consist of additional environmental controls (ie, negative pressure laboratories) and procedures, are intended for work involving indigenous or exotic agents that may cause serious or potentially lethal disease from inhalational exposure. Limited prophylactic vaccines and therapeutics may be available to treat exposed personnel in case of an accident. BSL-4 conditions are reserved for the most dangerous biological agents for which specific medical interventions are not available and an extreme risk for aerosol exposure exists. BSL-4 requires the use of negative pressure laboratories and one-piece, positive-pressure personnel suits ventilated by a life support system. Laboratory personnel should incorporate universal bloodborne pathogen precautions and follow the guidelines outlined in federal regulation 29 Code of Federal Regulations (CFR) 1910.1030, "Occupational Exposure to Blood-borne Pathogens."[6] Specific precautions for each of the highest priority biological threats can be found in the Basic Protocols for Level A (Sentinel) Laboratories (http://www.bt.cdc.gov or http://www.asm.org).

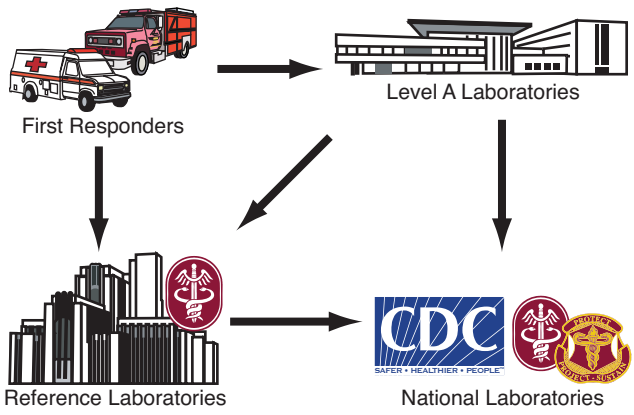

Fig. 18-1. The network of military laboratories with connections to federal and state civilian response systems provides unparalleled depth and resources to the biological threat response.

Biosurety

The 2001 anthrax letter attacks, which resulted in 22 cases of cutaneous or inhalational anthrax and five deaths, raised the national concern about the safety and security of laboratory stocks of biological threats in government, commercial, and academic laboratories.[7] As a result, the DHHS promulgated new regulations (42 CFR, Part 73) that provided substantial controls over access to biological select agents and toxins (BSATs), required registration of facilities, and established processes for screening and registering laboratory personnel.[8] DHHS and the US Department of Agriculture (USDA) identified over 80 biological agents that required these regulatory controls (see Exhibit 18-1). In addition to federal regulations, the US Department of Defense (DoD) directed additional controls for access to BSATs and required the establishment of biosurety programs. These actions were taken to foster public trust and assurance that BSATs are handled safely and securely in military laboratories. Among the services, the Army has established the most comprehensive set of draft regulations (AR 50-XX) with implementing memoranda.

At USAMRIID the framework for the military biosurety program was derived from the DoD's experience with chemical and nuclear surety programs.[9-11] These surety programs incorporate reliability, safety, and security controls to protect particular chemical and nuclear weapons. The DoD biological surety program applies many of the same controls as the chemical and nuclear surety programs to medical biological defense research and exceeds the standards of biosecurity programs in other federal and nonfederal laboratories.

Every military facility that stores and uses BSATs must be registered not only with the CDC (see 42 CFR Part 73) but also with the DoD.[8,9] In the case of Army laboratories, registrations are completed through the Assistant Secretary of the Army (Installation and Environment). Army clinical laboratories, especially those participating in the LRN triservice initiative, are coordinated through the Army Medical Command health policy and services. Not all clinical laboratories need to be registered. However, unregistered laboratories must follow the 42 CFR 73 "Clinical Laboratories Exemption," which states that clinical laboratories identifying select agents have 7 days to forward or destroy them. The transfer of BSAT cultures requires the exchange of transfer documents (ie, CDC/APHIS Form 2) between CDC-registered facilities.

Laboratory directors who supervise activities that stock BSATs must be prepared to implement a variety of stringent personnel, physical security, safety, and agent-inventory guidelines. The law established penalties of

TABLE 18-1

KEY IDENTITY MARKERS FOR SELECTED BIOLOGICAL SELECT AGENTS AND TOXINS

Biological Select Agent and Toxin	Key Identity Markers	Biosafety Level*	Confirmatory Methods
Anthrax	Gram-positive rod; spore-forming; aerobic; nonmotile; catalase positive; large, gray-white to white; nonhemolytic colonies on sheep blood agar plates	2	• Gamma phage sensitivity • Immunohistochemistry • PCR
Botulism	Gram-positive rod; spore-forming; obligate anaerobe catalase negative; lipase production on egg yolk agar; 150,000 dal protein toxin (types A,B,C,D,E,F,G); 2 subunits	2	• Immunoassay • Mouse neutralization assay • PCR
Plague	Gram-negative coccobacilli often pleomorphic; nonspore forming; facultative anaerobe; nonmotile beaten copper colonies (MacConkey's agar)	2	• Immunofluorescence assay • PCR
Smallpox	Large double-stranded DNA virus; enveloped, brick-shaped morphology; Guarnieri bodies (virus inclusions) under light microscopy	4	• PCR • EM • Immunohistochemistry • Immunoassay
Tularemia	Extremely small, pleomorphic, gram-negative coccobacilli; nonspore forming; facultative intracellular parasite; nonmotile; catalase positive opalescent smooth colonies on cysteine heart agar	2	• PCR • Immunoassay
Ebola	Linear, negative-sense single-stranded RNA virus; enveloped; filamentous or pleomorphic, with extensive branching, or U-shaped, 6-shaped, or circular forms; limited cytopathic effect in Vero cells	4	• PCR • EM • Immunoassay • Immunohistochemistry
Marburg	Morphologically identical to Ebola virus	4	• PCR • EM • Immunoassay • Immunohistochemistry
Viral encephalitides	Linear positive-sense single-stranded RNA virus; enveloped, spherical virions with distinct glycoprotein spikes; cytopathic effect in Vero cells	3	• PCR • EM • Immunoassay • Immunohistochemistry
Ricin toxin	60,000–65,000 dal protein toxin; 2 subunits castor bean origin	2	• Immunoassay

Data sources: (1) Burnett JC, Henchal EA, Schmaljohn AL, Bavari S. The evolving field of biodefense: therapeutic developments and diagnostics. *Nat Rev Drug Discov*. 2005;4:281–297. (2) Henchal EA, Teska JD, Ludwig GV, Shoemaker DR, Ezzell JW. Current laboratory methods for biological threat agent identification. *Clin Lab Med*. 2001;21:661–678.

*BSL-2 bacterial agents must be handled at BSL-3 with additional precautions or in a biological safety cabinet if laboratory procedures might generate aerosols.

