Chapter 26 LABORATORY IDENTIFICATION OF THREATS

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INTRODUCTION

Medical diagnosis is the process by which clinicians attempt to deduce the cause of a particular disease or disorder in a sick individual. The goal of diagnosis is to assist in making correct medical decisions about the patient's treatment and prognosis. For infectious diseases, a variety of medical information is used to make a diagnosis including a physical examination, interview with the patient, medical history of the patient, and clinical findings as reported by laboratory tests. The focus of this chapter will center on laboratory tests used to diagnose biological threat agents. These tests represent a piece of the diagnostic puzzle and should not be used solely for diagnosis and treatment. Physical and clinical findings as well as medical history are critical to an accurate diagnosis, and integrating all available medical information as well as all available laboratory information reduces the chance for misdiagnosis. Diagnosis requires the synthesis of multiple pieces of information into a medical judgment that will be used to affect patient care; therefore, getting the right answer must always take priority over getting a quick answer.

The content of this review will focus on the current and future state of in vitro diagnostics, as defined by the Food and Drug Administration (FDA):

Those reagents, instruments, and systems intended for use in diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body.¹

For the purposes of this chapter, a biological threat is any infectious disease entity or biological toxin encountered, either through natural distribution or intentionally delivered by an opposing force to deter, delay, or defeat US or allied military forces. The majority of biological threats of military and public health relevance are contained in the Department of Health and Human Services (DHHS) select agents and toxins list of regulated biological select agents and toxins (Table 26-1). Many of these biological threats were part of offensive biological weapons programs at one time. As with other infectious disease assays, biological threat laboratory assays are subject to the same requirements and regulations to be regulatory compliant, meaning that the test can be used for patient care.

For laboratory assays, two critical elements must meet minimal standards to be considered regulatory compliant: (1) the laboratory performing the test must be qualified, and (2) the test being performed must be validated. The Clinical Laboratory Improvement Amendments (CLIAs), which were passed in 1988 (CLIA '88), regulate the quality of the clinical laboratory performing the testing, whereas section 210(h) of the Federal Food, Drug, and Cosmetic Act regulates in vitro diagnostic tests. As such, the FDA has oversight and regulatory authority to clear in vitro diagnostic tests (medical devices) for commercial sale and use. The combination of a CLIA-accredited laboratory performing an FDA-cleared diagnostic test results in a regulatory compliant diagnostic result that can be used for the patient's treatment and prognosis. For the Department of Defense (DoD), maintaining regulatory compliance in performing in vitro diagnostic tests in a deployed environment poses a significant challenge.

CLIA sets the standards for any laboratory that performs testing on human samples for use in disease diagnosis and treatment. The goal of CLIA was to improve the quality of any testing conducted for medical purposes, and DoD facilities are not exempt from the requirements. However, the DoD was allowed to develop a separate plan for ensuring quality and standards in diagnostic testing, the Clinical Laboratory Improvement Program (CLIP; DoD Instruction 6440.2).2 CLIP is similar to CLIA with certain exceptions to meet military operational requirements. Both CLIA and CLIP govern the quality of the laboratory performing the diagnostic test and include standards for personnel, quality control, quality assurance, procedure manuals, proficiency testing, and inspections for adherence to the standards. CLIA and CLIP require laboratory registration to perform testing, and registrations are based on the level of test complexity that the laboratory is accredited to perform.

Minimal complexity tests (waived) are simple tests that do not require significant quality oversight, such as tests cleared by the FDA for home use. Moderate and high complexity tests require increased knowledge, training and experience, quality control, and interpretation and judgment. Moderate tests are typically more automated while high complexity tests require significant technical manipulation by personnel. The current FDA-cleared diagnostic system, the Joint Biological Agent Identification and Diagnostic System (JBAIDS), is a high complexity test, which can make it difficult to maintain a high complexity CLIA registration in a deployed setting.

Movement of laboratory diagnostic capabilities to forward locations is driving long-term goals for DoD medical diagnostic devices to be CLIA-waived devices. The Next Generation Diagnostic System (NGDS), the Biofire Defense FilmArray, will likely be a moderate complexity device, an incremental improvement over

TABLE 26-1

REGULATED BIOLOGICAL SELECT AGENTS AND TOXINS

HHS and USDA Select Agents and Toxins (7 CFR Part 331, 9 CFR Part 121, and 42 CFR Part 73)

HHS SELECT AGENTS AND TOXINS

- Abrin
- Botulinum neurotoxins*
- Botulinum neurotoxin-producing Clostridium*
- Conotoxins
- Coxiella burnetii
- Crimean-Congo hemorrhagic fever virus
- Diacetoxyscirpenol
- Eastern equine encephalitis virus
- Ebola virus*
- Francisella tularensis*
- Lassa fever virus
- Lujo virus
- Marburg virus*
- Monkeypox virus
- 1918 pandemic influenza virus
- Ricin
- Rickettsia prowazekii
- SARS-associated coronavirus (SARS-CoV)
- Saxitoxin
- South American hemorrhagic fever viruses: Chapare Guanarito
 - Junin Machupo
 - Sabia
- Staphylococcal enterotoxins A,B,C,D,&E subtypes
- T-2 toxin
- Tetrodotoxin
- Tick-borne encephalitis complex (flavi) viruses: Far Eastern subtype Siberian subtype
- Kyasanur Forest disease virus
- Omsk hemorrhagic fever virus
- Variola major virus (Smallpox virus)*
- Variola minor virus (Alastrim)*
- Yersinia pestis*