EM: electron microscopy

PCR: polymerase chain reaction

up to $250,000 (individual) or $500,000 (organization) for each violation. Enhanced safety procedures are required to work with or store BSATs. The DoD Biological Defense Safety Program is codified in Title 32 United States Code Part 627 and published as Army Regulation 385-69. Guidelines for the safe handling of BSATs can be found in CDC guidelines "Biosafety in Microbiological and Biomedical Laboratories."[5] Although many bacterial agents can be handled in the BSL-2 clinical laboratory (Table 18-1), most work

requires at least a class II biological safety cabinet or hood and BSL-3 practices if there is a potential to create aerosols.[5] Biosurety guidelines require that personnel complete biological safety training before having access to BSATs. A key goal of the guidelines is to prevent access to BSATs by unauthorized personnel. In addition to locked doors and freezers, continuous monitoring of areas where BSATs are held is required. Moreover, the capability to respond to the loss of agent must be incorporated into a response plan. Physical security of a facility by armed guards who can respond in minutes is a component of Army regulations.

Perhaps the most controversial of the DoD and Army guidelines is the requirement for a personnel reliability program, which requires that reviewing officials (usually the military unit commander, laboratory director, or otherwise delegated officer) aided by certifying officials (or employee supervisors) review the suitability of every staff member with access to BSATs with regard to behavioral tendencies, characteristics, medical history, financial history, work habits, attitude, training, and more. Additionally, employees are actively screened for illegal drug use through urinalysis and alcohol abuse by observation. The biosurety personnel reliability program incorporates the requirements of the chemical and nuclear surety programs, which were not incorporated into federal law (except for the need for national agency and credit checks). The DoD views the personnel reliability program as essential because threat assessments have identified the lone disgruntled insider as the most serious threat to the biodefense program. On-site and off-site contractors who support DoD programs must implement the same safeguards under the current policies. These regulations may seem excessive because many BSATs can be obtained from natural sources; however, the DoD and the Army provided these guidelines to minimize risks associated with the release of a high-consequence pathogen from military facilities.

IDENTIFICATION APPROACHES

Specimen Collection and Processing

Clinical specimens can be divided into three different categories based on the suspected disease course: (1) early postexposure, (2) clinical, and (3) convalescent.[12] The most common specimens collected include nasal and throat swabs, induced respiratory secretions, blood cultures, serum, sputum, urine, stool, skin scrapings, lesion aspirates, and biopsy materials.[2] Nasal swab samples should not be used for making decisions about individual medical care; however, they should support the rapid identification of a biological threat (post-attack) and subsequent epidemiological surveys.[13,14] After overt attacks with a suspected biological agent, baseline serum samples should be collected on all exposed personnel. In the case of suspicious deaths, pathology samples should be taken at autopsy to assist in outbreak investigations. Specimens and cultures containing possible select biological agents should be handled in accordance with established biosafety precautions. Specimens should be sent rapidly (within 24 hours) to the analytical laboratory on wet ice at 2°C to 8°C. Blood cultures should be collected before the administration of antibiotics and shipped to the laboratory within 24 hours at room temperature (21°C–23°C). Blood culture bottles incubated in continuous monitoring instrumentation should be received and placed within 8 hours of collection. Overseas (OCONUS) laboratories should not attempt to ship clinical specimens to CONUS reference laboratories using only wet ice. Shipments requiring more than 24 hours should be frozen on dry ice or liquid nitrogen. Specific shipping guidance should be obtained from the supporting laboratory before shipment. Specimens for complex analysis, such as gene amplification methods, should not be treated with permanent fixatives (eg, formalin or formaldehyde). International, US, and commercial regulations mandate the proper packing, documentation, and safe shipment of dangerous goods to protect the public, airline workers, couriers, and other persons who work for commercial shippers and who handle the dangerous goods within the many segments of the shipping process. In addition, proper packing and shipping of dangerous goods reduces the exposure of the shipper to the risks of criminal and civil liabilities associated with shipping dangerous goods, particularly infectious substances. Specific specimen collection and handling guidelines for the highest priority bioterrorism agents are available from CDC and the American Society for Microbiology (see http://www.bt.cdc.gov or http://www.asm.org).

Clinical Microbiological Methods

Laboratory methods for biological threat agent identification were previously reviewed in this chapter.[2,15] Specific LRN guidelines for identifying the highest priority (category A) bioterrorism agents can be obtained from the CDC (http:\www.bt.cdc.gov). The physician's clinical observations and direct smears of clinical specimens should guide the analytical plan (see Table 18-1).[2,15] Most aerobic bacterial threat agents can

be isolated by using four bacteriological media: (1) 5% sheep blood agar (SBA), (2) MacConkey agar (MAC), (3) chocolate agar (CHOC), and (4) cystine heart agar (CHA) supplemented with 5% sheep blood. Nonselective SBA supports the growth of *Bacillus anthracis*, *Brucella*, *Burkholderia*, and *Yersinia pestis*. MAC agar, which is the preferred selective medium for gram-negative *Enterobacteriaceae*, supports *Burkholderia* and *Y pestis*. CHA is the preferred medium for *Francisella tularensis*, but CHOC agar also suffices. A liquid medium, such as thioglycollate broth or trypticase soy broth, can also be used followed by subculturing to SBA or CHOC when solid medium initially fails to produce growth. The selection of culture medium can be modified when the target microorganism is known or highly suspected; however, in most cases, the use of multiple media options is recommended. Liquid samples can be directly inoculated onto solid agar and streaked to obtain isolated colonies. Specific culture details for the highest priority biological threats are available from the CDC (www.bt.cdc.gov).

Antibiotic Susceptibility Testing

Screening for unique antibiotic resistance or susceptibility may be critical to recognizing organisms that acquire natural or directed enhancements. Multiple drug-resistant *Y pestis, Brucella abortus, and Burkholderia* strains have been identified.[16-20] In addition to classical Kirby-Bauer disk diffusion antibiotic susceptibility tests or minimum inhibitory concentration determinations, a variety of commercial antibiotic susceptibility testing devices for use by community hospitals have been standardized to reduce the time required to achieve results.[21-24] Unfortunately, these more rapid tests may not always be optimum for detecting emerging resistance. Although standardization of protocols by the Clinical and Laboratory Standards Institute has ensured reproducibility of results, emerging technology for detecting resistance markers is not available in most clinical laboratories. In addition, detecting progressive stepwise resistance is limited to known and standardized techniques.[25] Molecular methods that could enhance screening for unique genetic markers of resistance have been developed[26-30]; however, genetic analysis approaches can be cumbersome when multiple loci are involved, as in the case of resistance to antibiotics related to tetracycline or penicillin.[29,30] DNA microarrays offer the potential for simultaneous testing for specific antibiotic resistance genes, loci, and markers.[28,29] Grimm et al differentiated 102 of 106 different TEM beta-lactamase variant sequences by using DNA microarray analysis.[29] However, a comprehensive database of resistance genetic determinants for many biological threats is not available, and new loci may emerge. In response to the problem of emerging enteric diseases, an electronic network has been established to detect outbreaks of selected foodborne illnesses by using pulsed-field gel electrophoresis.[31,32] Fontana et al demonstrated pulsed-field gel electrophoresis combined with ribotyping (a molecular method based on the analysis of restriction fragment length polymorphisms of ribosomal RNA genes) as an effective approach for detecting multidrug-resistant *Salmonella*.[32] Applying these methods to the broader array of potential threats should be an intensive future research effort.

Immunodiagnostic Methods

An integrated approach to agent detection and identification, which is essential for a complete and accurate disease diagnosis, provides the most reliable laboratory data.[2] Immunodiagnostic techniques may play a key role in diagnosing disease by detection of agent-specific antigens and/or antibodies present in clinical samples. The most significant problem associated with the development of an integrated diagnostic system has been the inability of such technologies to detect agents with sensitivities approaching those of more sensitive nucleic-acid–detection technologies. These differences in assay sensitivity increase the probability of obtaining disparate results, which could complicate medical decisions. However, recent advances in immunodiagnostic technologies provide the basis for developing antigen- and antibody-detection platforms capable of meeting requirements for sensitivity, specificity, assay speed, robustness, and simplicity.

Detecting specific protein or other antigens or host-produced antibodies directed against such antigens constitutes one of the most widely used and successful methods for identifying biological agents and diagnosing the diseases they cause. Nearly all methods for detecting antigens and antibodies rely on the production of complexes made of one or more receptor molecules and the entity being detected.

Traditionally, assays for detecting proteins and other non-nucleic acid targets, including antigens, antibodies, carbohydrates, and other organic molecules, were conducted using antibodies produced in appropriate host animals. As a result, these assays were generically referred to as immunodiagnostic or immunodetection methods. In reality, numerous other nonantibody molecules, including aptamers, peptides, and engineered antibody fragments, are now being used in affinity-based detection technologies.[33-42]

Diagnosing disease by immunodiagnostic technologies is a multistep process involving formation of complexes bound to a solid substrate. This process is like making a sandwich: detecting the biological agent or antibody depends on incorporating all the "sandwich" components. Elimination of any one part of the sandwich results in a negative response (Figure 18-2). The primary ligands used in most immunoassays are polyclonal or monoclonal antibodies or antibody fragments.

Binding one or more of the antibodies onto a solid substrate is usually the first event of the assay reaction cascade. Immunoassays can generally be termed as either heterogeneous or homogeneous, depending on the nature of the solid substrate. A heterogeneous assay requires physical separation of bound from unbound reactants by using techniques such as washing or centrifugation. These types of assays can remove interfering substances and are, therefore, usually more specific. However, heterogeneous assays require more steps and increased manipulation that cumulatively affect assay precision. A homogeneous assay requires no physical separation but may require pretreatment steps to remove interfering substances. Homogeneous assays are usually faster and more conducive to automation because of their simplicity. However, the cost of these assays is usually greater because of the types of reagents and equipment required.

The final step in any immunoassay is the detection of a signal generated by one or more assay components. This detection step is typically accomplished by using antibodies bound to (or labeled with) inorganic or organic molecules that produce a detectable signal under specific chemical or environmental conditions. The earliest labels used were molecules containing radioactive isotopes; however, radioisotope labels have generally been replaced with less cumbersome labels such as enzymes. Enzymes are effective labels because they catalyze chemical reactions, which can produce a signal. Depending on the nature of the signal, the reactants may be detected visually, electronically, chemically, or physically. Because a single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction, these labels are effective at amplifying assay signals. Most common enzyme-substrate reactions used in immunodiagnostics produce a visual signal that can be detected with the naked eye or by a spectrophotometer.

Fluorescent dyes and other organic and inorganic molecules capable of generating luminescent signals are also commonly used labels in immunoassays. Assays using these molecules are often more sensitive than enzyme immunoassays but require specialized instrumentation and often suffer from high background contamination from the intrinsic fluorescent

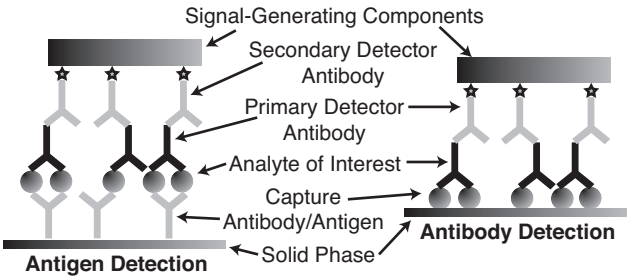

Fig. 18-2. Standard Sandwich Immunoassay. Detecting the biological agent or antibody depends on incorporating all the "sandwich" components. Elimination of any one part of the sandwich results in a negative response.

and luminescent qualities of some proteins and light-scattering effects. Signals in assays using these types of labels are amplified by integrating light signals over time and cyclic generation of photons. Other commonly used labels include gold, latex, and magnetic or paramagnetic particles. Each of these labels, which can be visualized by the naked eye or by instruments, are stable under a variety of environmental conditions. However, because these labels are essentially inert, they do not produce an amplified signal. Signal amplification is useful and desirable because it results in increased assay sensitivity.

Advances in biomedical engineering, chemistry, physics, and biology have led to an explosion of new diagnostic platforms and assay systems that offer great promise for improving diagnostic capabilities. The following overview discusses technologies currently used for identifying biological agents and also used (or under development) for diagnosing the diseases caused by these agents.

Enzyme-Linked Immunosorbent Assay

Since the 1970s the ELISA has remained a core technology for diagnosing disease caused by a wide variety of infectious and noninfectious agents. As a result, the ELISA is perhaps the most widely used and best understood immunoassay technology. Developed in many formats, assays can be designed to detect either antibodies produced in response to infection or antigens associated with the agents themselves. ELISAs that detect biological agents or agent-specific antibodies are heterogeneous assays in which an agent-specific antigen or host-derived antibody is captured onto a plastic multi-well plate by an antibody or antigen previously bound to the plate surface (capture moiety). Bound antigen or antibody is then detected using a secondary antibody (the detector antibody). The detector antibody can be directly labeled with a

signal-generating molecule or it can be detected with another antibody labeled with an enzyme. These enzymes catalyze a chemical reaction with substrate, which results in a colorimetric change. The intensity of this color can be measured by a modified spectrophotometer that determines the optical density of the reaction by using a specific wavelength of light. In many cases, the assay can be interpreted without instrumentation by simply viewing the color that appears in the reaction vessel.

The major advantage of ELISAs is their ability to be configured for a variety of uses and applications. Use of ELISAs in field laboratory settings is possible but does require certain fixed-site logistical needs, such as controlled temperature incubators and refrigerators, the power needed to run them, and other ancillary equipment needs. In addition, ELISAs are commonly used and understood by clinical laboratories and physicians, are amenable to high-throughput laboratory use and automation, do not require highly purified antibodies, and are relatively inexpensive to perform. The major disadvantages are that they are labor intensive, temperature dependent, have a narrow antigen concentration dynamic range that makes quantification difficult, and are relatively slow.