OVERLAP SELECT AGENTS AND TOXINS

- Bacillus anthracis*
- Bacillus anthracis Pasteur strain
- Brucella abortus
- Brucella melitensis
- Brucella suis
- Burkholderia mallei*
- Burkholderia pseudomallei*
- Hendra virus
- Nipah virus
- Rift Valley fever virus
- Venezuelan equine encephalitis virus

USDA SELECT AGENTS AND TOXINS

- African horse sickness virus
- African swine fever virus
- Avian influenza virus
- Classical swine fever virus
- Foot-and-mouth disease virus*
- Goat pox virus
- Lumpy skin disease virus
- Mycoplasma capricolum
- Mycoplasma mycoides
- Newcastle disease virus
- Peste des petits ruminants virus
- Rinderpest virus*
- Sheep pox virus
- Swine vesicular disease virus

USDA PLANT PROTECTION AND QUARANTINE SELECT AGENTS AND TOXINS

- Peronosclerospora philippinensis
- Peronosclerospora sacchari
- Phoma glycinicola
- Ralstonia solanacearum
- Rathavibacter toxicus
- Sclerophthora rayssiae
- Synchytrium endobioticum
- Xanthomonas oryzae

*Denotes Tier 1 agent.

CFR: Code of Federal Regulations; HHS: Health and Human Services; SARS: severe acute respiratory syndrome; USDA: US Department of Agriculture.

Data source: http://www.selectagents.gov/SelectAgentsandToxinsList.html (valid June 2016).

the currently deployed system. The system is already FDA cleared for several infectious disease diagnostics and received an Emergency Use Authorization (EUA) during the 2014 Ebola outbreak in West Africa. FDAcleared assays for biological threats on the FilmArray will likely be available in 2017 and will replace the JBAIDS. Although CLIA/CLIP requirements are based on test complexity, FDA requirements for clearance are based on the risk associated with the test, and risk is dependent on the potential harm associated with obtaining the wrong diagnostic result. The FDA classifies in vitro diagnostic tests as either class I (lowest risk), class II (moderate to high risk), or class III (highest risk) medical devices. The currently fielded JBAIDS system is an FDA regulated class II device and currently resides in combat support hospitals within the US Army and within other medical treatment facilities for the Air Force and Navy. For the DoD, the challenge remains maintaining regulatory compliance in far forward operational settings.

The availability of FDA-cleared assays for biological threats remains somewhat limited. In vitro diagnostic tests for biological threats are orphan products, that is, there is not a large enough market to incentivize private industry to develop the tests because they are performed on an infrequent basis and, therefore, sales are limited. Consequently, most of the cleared diagnostic tests for biological threats have resulted from DoD acquisition programs. Currently cleared biological threat assays on the JBAIDS system include tests for Bacillus anthracis, Yersinia pestis, Francisella tularensis, and Coxiella burnetii. The absence of useful and cleared infectious disease assays on JBAIDS hampers the utility of the system for clinicians. Future diagnostic devices for DoD would benefit from expanded capabilities for common infectious diseases of military relevance, not just those that are most likely to be used in a biological attack.

Although biological science technology continues to advance, it must be emphasized that the DoD currently fielded and regulatory compliant in vitro diagnostic tests for biological threat agents are based on nucleic acid amplification chemistry that is 30 years old and a rapid cycling polymerase chain reaction (PCR) platform that is more than 10 years old. With the NGDS acquisition program underway, the platform is likely to be only an incremental improvement over the currently fielded system. The two most likely improvements will be onboard integrated sample processing and a sample in/answer out analysis flow. In essence, the system is likely to be an automated nucleic acid amplification in vitro diagnostic platform. During this time, microarrays, mass spectrometry, and DNA sequencing have advanced significantly for the identification of infectious agents.³ Yet none of these approaches has matured to the point of receiving FDA clearance for medical diagnostic use or offer the hope of a simplified test that can be performed in a deployed setting for biological threat agents.

Unlike technology, the ability of military laboratories to identify and confirm the presence of biological threats using regulatory compliant diagnostics matures at a much slower rate. This is not to discount the use of newer technologies by the DoD for environmental testing, vector surveillance, and population surveillance. These results can be used to make operational decisions, but they cannot be used for individual patient treatment, a concept often lost within the research and development community. The combination of using multiple diagnostic devices, multiple diagnostic markers, medical intelligence, medical acumen clinical signs and symptoms, and classical microbiology (Figure 26-1) still provides the most reliable approach for medical diagnosis of diseases to affect medical treatment or response after a biological threat attack.

To enhance readiness and ensure the availability of laboratory testing capabilities, military and civilian clinical laboratories are linked into a series of laboratory response networks. The Centers for Disease Control and Prevention (CDC) sponsors the preeminent Laboratory Response Network (LRN) for bioterrorism. More recently, the DoD also has established the Defense Laboratory Network to further enhance military readiness. Together, these efforts have improved the national preparedness for biological threat identification, 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 currently available and future capabilities for agent identification and diagnostic technologies to protect and sustain the health of military personnel.