The DoD has successfully developed antigen-detection ELISAs for nearly 40 different biological agents and antibody-detection ELISAs for nearly 90 different agents. All of these assays were developed by using the same solid phase buffers and other reagents, incubation periods, incubation temperatures, and general procedures (Table 18-2). Although there is significant variation in assay limits of detection, ELISAs typically are capable of detecting as little as 1 ng of antigen per mL of sample.

Electrochemiluminescence

Among the most promising new immunodiagnostic technologies is a method based on electrochemiluminescence (ECL) detection. One ECL system makes use of antigen-capture assays and a chemiluminescent label (ruthenium [Ru]) and includes magnetic beads to concentrate target agents. These beads are coated with capture antibody, and in the presence of biological agent, immune complexes are formed between the agent and the labeled detector antibody. Because of its small size (1,057 kDa), Ru can be easily conjugated to any protein ligand by using standard chemistries without affecting immunoreactivity or solubility of the protein. The heart of the ECL analyzer is an electrochemical flow cell with a photodetector placed just above the electrode. A magnet positioned just below the electrode captures the magnetic-bead-Ru-tagged

TABLE 18-2

COMPARISON OF IMMUNODIAGNOSTIC METHODS

	Enzyme-Linked Immunosorbent Assay	Dissociation-enhanced lanthanide fluorescence immunoassay time-resolved fluorescence	Electrochemi-luminescence	Flow-Based	Hand-Held Assay
Assay Parameters					
Incubation time	3.5 h	2.2 h	15 min	30 min	15 min
Number of steps	5	4	1	1	1
Detection method	Colorimetric	Fluorescence	Chemiluminescence	Fluorescence	Visual
Multiplexing	No	Potential	No	Yes	Potential
Key Performance Parameters					
Intra-assay variation (%)	15–20	20–50	2–12	10–25	Undetermined
Limit of detection: *Yersinia pestis* F1 (colony-forming units)	250,000	250	500	62,500	125,000
Limit of detection: Staphylococcal enterotoxin B (ng)	0.63	0.04	0.05	3.13	6.25
Limit of detection: Venezuelan equine encephalitis virus (plaque-forming units)	1.25 x 107	3.13 x 106	1.0 x 107	3.13 x 108	6.25 x 108

immune complex and holds it against the electrode. The application of an electric field results in a rapid electron transfer reaction between the substrate (tripropylamine) and the Ru. Excitation with as little as 1.5 v results in light emission, which in turn is detected. The magnetic beads provide a greater surface area than conventional surface-binding assays like the ELISA. The reaction does not suffer from the surface steric and diffusion limitations encountered in solid-phase immunoassays; instead, it occurs in a turbulent bead suspension, thus allowing for rapid-reaction kinetics and short incubation time. Detection limits as low as 200 fmol/L with a linear dynamic range can span six orders of magnitude.[43-44]

A field-ready ECL system consists of an analyzer and a personal computer with software. ECL systems possess several advantages, including speed, sensitivity, accuracy, and precision over a wide dynamic range. In a typical agent-detection assay, sample is added to reagents consisting of capture antibody-coated paramagnetic beads and a Ru-conjugated detector antibody. Reagents can be lyophilized. After a short, 15-minute incubation period, the analyzer draws the sample into the flow cell, captures and washes the magnetic beads, and measures the electrochemiluminescent signal (up to 1 min per sample cleaning and reading time). The system uses 96-well plates and is therefore able to handle large sample throughput requirements.

The ECL system has been demonstrated to be effective for detecting staphylococcal enterotoxin B, ricin toxin, botulinum toxin, *F tularensis*, *Y pestis* F1 antigen, *B anthracis* protective antigen, and Venezuelan equine encephalitis virus.[2,45,46] The ECL system, which has been demonstrated in field settings, is used as one part of an integrated diagnostic system in several deployable and deployed laboratories. Critical assay performance characteristics and detection limits from three typical ECL agent-detection assays are shown in Table 18-2.

Time-Resolved Fluorescence

Time-resolved fluorescence (TRF) is an immunodiagnostic technology with assays available for detecting agent-specific antibodies, microorganisms, drugs, and therapeutic agents.[47-49] In practice, TRF-based assays are sandwich-type assays similar to those used for ELISA. The solid phase is a micro-well plate coated in some manner with specific capture antibody (similar to that used with colorimetric ELISA platforms). However, instead of being labeled with enzymes, detector antibodies are labeled with lanthanide chelates. The technology takes advantage of the differential fluorescence lifespan of lanthanide chelate labels compared to background fluorescence. The labels have an intense, long-lived fluorescence signal and a large Stokes shift, which result in an assay with a very high signal-to-noise ratio and high sensitivity.[50] Unlike ECL, TRF produces detectable fluorescence through the excitation of the lanthanide chelate by a specific wavelength of light. Fluorescence is initiated in TRF with a pulse of excitation energy, repeatedly and reproducibly. In 1 second, the fluorescent material can be pulse-excited 1,000 times with an accumulation of the generated signal. One TRF format is dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) in which dissociation of the complex-bound chelate caused by adding a low-pH enhancement solution forms long-lasting fluorescent micelles. Detection limits as low as 10^{-17} moles of europium per well with a dynamic range of at least four orders of magnitude have been demonstrated.

The strength of DELFIA assays derives from their sensitivity, similarity to the commonly used ELISA techniques, and potential for multiplexing. Four different lanthanides are available (europium, samarium, terbium, and dysprosium), and each has its own unique narrow emission spectrum.[51] Both immunoassays and nucleic acid detection assays are compatible with this platform. Like the ECL assays, DELFIA assays require purified high-quality antibodies. Critical assay performance characteristics and assay limits of detection from three typical DELFIA agent detection assays are shown in Table 18-2. Although a field-ready version of this instrument is not available, the system is common to clinical laboratories and is used by the CDC-sponsored LRN.

Flow Cytometry

Flow cytometry, the measurement of physical and chemical characteristics of small particles, has many current research and healthcare applications and is commonplace in most large clinical laboratories. Applications include cytokine detection, cell differentiation, chromosome analysis, cell sorting and typing, bacterial counting, hematology, DNA content, and drug discovery. The technique involves placing biological samples (ie, cells or other particles) into a liquid suspension. A fluorescent dye, the choice of which is based on its ability to bind to the particles of interest, is added to the solution. The suspension is made to flow in a stream past a laser beam. The light is scattered, showing distribution and intensity characteristic of the particular sample. A wavelength of the light is selected that causes the dye, bound to the particle of interest, to fluoresce, and a computer counts or analyzes the fluorescent sample as it passes through the laser beam.

Using the same excitation source, the fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and the signals interpreted by specialized software. A number of multiplexed flow cytometry assays have been demonstrated.[52] Particles can also be sorted from the stream and diverted into separate containers by applying a charge to the particles of interest.

One commercially available platform is a rapid assay system that reportedly can perform up to 100 tests simultaneously on a single sample. This system incorporates three familiar technologies: (1) bioassays, (2) microspheres, and (3) fluorescence. The system consists of a flow cytometer with a specific digital signal processing board and control software. Assays occur in solution, thus allowing for rapid reaction kinetics and shorter incubation times. Capture antibodies or ligands are bound to microspheres labeled with two spectrally distinct fluorochromes. By adjusting the ratio of each fluorochrome, microspheres can be distinguished based on their spectral address. Bioassays are conducted on the surfaces of these microspheres. Detector antibodies are labeled with any of a number of different green fluorescent dyes. This detector-bound fluorochrome measures the extent of interaction that occurs at the microsphere surface, ie, it detects antigen in a typical antigen-detection assay. The instrument uses two lasers: one for detecting the microsphere itself, and the other for the detector. Microspheres, which are analyzed individually as they pass by two separate laser beams, are classified based on their spectral address and are measured in real time. Thousands (20,000) of microspheres are processed per second, resulting in an assay system theoretically capable of analyzing up to 100 different reactions on a single sample in just seconds. The manufacturer reports assay sensitivities in the femtomole level, a dynamic range of three to four orders of magnitude, and highly consistent and reproducible results.[53] Because the intensity of the fluorescent label is read only at the surface of each microsphere, any unbound reporter molecules remaining in solution do not affect the assay, making homogeneous assay formats possible. The system, which can be automated, can use tubes as well as 96- and 384-well plates. Many multiplexed assay kits are commercially available from a number of manufacturers for various cytokines, phosphoproteins, and hormones.

Critical assay performance characteristics and limits of detection from three typical flow-based agent-detection assays are shown in Table 18-2. No field-ready versions of these instruments are available, however, limiting the practical use of this platform in deployment situations, and no commercial or DoD sources for biothreat agent assays are available for this platform.

Lateral Flow Assays

Commercially produced lateral flow assays, which have been on the market for many years, are so simple to use and interpret that some types are approved for over-the-counter use by the US Food and Drug Administration. Lateral flow assays are typically designed on natural or synthetic membranes contained within a plastic or cardboard housing. A capture antibody (for antigen detection) or antigen (for antibody detection) is bound to the membrane, and a second antibody labeled with a visible marker element is placed on a sample application pad. As the sample flows across the membrane, antigen or antibody present in the sample binds to the labeled antibody and is captured as the complex passes the bound antibody or antigen (Figure 18-3). Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane.

One of the greatest advantages of lateral flow assays is their lack of reliance on instrumentation and the associated logistical needs. However, this lack of instrumentation decreases the utility of the tests because results cannot be quantified. To respond to this deficiency, several technologies are being developed to make these assays more quantitative (they also increase the assays' sensitivity). One technology allows for quantitative interpretation of the lateral flow assay.[54] Another method for quantitative detection of antibody/antigen complex formation in lateral flow assays uses up-converting phosphors.[55,56] Paramagnetic particles have similarly been used in assays and instruments capable of detecting changes in magnetic flux within the capture zone, improving sensitivity by as much as several orders of magnitude over more traditional lateral flow assays.

Lateral flow assays are commonly used by the DoD for detecting biological threat agents. In addition, several companies have begun to market a variety of threat agent tests for use by first responders. However, independent evaluation of these assays has not typically been performed, so data acquired from the use of these assays must be interpreted carefully. Another common disadvantage of lateral flow assays is their inability to run a full spectrum of control assays on a single strip assay. Only flow controls are included with most lateral flow assays. These controls show that the conditions were correct for reagent flow across the membrane but do not indicate the ability of the assay to appropriately capture antigen.

Molecular Detection Methods

Polymerase Chain Reaction

Originally conceived in 1983 by Kary Mullis at the Cetus Corporation,[57] polymerase chain reaction (PCR) became a reality only 2 years later with the publication by Saiki et al of its first practical application.[58] This first description of PCR by Mullis et al marked a milestone in biotechnology and the beginning of the field now known as molecular diagnostics. PCR is a simple, in-vitro chemical reaction that permits the synthesis of almost limitless quantities of a targeted nucleic acid sequence. At its simplest, the PCR consists of target DNA (also called template DNA), two oligonucleotide primers that flank the target DNA sequence to be amplified, a heat-stable DNA polymerase, a defined solution of salts, and an equimolar mixture of deoxyribonucleotide triphosphates (dNTPs). The mixture is then subjected to repeated cycles of defined temperature changes that help to facilitate denaturation of the template DNA, annealing of the primers to the target DNA, and extension of the primers so that the target DNA sequence is replicated. A typical PCR protocol comprises 30 to 50 thermal cycles. Each time a cycle is completed, there is a theoretical doubling of the target sequence. Therefore, under ideal conditions, a single copy of a nucleic acid target can be multiplied over a billion-fold after 30 cycles. The whole procedure is carried out in a programmable thermal cycler that precisely controls the temperature at which the steps occur, the length of time the reaction is held at the different temperatures, and the number of cycles. The PCR products are typically visualized as bands on an agarose gel after electrophoresis and staining with a DNA intercalating dye such as ethidium bromide or Sybr green.

In multiplex PCR, two or more sets of primers specific for different targets are included in the same reaction mixture, allowing for multiple target sequences to be amplified simultaneously.[59] The primers used in multiplexed reactions must be carefully designed to have similar annealing temperatures and lack complementarity. Multiplex PCR assays have played a larger role in human and cancer genetics than in the detection of infectious organisms, where they have proven more complicated to develop and often result in lower sensitivity than PCR assays using single primer sets.

Reverse Transcriptase-PCR

The PCR method described previously was designed to amplify DNA. However, many important human diseases are caused by viruses with an RNA genome. Therefore, reverse transcriptase PCR (RT-PCR) was developed to amplify specific RNA targets. In this process, extracted RNA is first converted to complementary