Figure 26-1. Orthogonal diagnostic testing uses an integrated testing strategy where more than one technology, technique, or biomarker is used to produce diagnostic results, which are then interpreted collectively. Although orthogonal diagnostic testing is a statistically independent approach, the combination of independent sensitivity and specificity values becomes highly valuable when combined. Orthogonal diagnostic testing improves the probability of reaching a "correct" result when the assays are less than 100% specific independently.

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. Intentionally delivered 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 laboratories is to provide data to support a clinical diagnosis, laboratory staff also provides subject matter expertise in theaters of operation on the handling and identification of hazardous microorganisms and biological toxins. In addition, these laboratories have a global view of disease in the theater and 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 force health protection or a preventive medicine surveillance system in a theater of operations.⁴ Military biological laboratory capabilities also exist to provide chemical, biological, radiological, and nuclear (CBRN) response, and elimination and remediation activities.⁵

Military Field Laboratories

Military field laboratories, which have many different configurations, are often incorporated into most of the services' basic deployable treatment facilities. If a complete medical treatment facility (MTF) 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 this case, medical laboratory support would be provided by a facility outside the area of operations.⁶ A typical Army MTF in a theater of operations will have a limited initial microbiology capability even with the intrinsic laboratory component.

Following the removal of the microbiology capability from most Army medical treatment facilities under the 1994 Medical Reengineering Initiative, the capability has been restored with adding a microbiology augmentation set (Medical Materiel Set, laboratory [microbiology] augmentation UA N403 NSN 6545-01-505-2714 LIN M48987) and JBAIDS (UA 9409 NSN 6545-01-537-1100 LIN J00447). The N403 set contains necessary equipment and reagents to identify commonly encountered pathogenic bacteria. Susceptibility testing is not included. Although this medical set supports diagnostics of common bacterial infections, it does not contain an authoritative capability for identifying biological warfare agents. At the time of publication, the capability for biological threat agent detection is a mission primarily accomplished using JBAIDS. Specimens requiring more comprehensive analysis still require forwarding to the nearest reference or confirmatory laboratory, including the currently deployable assets for each service.

Army

The Area Medical Laboratory (AML) is a modular, task-organized, and corps-level asset providing comprehensive laboratory support to theater commanders.^{5,7} The AML has transitioned from the original mission of testing primarily clinical specimens, with a capability for environmental samples (supporting force health protection) to being strictly an environmental sample testing lab. The AML can test for a broad range of biological, chemical, and radiological hazards. For biological agents, the laboratory uses a variety of rapid analytical methods, including molecular methods (such as real-time PCR), immunoassays (such as electrochemiluminescence [ECL] and enzyme-linked immunosorbent assay [ELISA]), and more advanced analyses involving bacterial culture, fatty acid profiling, and immunohistochemistry. The AML, which is the largest of the service deployable laboratories, can typically staff missions with a mix of microbiologists, biochemists, veterinary pathologists, and physicians. The AML maintains a degree of redundant equipment for long-term or split-base operations.

The 20th CBRNE Command (CBRNE-Chemical, Biological, Radiological, Nuclear, and Explosives), previously called the 20th Support Command CBRNE, fields a multitude of assets under a single operational headquarters. Its mission is to detect, identify, assess, render safe, dismantle, transfer, and dispose of unexploded ordnance, improvised explosive devices, and other CBRNE hazards, including biological warfare agents (see reference 14). The CBRNE Analytical & Remediation Activity Mobile Expeditionary Laboratory (CARA MEL), a unit within the 20th, provides high-throughput chemical, explosives, and biological sample analysis. It also has three mobile lab packages (a light mobile expeditionary lab, a heavy mobile expeditionary lab, and a chemical air monitoring system platform) that deploy to support weapons of mass destruction elimination and remediation efforts in forward deployed areas.

Navy

The Navy's forward deployable preventive medicine units are medium-sized mobile laboratories that use multiple rapid techniques (to include PCR and ELISA) for identifying biological warfare agents on the battlefield. The forward deployable preventive medicine units are modular and can analyze samples containing chemical and radiological hazards. These laboratories specialize in providing high confidence identification of biological threat agents in concentrated environmental samples, and they can identify endemic infectious disease in clinically relevant specimens.

Air Force

Air Force biological augmentation teams (unit type code FFBAT) and home station medical response laboratory biodetection teams use rapid analytical methods (such as real-time PCR) and immunological methods to screen environmental and clinical samples for threat agents.^{8,9} The biological augmentation teams are small (two members), easily deployed, and typically housed in a separate facility designed to be collocated with preexisting or planned medical facilities. The units are capable of providing early warning to commanders about the potential presence of biological threat agents, typically in support of installation protection programs. 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.

Defense Laboratory Network

The response to future CBRN threats will require an integrated military laboratory network that can respond with agility and competence. 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 provide rapid and accurate laboratory support for medical diagnosis, rule out the most common threats, and alert the command about suspicious disease occurrences. The military Defense Laboratory Network consists of the front-line MTF clinical laboratories or deployed military laboratories backed by regional MTFs 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 capability are research laboratories, such as the US Army Medical Research Institute of Infectious Diseases (USAMRIID; Fort Detrick, MD) and the Naval Medical Research Center (Silver Spring, MD). Other laboratories, such as the US Air Force Institute for Operational Health (Wright Patterson Air Force Base, OH) and the Naval Health Research Center (San Diego, CA), also provide reference laboratory services for a myriad of endemic infectious diseases. Military research laboratories have traditionally solved some of the most complex and difficult diagnostic problems, but they are not routinely organized to perform high-throughput clinical sample processing and evaluation. Sentinel laboratories are generally supported by the network's designated confirmatory laboratories, but they may communicate directly with national laboratories if necessary.