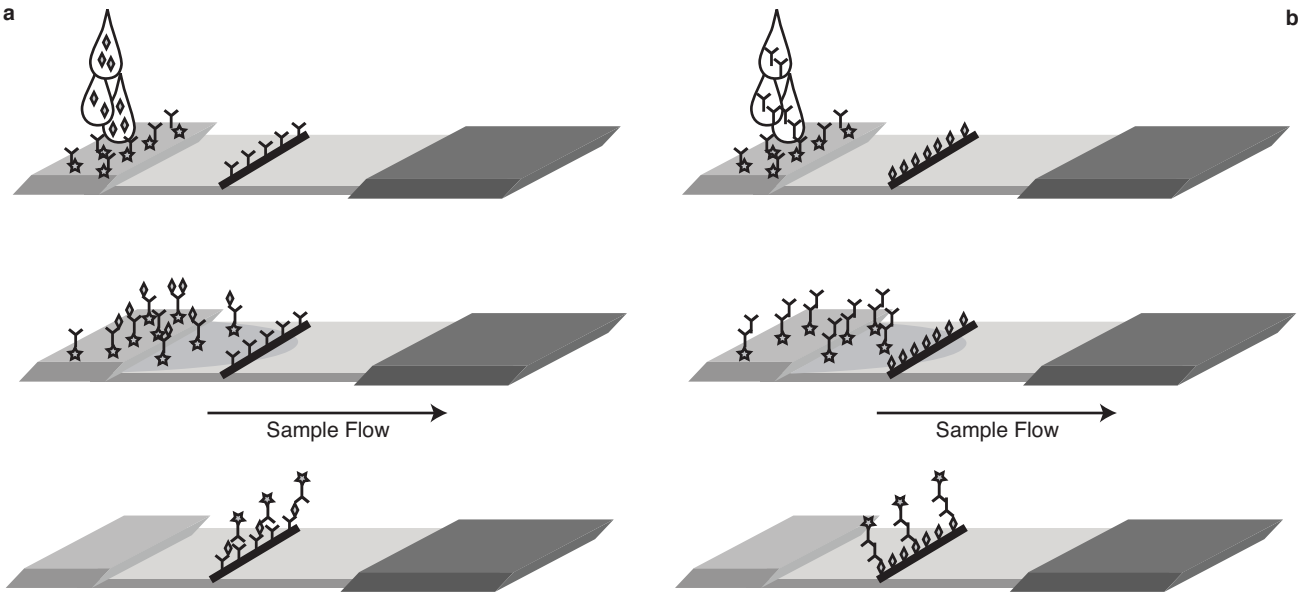

Fig. 18-3. Lateral flow assay format: A capture antibody (for antigen detection [a]) or antigen (for antibody detection [b]) is bound to the membrane, and a second antibody labeled with a visible marker element is placed on a sample application pad. As the sample flows across the membrane, antigen or antibody present in the sample binds to the labeled antibody and is captured as the complex passes the bound antibody or antigen.

DNA (cDNA) by reverse transcription, and then the cDNA is amplified by PCR. As originally described, reverse transcription of RNA into cDNA was carried out using retroviral RT enzymes from either avian myeloblastosis virus or Moloney murine leukemia virus. These enzymes are heat-labile and cannot be used at temperatures above about 42°C, which presents problems in terms of both nonspecific primer annealing and inefficient primer extension resulting from the potential formation of RNA secondary structures. These problems have largely been overcome by the development of a thermostable DNA polymerase derived from *Thermus thermophilus,* which, under the right conditions, can act as both a reverse transcriptase and a DNA polymerase.[60,61] These and other similar enzymes can amplify RNA targets without the need for a separate RT step. Thus, this so-called "one-step" RT-PCR eliminates the need for the cumbersome, time consuming, and contamination-prone transfer of RT products to a separate PCR tube. Commercial RT-PCR assays are available for detecting a few important RNA viruses such as hepatitis C virus and human immunodeficiency virus, with numerous others published in the scientific literature as in-house or "home-brew" assays.

Real-Time PCR

By far the most important development in rapid identification of biological agents has been the development of "real-time" PCR methods. Although traditional PCR was a powerful analytical tool that launched a revolution in molecular biology, it was difficult to use in clinical and field laboratories. As originally conceived, gene amplification assays could take more than 5 to 6 hours to complete, not including the sample processing required before amplification. The improvement of assay throughput came with the development of assay chemistries that allowed the PCR reaction to be monitored during the exponential amplification phase on fast thermocyclers. Lee et al and Livak et al demonstrated assays based on the detection and quantification of fluorescent reporters that increased in direct proportion to the amount of PCR product in a reaction.[62,63] By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase, in which the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during cycles 3 through 15 indicates the detection of accumulated PCR product. There are three main probe-based fluorescence-monitoring systems for DNA amplification: (1) hydrolysis probes, (2) hybridization probes, and (3) DNA-binding agents. Hydrolysis probes most exemplified by TaqMan (Applied Biosystems, Foster City, Calif) chemistries have been the most successful for rapidly identifying biological threats. Probe hydrolysis assays use the fluorogenic 5′ exonuclease activity of Taq polymerase.

Fast thermocycling was achieved first by using small volume assays in sealed capillary tubes placed in convection ovens and later by solid-state electronic modules.[64,65] Optimal assay development coupled to instrument improvements has allowed the identification of selected biological agents within 20 to 40 minutes after specimen processing. Over 50 assays against 26 infectious agents have been developed using these approaches by the DoD, the CDC, and the US Department of Energy.[2] Commercially available rapid thermocycling instruments that can detect the fluorescent signals are now available from several sources, including Applied Biosystems (Foster City, Calif), Roche Diagnostics (Indianapolis, Ind), Idaho Technologies (Salt Lake City, Utah), Cepheid (Sunnyvale, Calif), and Bio-Rad (Hercules, Calif). The Idaho Technologies Ruggedized Advanced Pathogen Identification Device (RAPID) instrument has been incorporated into the first generation of the JBAIDS for use in field medical laboratories. By using new sample-processing techniques, the presumptive identification of most biological agents can be completed in 3 hours or less with rapid fluorescent-probe–based methods, compared to approximately 6 hours with older PCR methods. Other assay formats, such as fluorescent resonance energy transfer, have allowed the resolution of closely related species and mutation detection by characterizing the melting point of the detection probe.[66,67] The demonstration of integrated sample preparation and gene amplification cartridges (such as Genexpert; Cepheid, Sunnyvale, Calif) has the potential to improve the reliability of PCR identification of biothreats by decreasing the need for extensive operator training and assay contamination.[68] Integrated cartridge gene amplification systems have been incorporated into the biohazard detection systems deployed to protect the US Postal Service.[69]

TIGER

A significant obstacle for detecting future biothreats is the requirement of many technologies, such as immunoassays and most gene amplification methods, to have identified target biomarkers ahead of time. A unique coupling of broadly targeted gene amplification with mass-based detection of amplified products may allow for early recognition of replicating etiological agents without any preknowledge of

the targets. Sampath and Ecker have described the amplification of variable gene regions flanked by conserved sequences, followed by electrospray ionization mass spectrometry and base composition analysis of the products.[70,71] This method, known as TIGER (triangulation identification for genetic evaluation of risks), provides for a high-throughput, multiple detection and identification system for nearly all known, newly emergent, and bioengineered agents in a single test (http://www.ibisrna.com/; valid August 8, 2004). This rapid, robust, and culture-free system could have been used to identify agents such as SARS-related coronaviruses, before their recognition and characterization by traditional methods.[71] Robust and portable TIGER systems are being developed for civilian and military applications.