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 26-2). The Defense Laboratory Network is a standing member of the federal Integrated Consortium of Laboratory Networks (ICLN). The ICLN was established in 2005 under a memorandum of agreement signed by senior officials of federal agencies including the Departments of Agriculture, Defense, Commerce, Energy, Health and



Figure 26-2. The network of military laboratories with connections to federal and state civilian response systems provides unparalleled depth and resources to the biological threat response. National Laboratory Response Network for Bioterrorism. CDC: Centers for Disease Control and Prevention; NMRC: Naval Medical Research Center; USAMRIID: US Army Medical Research Institute of Infectious Diseases.

Human Services, Homeland Security, Interior, Justice, and State, and the Environmental Protection Agency (https://www.icln.org/valid February 2014). The ICLN was charged with promoting enhanced commonality and integration of network functions. Although the ICLN does not direct resources or operations, it does provide an environment for integrating network operations and strategies. The Department of Homeland Security (DHS) is charged with overall leadership and coordination.

Identification Levels

Rapid infectious disease diagnostics are not quantitative, not linked to traceable standards, and, overall, are not as well developed as other laboratory technologies. The inherent biological variability that exists between any two organisms (mammalian and microbial) complicates the ability to discern with absolute certainty the perpetrator of an infectious disease event. Laboratory tests for many infectious agents are not highly automated and still rely on decades-old technologies and techniques. Culture remains the gold standard for identifying organisms, but not all infectious disease agents can be grown in culture, or are difficult to culture in routine microbiology laboratories, making alternative methods necessary. These constraints significantly affect the confidence at which results on diagnostic or detection assays for infectious agents can be reported. It often goes unstated that the best that can be done in biology is that, with high confidence, what is incriminated as the infectious disease agent has high probability of being correct.

When microbiology culture capability is difficult or not available (eg, virus cultures in field laboratories), serological diagnosis (use of the antibody response) to the organism is still a useful method and sometimes the only way to discern some infections. The problem with both traditional culture and serodiagnosis is the time required to obtain results. Culture may take several days and serodiagnosis is constrained by the time required to mount an antibody response, which can exceed a week or more (Figure 26-3).

Within the past few decades molecular and immunodiagnostic technologies have been developed to improve the specificity and time to obtain diagnostic and detection information on infectious agents. Immunodiagnostic technologies are based on the use of antibodies as diagnostic reagents. Diagnostic and detection assays have been developed that can decrease detection times down to the range of minutes. Molecular diagnostics are based on the detection of specific nucleic acids characteristic of the infectious disease agent. Often the molecular diagnostic assay has to rely on the amplification of specific DNA sequences from extracted nucleic acids, DNA or RNA. Amplification techniques take tiny amounts of nucleic acid material and replicate them many times through enzymatic reactions, some that occur through cycles of heating and cooling. These techniques may bring more ambiguity on interpreting the results of the assays. Unlike cultured microbial agents, which can provide definitive results, immunodiagnostic and molecular diagnostic assays have various levels of false-positive and false-negative results. Discerning false-positive and false-negative results from true results becomes a risk management effort, aided by different levels of identification to express the degree of confidence associated with various testing methodologies.

Civilian

The CDC LRN uses two levels of identification: (1) presumptive and (2) confirmed.¹⁰ In 1998,¹¹ following a demonstration that Iraq sponsored state activities involving production and use of biological weapons, President Clinton issued Presidential Decision Directive 62, Combating Terrorism, and assigned specific missions to federal departments and agencies. The



Figure 26-3. The typical infection and response time course begins with the initial pathogen encounter and leads to the formation and maintenance of active immunological memory (IgM and IgG) where serological detection is useful. Clinical disease, however, typically occurs around days 3 to 5 where detection of the infectious agent is possible. Often, by the time clinical disease is manifest, especially for the biological threat agents, clinical intervention to ensure survivability is not as effective as desired. To provide the most effective medical intervention on infectious agents, the closer to time 0 laboratory data is available, the more successful the outcome. IgG: immunoglobulin G; IgM: immunoglobulin M.

directive included a request to Congress to provide funding to the DHHS to support a renewed program of public health preparedness. In 1999, the LRN was established by the DHHS, the CDC in collaboration with founding partners, the Federal Bureau of Investigation, DoD, and the Association of Public Health Laboratories. The mission statement for the LRN is as follows:

The LRN is a critical national security infrastructure asset that, with its partners, will develop, maintain, and strengthen an integrated domestic and international network of laboratories to respond rapidly to biological, chemical, and radiological threats and other high priority public health emergencies through training, rapid testing, timely notification and secure messaging of laboratory results.¹¹

The LRN includes a biological network (LRN-B) and a chemical network. Identification of biological threat agents within LRN-B is-in part-based on the level of testing, which is based on the level of the laboratories where testing is performed. The different levels of laboratories within the LRN are sentinel, reference, and national laboratories (Figure 26-2). Sentinel laboratories represent the thousands of community based hospital laboratories that have direct contact with patients and may be the first to spot atypical infectious disease presentations. Sentinel laboratories do not actually confirm the presence of biological agents but rather are trained to recognize and appropriately handle biological agents that could potentially be extremely dangerous pathogens. Sentinel laboratories then refer these presumptive cultures to their closest LRN reference laboratory for more definitive testing. These reference laboratories perform standardized tests to detect, and typically confirm, the presence of biological agents that may represent a biological threat.