EMERGING THREATS

The emergence of new biological threats is a particular challenge for the military clinical or field laboratory. For the past 50 years, the biological defense research program has focused on known or hypothesized collections of biological threats in the biological weapons program of the United States (ended in 1969) or of the former Soviet Union.[72,73] However, several critical events have broadened the scope of the biological threat since 1984. First was the recognition after 1984 that nonstate actors might use biological agents in terrorist scenarios to advance political, religious, or social agendas (Table 18-3).[74-80] These demonstrations suggest a more dangerous future because individuals or groups without any national allegiance use biological threats in small-scale scenarios outside of battlefield boundaries. Second, the discovery of an emerging biological weapons program in Iraq after the Persian Gulf War included several unexpected new threats, including aflatoxins, *Shigella,* and camelpox virus, in conjunction with historical biological threats, such as anthrax, ricin toxin, cholera, *Clostridium perfringens* and *C botulinum* neurotoxins.[81] This discovery suggested that any etiological agent or combinations of biological agents, beyond those identified previously as optimal for past biological weapons of mass destruction, could be used by US adversaries to create fear and confusion. Third, the maturation and proliferation of biotechnology have resulted in several laboratory demonstrations of genetically engineered threats with new, potentially lethal characteristics.[81-85] Jackson et al demonstrated the virulence of orthopoxviruses enhanced by the insertion of immunoregulatory genes, such as interleukin-4.[82] In other work, Athamna et al demonstrated the intentional selection of antibiotic-resistant *B anthracis*.[83] Borzenkov et al modified *Francisella, Brucella, and Yersinia* species by inserting beta-endorphin genes.[84,85] As a result of the proliferation of these biotechniques, public health officials can no longer depend on an adversary choosing any of the 15 to 20 biological threats of past generations, but now must prepare for a future of an infinite number of threats, some of which may have been genetically engineered to enhance virulence or avoid detection.

TABLE 18-3

BIOTERRORISM INCIDENTS, 1984–2004

Biological Agent	Description
Salmonella typhimurium	Rajneeshee cult, The Dalles, Oregon, 1984[1]
Ricin toxin	Patriots Council, Minnesota; Canada, 1991–1997[2,3]
Bacillus anthracis	Aum Shinrikyo cult, Tokyo, Japan, 1995[4]
Shigella dysenteriae	Clinical lab, 1996[5]
Various	Hoax incidents, Nevada, 1997–1998[6]
B anthracis	Letters, Palm Beach, Florida; civilian news operations in New York City and in the Hart Senate Office Building, Washington, DC; also US postal facilities in the national capital area and in Trenton, NJ; 2001[7]
Ricin toxin	Manchester, England, 2002[3]; Possible Chechen separatist plan to attack the Russian embassy, London, England, 2003
Ricin toxin	Dirksen Senate Office Building, Mailroom serving Senate Majority Leader Bill Frist's office, Washington, DC, 2004[3]

Data sources: (1) Torok TJ, Tauxe RV, Wise RP, et al. A large community outbreak of salmonellosis caused by intentional contamination of restaurant salad bars. *JAMA*. 1997;278:389–395. (2) Mirarchi FL, Allswede M. CBRNE–ricin. *eMedicine* [serial online]. Available at: http://www.emedicine.com/emerg/topic889.htm. Accessed March 16, 2005. (3) Shea D, Gottron F. *Ricin: technical background and potential role in terrorism*. Washington, DC: Congressional Printing Office; February 4, 2004. Congressional Research Service Report RS21383. (4) Keim P, Smith KL, Keys C, Takahashi H, Kurata T, Kaufmann A. Molecular investigation of the Aum Shinrikyo anthrax release in Kameido, Japan. *J Clin Microbiol*. 2001;39:4566–4567. (5) Kolavic SA, Kimura A, Simons SL, Slutsker L, Barth S, Haley CE. An outbreak of Shigella dysenteriae type 2 among laboratory workers due to intentional food contamination. *JAMA*. 1997;278:396–398. (6) Tucker JB. Historical trends related to bioterrorism: an empirical analysis. *Emerg Infect Dis*. 1999;5:498–504. (7) Bush LM, Abrams BH, Beall A, Johnson CC. Index case of fatal inhalational anthrax due to bioterrorism in the United States. *N Engl J Med*. 2001;345:1607–1610.

These new threats will require the development of identification and diagnostic systems that can be flexibly used to allow early recognition of a unique biothreat, representing one of the next major research and development challenges of the DoD and the National Institutes of Health.

BIOFORENSICS

Military clinical and field laboratories are not responsible for forensics protocols, which are required to support biocrime investigations and identify the origins of a biological threat. However, law enforcement personnel and military unit commanders may request the support of clinical laboratory experts and microbiologists to protect the nation's health and safety immediately after an attack. When allowed by command policy, military laboratories may assist in the evaluation of suspicious materials and rule out hoax materials if they use approved agent-identification protocols. Laboratories should not attempt to perform independent forensic analyses unless requested and supervised by appropriate law enforcement authorities. In CONUS, the intentional release of a biological threat is a crime and therefore is investigated by local and federal law enforcement agencies. OCONUS laboratories should coordinate closely with theater command staff and regional reference centers before conducting any analyses. At the national level, the US Department of Homeland Security National Bioforensic Analysis Center is responsible for providing highly regulated evaluations of biological threat materials from civilian and military sources. The Center also is responsible for establishing standards and coordinating analyses performed in supporting laboratories.

Although many clinical laboratories may be familiar with epidemiological investigations, bioforensic activities require a strict chain-of-custody and documentation process. Standards for analysis have been established by the American Society of Crime Laboratory Directors (see http://www.fbi.gov/hq/lab/codis/forensic.htm; accessed September 23, 2005). Related guidance can be found in International Organization for Standardization 17025 (Guide 25).[86] All laboratory activities must be directed to preserving the original evidence. Only validated analysis methods, in which the performance variables such as sensitivity, specificity, precision, robustness, and reliability have been scientifically peer reviewed, should be used. Laboratory protocols used in the CDC-sponsored LRN have been accepted by law enforcement officials for the analysis of evidentiary materials.

The biological and ecological complexities of most biothreat agents present forensic microbiologists with a number of significant analytical and interpretive challenges. Several available methods would be useful in characterizing biocrime evidence. Classical phenotypic assays for physiological properties are among the most basic. Other methods include

- sequencing of DNA/RNA in samples and genomic sequencing of culture isolates;
- determination of phylogenetic patterns of single nucleotide polymorphisms from sequence data;
- association of microorganism genotypes with phenotypes;
- use of pathogenicity arrays (including 16S rRNA probes) to detect artificially constructed hybrid microorganisms; and
- use of screening tests for detection of antimicrobial resistance markers.