Reference laboratories, which are normally located within the respective state public health laboratories, perform reference-level tasks in biological safety level 3 (BSL-3) facilities.¹² Some LRN reference laboratories are located at county public health laboratories, animal health/veterinarian laboratories, military medical treatment facilities, and food safety laboratories. Public health directors can use LRN reference laboratory results to determine when a broad range of public health responses can be implemented. The CDC LRN protocols are currently limited to several bacterial agents, orthopoxviruses, and a couple of biologic toxins, and not all of the protocols have full confirmation methods for reference laboratory use (Table 26-2).

A recent programmatic change to the LRN system will subdivide the network configuration for the reference laboratories. The LRN reference laboratories will be broken down into limited (RL3), standard (RL2), and advanced levels (RL1). The standards for each level will be based in part on the minimum operational BSL, the core instrumentation and equipment available (including advanced platforms), and testing capabilities (number of agents and technologies). Limited laboratories (RL3) will typically do limited, specialized testing not meeting RL2 standards. Standard laboratories (RL2) will be typical state public health laboratories capable of the full agent testing capability on clinical and high risk environmental samples. Advanced laboratories (RL1), typically state public health laboratories in regional locations that cover a risk-based, priority population center (under the DHS Urban Area Security Initiative¹³), will be capable of additional testing capabilities using advanced detection and characterization methods such as mass spectrometry. The three national laboratories have unique resources to handle highly infectious agents (typically at BSL-3 and BSL-4¹⁴) and the ability to identify and characterize more agents, including BSL-4 viruses.

The CDC LRN, as a network, includes laboratories, secure communications, training, protocols, reagents, and proficiency testing. LRN member laboratories encompass federal laboratories (including laboratories at CDC, the US Department of Agriculture [USDA], the FDA, and other facilities run by federal agencies), state and local public health, military (DoD laboratories located both within the United States and abroad), food testing (FDA and USDA laboratories), environmental (water and other environmental samples), veterinary (USDA and state), and international laboratories (Canada, the United Kingdom, Australia, Mexico, and South Korea). As the LRN-B continues programmatic maturation, it will not only continue to address biological terrorism preparedness and response (national security and public health emergency preparedness), but also address emerging infectious disease preparedness and response (eg, newly emerging viruses such as Middle East respiratory syndrome coronavirus) and biosurveillance.

Military

Military identification levels differ from the civilian system in two specific aspects:

- 1. Current military doctrine includes four levels of identification (presumptive, field confirmation, theater level validation, and definitive) based, in part, on what level or what unit does the testing; and
- 2. Testing algorithms are based on the concept of testing for biological markers (biomarker) rather than culturing the specific agents.

TABLE 26-2

Organism	Disease*	Presumptive ⁺	Confirmatory (LRN) [‡]	Key Identity Markers	BSL-2	BSL-3
Bacillus anthracis	Anthrax	Nucleic acid amplification (PCR) Immunoassay (spore vs cell) - HHA or plate based	Culture and gamma phage with capsule or PCR of a culture with three assays	<i>B</i> anthracis is one of more than 260 different <i>Bacillus</i> spp. but is readily distinguishable from the others by the production of beta- hemolysin that is readily apparent on blood agar plates. <i>B</i> anthracis exists as both a vegetative cell and as an environmentally stable spore. <i>B</i> anthracis contains 2 plasmids, pXO1 and pXO2 that impart virulence characteristics and serve as diagnostic markers for both immunoassay and nucleic acid assays. Immunoassays will differ when testing for the vegetative cell or the spore. Whereas immunoassay and nucleic acid analysis can be used for diagnostic confirmation, culture is required for confirmation. Gram-positive rod; spore-forming; aerobic; nonmotile catalase positive; large, gray-white to white; nonhemolytic colonies on sheep blood agar plates.	Culture; PCR; immunoassay	Not needed

PRESUMPTIVE AND CONFIRMATION METHODS

(Table 26-2 continues)

Organism	Disease [*]	Presumptive ⁺	Confirmatory (LRN) [‡]	Key Identity Markers	BSL-2	BSL-3
Brucella abortus Brucella melitensis Brucella suis	Brucellosis Brucellosis	Nucleic acid amplification (PCR) (not species specific) Immunoassay (not species specific) - HHA or plate based	Culture with biochemical testing	Depending on the tax- onomy being used, brucellae contain 10 recognized spe- cies that include <i>B</i> <i>abortus</i> , <i>B melitensis</i> , and <i>B suis</i> , the most common and impor- tant human patho- gens. Differentiating the human patho- genic species from the other brucellae, however, is not easy and requires several growth and biochemical deter- minations. Immu- noassay and nucleic acid assays are currently not helpful in distinguishing the pathogens from the nonpatho- gens. Culture and biochemical testing are required for confirmation. Gram-negative cocco- bacilli or short rods; white, nonmotile, nonencapsulated, nonspore forming, slow-growing, non- hemolytic colonies on sheep blood agar plates; some species require enhanced CO ₂ for growth.	Initial culture; PCR; immunoassay	Culture confirma- tion

Organism	Disease [*]	Presumptive ⁺	Confirmatory (LRN) [‡]	Key Identity Markers	BSL-2	BSL-3
Burkholde- ria mallei Burkholde- ria pseu- domallei	Glanders Melioidosis	Nucleic acid amplification (PCR)	Culture with biochemical testing	<i>B mallei</i> and <i>B</i> <i>pseudomallei</i> are two of the 60 currently recognized species that include other human pathogens. As part of their environmental saprophytic lifestyle, the <i>Burkholderia</i> are complex organisms that are readily culturable, but often display colony morphology variations that confound routine microbiological analysis. Biochemical differentiation, including gentamicin and polymyxin susceptibility, determination of arginine dihydrolase and lysine decarboxylase, and arabinose fermentation are required for differentiation and confirmation. Gram-negative rod; oxidase-positive to variable, small, nonmotile, nonsporulating, nonencapsulated; primary isolation requires 48–72 h at 37°C; nonhemolytic, typically about 1 mm in width, white (turning yellow with age), <i>B</i> <i>pseudomallei</i> grows well on MacConkey agar, but <i>B mallei</i> does not.	Initial culture; PCR	Culture confirma- tion

(Table 26-2 continues)

Organism	Disease [*]	Presumptive ⁺	Confirmatory (LRN) [‡]	Key Identity Markers	BSL-2	BSL-3
Clostri- dium bo- tulinum	Botulism A–E	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Mouse testing	Gram-positive rod; spore-forming; obligate anaerobe catalase negative; lipase production on egg yolk agar; 150,000 Da protein toxin (types A–G); 2 subunits.	Initial culture; PCR; im- munoassay; toxin-antitox- in neutraliza- tion test	Not needed
Clostridium perfrin- gens	Epsilon toxin	Nucleic acid amplification (PCR)	Not in LRN	Gram-positive rod; spore-forming; obligate anaerobe catalase negative; 5 types (A-E), but only types B and D produce the epsilon toxin; on a blood agar plate produces double zone beta hemolysis.	Initial culture; PCR; immu- noassay	Not needed
Coxiella burnetii	Q fever	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Send to CDC	<i>C burnetii</i> is an obligate intracel- lular parasite that makes routine culture difficult. Culture in eggs or cells has previously been required so routine laboratory diagnostics are not common. Although highly infectious, <i>C</i> <i>burnetii</i> is typically not fatal and often serology is used for diagnosis. Direct fluorescent antibody and nucleic acid as- says are often used for presumptive and confirmatory diagnostics.	PCR; immuno- assay	Culture confirma- tion

Organism	Disease [*]	Presumptive ⁺	Confirmatory (LRN) [‡]	Key Identity Markers	BSL-2	BSL-3
Francisella tularensis	Tularemia	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Culture with direct fluorescent antibody stain	<i>F tularensis</i> subspecies tularensis (type A) and <i>F tularensis</i> sub- species holarctica (type B) are the two most virulent strains of this expanding group of organ- isms. Until recently, <i>F tularensis</i> type A or B was restricted to the Northern Hemisphere where <i>F tularensis</i> type A or B is common in North America, but only <i>F tularensis</i> type B is typically found in Europe and Asia. <i>F tularensis</i> is relatively easy to grow and growth is required for confir- mation, typically by the direct fluores- cent antibody assay. Extremely small, pleomorphic, gram-negative coc- cobacilli; nonspore forming; faculta- tive intracellular parasite; nonmotile; catalase positive opalescent smooth colonies on cysteine heart agar.	Initial culture; PCR; immu- noassay	Culture confirma- tion
Rickettsia prowa- zekii	Louse- borne typhus, <i>Typhus</i> exanthe- maticus	JBAIDS nucleic acid amplifica- tion (PCR)	Not in LRN	Gram-negative, obligate intracellular parasitic, aerobic bacteria.	PCR	Culture confirma- tion
Rickettsia rickettsii	Spotted fever	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based				

(Table 26-2 continues)

Staphylo-	Enterotoxins A and B (SEA & SEB)	Immunoassay - HHA or plate based	Not in LRN	Gram-positive, cocci; facultative anaero- bic, large round white to yellow, beta-hemolytic colo- nies on sheep blood agar; characteristic "grape-cluster" on Gram stain; catalase and coagulase-posi- tive; multiple toxins depend on strain.	Initial culture; immunoas- say	Not needed
Yersinia pestis	Plague	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Culture with phage testing	<i>Y pestis</i> belongs to a smaller group of organisms, but is much more difficult to correctly identify. <i>Y pestis</i> has several plasmids that confer various virulence traits and are useful diagnostic assay targets, but the plasmids are promiscuous and can be found in non- <i>Y pestis</i> causing the potential for false-positive assays. Capsule (F1) is a good marker for the diagnosis of <i>Y pestis</i> , but does not get produced at the optimal growth temperature for <i>Y pestis</i> (28°C). Instead, it is produced at 35°–37°C, making this marker less reliable for environmental <i>Y pestis</i> detection. Immunoassay and nucleic acid assays are available for diagnositics, but confirmation of <i>Y pestis</i> is done using phage on cultural growth. Gram-negative coccobacilli often pleomorphic; nonspore forming; facultative anaerobe; nonmotile beaten copper colonies (MacConkey agar).	(Table 26	Culture confirma- tion