Use of multiple test methods is desirable to avoid misidentification of agents caused by induced or engineered mutations. To this end, portions of samples should be saved for additional investigation or confirmatory testing. Blind, barcoded sample replicates (eg, 10% of the replicates) are recommended.[87]

Although the number of bioterrorism incidents has been small, integrated forensic and epidemiological approaches have assisted in past investigations. For example, a combination of epidemiological methods, classical phenotyping, and restriction endonuclease digest of marker plasmids contributed to the identification of a large community outbreak of salmonellosis caused by intentional contamination of restaurant salad bars.[74] The introduction of pulse field analysis of DNA from culture isolates helped to determine the magnitude and source of an outbreak of *Salmonella dysenteriae* type 2 among laboratory workers resulting from intentional food contamination.[76]

Differentiation of *B anthracis* strains has been problematic because phenotypic and genetic markers are shared among the members of the *B cereus* family.[88] Worldwide clone-based diversity patterns have been demonstrated for *B anthracis*.[89] With the identification of variable number tandem repeats, identifying strains (unique genotypes) by multiple locus variable number tandem repeats analysis is now possible. Keim et al have suggested that there are about six major worldwide clonal lineages and nearly 100 unique types.[89,90] Using these methods on *B anthracis*

spores that were aerosolized over Kameido, Japan, by the Aum Shinrikyo cult were identified as consistent with strain Sterne 34F2, which was used in Japan for protecting animals against anthrax.[79] Molecular subtyping of *B anthracis* played an important role in differentiating and identifying strains during the 2001 bioterrorism-associated outbreak.[91] Because phylogenetic reconstruction using molecular data is often subject to inaccurate conclusions about phylogenetic relationships among operational taxonomic units, the analysis of single nucleotide polymorphisms, which exhibit extremely low mutation rates, may be more valuable for phylogenetic analyses. Using a remarkable set of 990 single nucleotide polymorphisms, Pearson et al demonstrated that nonhomoplastic, whole genome single nucleotide polymorphism characters allowed branch points and clade membership for *B anthracis* laboratory reference strains to be estimated with great precision, providing greater insight into epidemiological, ecological, and forensic questions.[92] These investigators determined the ancestral root of *B anthracis*, showing that it lies closer to a newly described "C" phylogenetic branch than to either of two previously described "A" or "B" branches. Similar analytical methods are evolving for characterizing strains of *Y pestis* and *F tularensis*.[93,94] Continued maturation of genetic fingerprinting methods in the forensic environment can significantly deter biocrime and biological warfare in the future and result in more rapid identification of perpetrators.

FUTURE APPROACHES

Early Recognition of the Host Response

The host responds to microbial invasion immunologically and also responds to pathological factors expressed by the foreign organism or toxin. Identifying early changes in the host gene response may provide an immediate indication of exposure to an agent and subsequently lead to early identification of the specific agent, before the onset of disease. Several biological agents and toxins directly affect components important for innate immunity, such as macrophage or dendritic cell functions or immunomodulator expression. Studies suggest that anthrax lethal factor may induce apoptosis in peripheral blood mononuclear cells, inhibit production of proinflammatory cytokines in peripheral blood mononuclear cells, and impair dendritic cells.[95,96] Poxviruses may possess several mechanisms to inhibit innate immunity.[97] Gibb et al reported that alveolar macrophages infected with Ebola virus demonstrated transient increases in cytokine and chemokine mRNA levels that were markedly reduced after 2 hours postexposure.[98] Others have shown that Ebola virus infections are characterized by dysregulation of normal host immune responses.[99] However, directly detecting these effects, especially inhibition of cytokine expression, is technically difficult to measure in potentially exposed populations.

New approaches that evaluate the regulation of host genes in microarrays may allow for early disease recognition.[100,101] A complicated picture is emerging that goes beyond dysregulation of genes related to innate immunity. Relman et al suggested that there are genome-wide responses to pathogenic agents.[102] Mendis et al identified cDNA fragments that were differentially expressed after 16 hours of in-vitro exposure of human peripheral blood mononuclear cells to staphylococcal enterotoxin B.[103] By using custom cDNA microarrays and RT-PCR analysis, these investigators found a unique set of genes associated with staphylococcal enterotoxin B exposure. By 16 hours, there was a convergence of some gene expression responses, and many of those genes code for proteins such as proteinases, transcription factors, vascular tone regulators, and respiratory distress. Additional studies are needed to characterize normal baseline parameters from a diverse group of individuals undergoing common physiological responses to the environment, as well as responses to the highest priority biological agents and toxins in appropriate animal models. Approaches that integrate detection of early host responses with the sensitive detection of biological agent markers can decrease morbidity and mortality by encouraging optimal therapeutic intervention.

Joint Biological Agent Identification and Diagnostic System

An integrated diagnostic approach is required to recognize the biological threats of the future.[2] No single technology is sufficient to definitively identify any biological threat; thus, diagnostic systems must be able to detect multiple biological markers. Future systems must use a combination of immunological, gene amplification, and classical identification methods to identify important virulence factors, genus and species markers, common pathogenic markers, and antibiotic markers (Figure 18-4). The DoD is developing the JBAIDS as a flexible diagnostic platform that can incorporate a variety of new technologies.[104] JBAIDS will be a comprehensive integrated diagnostic

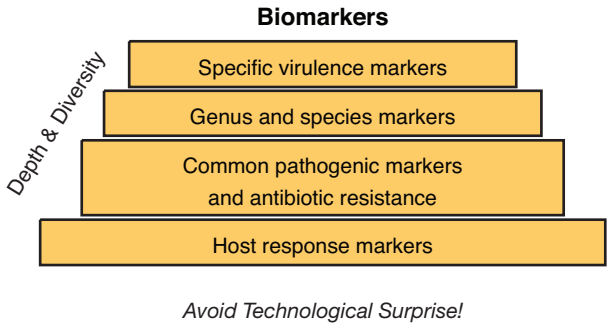

Fig. 18-4. Diagnostic systems must be able to detect multiple biological markers. No single technology is sufficient to definitively identify any biological threat. Future systems must use a combination of immunological, gene amplification, and classical identification methods to identify important virulence factors, genus and species markers, common pathogenic markers, and antibiotic markers.

platform capable of reliably identifying multiple biological threat agents and endemic infectious diseases. An acquisition strategy has been developed that will allow the integration of identification technologies into a single platform. Initial systems will include gene and antigen-detection systems linked to an interactive information-management framework. JBAIDS will support reliable, fast, and specific identification of biological agents from a variety of clinical and environmental sources and samples. JBAIDS will enhance healthcare by guiding the choice of appropriate treatments, effective preventive measures, and prophylaxis at the earliest stage of disease. In addition, JBAIDS will identify and quantify biological agents that could affect military readiness and effectiveness. Reliability, technological maturity, and supportability are the primary criteria used for selecting technologies included in JBAIDS.

SUMMARY

Protection of service members and their families from the effects of attack by biological agents requires the combined resources of the US military healthcare system and coordination with civilian public health officials. Military clinical and field laboratories serve as unique sentinels in CONUS and OCONUS areas for biological threats and emerging infectious diseases. Field laboratories in forward areas, which are equipped with the basic tools necessary to rule out endemic infectious diseases, can be augmented with the capability to identify the most likely biological warfare agents. CONUS military laboratories conform to standards and protocols established for the CDC-sponsored LRN for the identification of biological threats. This response is supplemented by the comprehensive capabilities of the national laboratories, such as the CDC and USAMRIID, and military reference centers. Classical microbiology methods will remain as part of the core capability, which is being expanded to include integrated rapid immunodiagnostics and gene analysis technologies. The laboratory response for biological threats must be flexible to accommodate emerging and "nonclassical" agents. Future research will continue to develop real-time, simple, reliable, and robust methods that will be useable throughout the military healthcare and surveillance system.

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