Organism	Disease [*]	Presumptive ⁺	Confirmatory (LRN) [‡]	Key Identity Markers	BSL-2	BSL-3
Crimean- Congo hemor- rhagic fever virus/ bunyavi- ruses	Viral hem- orrhagic fevers	Nucleic acid amplification (PCR)	Not in LRN	Single negative- stranded, tripartite genomes (large [RNA-polymerase], medium [glycopro- teins], small [nu- cleocapsid protein]) exist in a helical/ pseudo-circular structure; enveloped RNA viruses.	PCR	Culture confir- mation/ BSL-4
Ebola, Marburg virus/fi- loviridae viruses	Viral hem- orrhagic fevers	Nucleic acid amplification (PCR)	Not in LRN	Linear, negative-sense single-stranded RNA virus; envel- oped; filamentous or pleomorphic, with extensive branch- ing, or U-shaped, 6-shaped, or circular forms; limited cyto- pathic effect in Vero cells.	PCR	BSL-4
Lassa/ arenavi- ruses	Viral hem- orrhagic fevers	Nucleic acid amplification (PCR)	Not in LRN	Two single-stranded RNA segments am- bisense RNA virus; beaded nucleocap- sid, spherical with glycoprotein spikes.	PCR	BSL-4
Variola major	Smallpox	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Send to CDC	Large double-strand- ed DNA virus; enveloped, brick- shaped morphology; Guarnieri bodies (virus inclusions) under light micros- copy.	PCR; immuno- assay	BSL-4 (CDC ONLY)
Venezuelan equine encepha- litis virus/ alpha viruses	Viral en- cephalitic disease	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Not in LRN	Linear positive-sense single-stranded RNA virus; envel- oped, spherical virions with distinct glycoprotein spikes; cytopathic effect in Vero cells.	PCR	Culture confirma- tion
Yellow fever virus/fla- viruses	Viral en- cephalitic disease	Nucleic acid amplification (PCR)	Not in LRN	Linear positive-sense single-stranded RNA virus; envel- oped, icosahedral nucleocapsid; cyto- pathic effect in Vero cells.	PCR	Culture confirma- tion

(Table 26-2 continues)

Table 26-2 c	ontinued
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Organism	Disease *	Presumptive ⁺	Confirmatory (LRN) [‡]	Key Identity Markers	BSL-2	BSL-3
Ricin toxin	Ricin intoxi- cation	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Send to CDC	60,000–65,000 Da protein toxin; 2 subunits castor bean origin.	PCR; immuno- assay	Not needed

*Disease refers to the disease state induced by the agent or the disease-causing entity of the agent.

[†]Presumptive refers to typical diagnostic assay techniques used for reporting the presumptive evidence of a disease-causing agent. [‡]Confirmatory (LRN) refers to the diagnostic assay techniques used for reporting the confirmed evidence of a disease-causing agent being

present

BSL: biological safety level

CDC: Centers for Disease Control and Prevention

HHA: hand held immunoassay

JBAIDS: Joint Biological Agent Identification and Diagnostic System

LRN: Laboratory Response Network

PCR: polymerase chain reaction

RNA: ribonucleic acid

SEA: Staphylococcal enterotoxin A

SEB: Staphylococcal enterotoxin B

The military concept of testing for biomarkers follows the logic that if the biomarker is present, then the agent of interest is also present. Some complications exist with using biomarkers. One problem is defining a biomarker. *Multiservice Tactics, Techniques, and Procedures (MTTP) for Biological Surveillance Editor*¹⁵ provided a definition, but that document was replaced by Army Techniques Publication 3-11.37⁷ in 2013 and the definition was lost. The current doctrinal revision to Army Techniques Publication ATP 4-02.7/MCRP 4-11.1F/NTTP 4-02.7/AFTTP 3-42.3 15 March 2016, *MTTP for Health Services Support in a CBRN Environment*, reestablishes the definition as:

A biomarker refers to a detectable/measurable substance that is correlated with the presence of a BW [biological warfare] agent (bacteria, virus, or toxin). Biomarkers should be unique to the biological agent, often associated with virulence, and can be independent of the biological agent's viability/infectivity/ functionality.

The types of biomarkers listed included nucleic acid sequences, antigens or toxins for immunological methods, growth properties (as demonstrated on biochemical tests or selective media), and microscopic characteristics. The revised doctrinal definition will help guide correct application in the absence of specific details. Another scientific concern with the use of biomarkers is that some biomarkers are present due to nonthreat infectious agents inducing similar biomarker profiles to threat agents. Although these results are considered false-positives for biothreats, induction of disease specific profiles still indicates infection and therefore can remain useful in the overall determination of the etiologic agent.

Biomarkers also do not necessarily reflect viability of the infectious agent. Although the simple presence or absence of an agent can be important, determination of viability may be a significant component, especially in nonclinical samples where the biomarker could be simply background flora. When laboratories rely on biomarkers in lieu of culture, the ability to determine other critical information is often lost, such as antimicrobial resistance, epidemiological strain typing, or legal evidence for forensic science and attribution purposes. Concentrating on biomarkers may lead to a myopic result that limits the full understanding of medical implications for an incident or outbreak.

The military identification levels are well defined in doctrine as follows^{5,7} (Figure 26-4). Presumptive identification of a biological threat agent is achieved by the detection of a biological marker using a single test methodology (eg, hand held assay [HHA]). Presumptive identification uses technologies with limited specificity and sensitivity by general purpose forces in a field environment to determine the presence of a biological hazard with a low level of confidence but with a degree of certainty necessary to support immediate tactical decisions. Since identification at this level is based on specific technologies, it is limited to the assays deployed and cannot detect or identify new or emerging infectious disease agents for which the technologies assays are not available.

Field confirmatory identification is achieved when two or more independent technologies confirm the identification of a biological agent. This may be an immunoassay (eg, HHA, ECL, ELISA, nucleic acid amplification result, and/or culture growth/microscopy). According to doctrine, a single result from JBAIDS can be used as a field confirmatory identification. A genomic biomarker must be included. Field confirmatory identification uses technologies with increased specificity and sensitivity, by technical forces in a field environment, to identify the presence of biological agents with a moderate level of confidence and a degree of certainty necessary to support follow-on tactical decisions. Depending on the technologies deployed (eg, culture), some limited ability exists to detect or identify infectious disease agents beyond the limits of deployed assays.

Theater validation is achieved using devices, materials, or technologies that detect biomarkers using two or more independent biomarker results (ie, one biomarker is detected by two or more independent methodologies or more than one biomarker is detected by a single methodology). Examples are: (1) hand held immunological assay plus nucleic acid amplification or (2) nucleic acid amplification using two different biomarkers (eg, gene targets). Theater validation identification uses multiple independent, established protocols and technologies by scientific experts in a controlled environment of a fixed or mobile/transportable laboratory to characterize biological materials with a high level of confidence and the degree of certainty necessary to support operational level decisions. After a preventive medicine detachment, a combat support hospital or CBRN reconnaissance assets identify a biological/clinical specimen as a biological threat agent, the specimen is sent by courier to those specialized laboratories/teams with advanced microbiological capabilities and highly skilled medical personnel. These could include laboratories/teams such as an AML, 20th CBRNE CARA MEL, the US Air Force biological augmentation team, or the US Navy forward



Figure 26-4. Military identification levels correspond with both the technology used and the facility doing the laboratory analysis. Although there is some correspondence to the civilian Centers for Disease Control and Prevention's Laboratory Response Network system, the military system has significantly unique aspects.

AML: area medical laboratory; BAT: biological augmentation team; CARA: CBRNE analytical remediation activity; CBRN: chemical, biological, radiological, and nuclear; CBRNE: chemical, biological, radiological, nuclear, and explosives; CONUS: continental United States; CVN: aircraft carrier, nuclear; FDPMU: forward deployed preventive medicine unit; Hosp: hospital; LHA: amphibious assault ship (general purpose); LHD: amphibious assault ship (multipurpose); LRN: Laboratory Response Network; NEPMU: Navy environmental and preventive medicine unit; OCONUS: outside the continental United States; PVNTMED: preventive medicine; T-AH: hospital ship; VET: veterinary

deployable preventive medicine unit when available in the operational area. Although the units listed here have the potential to produce theater validation level results, they may not inherently have that capability deployed in all circumstances.

The theater validation laboratories must implement a quality assurance program, preferably with independent audits, proficiency testing, scientist level data review, document control, demonstration of procedure traceability, some level of electronic sample management, documentation of personnel training, and accreditation (if available). These laboratories would typically conduct initial field confirmatory analysis (quick report) followed by theater validation (more testing and time). If these specialized laboratories/ teams are unavailable, biological specimens that are presumptively positive for a biological threat agent will have to be forwarded to the nearest reference laboratory, even if this is in the continental United States (CONUS).

Definitive identification is the correlation of a biological agent to a known substance, or in the case where the substance is previously unknown, the substance is type classified and analyzed. Definitive identification is the use of multiple state-of-the-art, independent, established protocols and technologies by scientific experts in a nationally recognized laboratory to determine the unambiguous identity of a biological agent with the highest level of confidence and degree of certainty necessary to support strategic level decisions. It also supports the initiation of attribution to implicate or point to the source of the identified material. In all cases a definitive identification occurs at a US-based and sanctioned reference laboratory specifically equipped to perform detailed analysis on the type of suspect material to be identified. Definitive identification typically includes the ability to propagate the biological agent so that there is sufficient material available for analysis by the multiple methods and protocols, and the ability to look at strains by epidemiological methods, but also so material is available to initiate attribution analysis. Definitive identification is performed using the highest level quality assurance measures in a controlled laboratory. Definitive identification or "confirmation" testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC LRN as appropriate. Specific LRN protocols and reagents are proprietary, but any definitive identification or confirmation typically follows a well-established scheme, including the use of well characterized reagents by well-practiced personnel.

Like biomarkers, there are also inherent problems with the application and details involved in the identification levels that need to be understood to correctly apply the inherent concepts contained within the definitions and an appropriate application of the term confirmed. In one definition of confirmation, it states "the occurrence of two or more indicators corresponding with one another and thereby corroborating the predicted outcome." Confirmation of an identification of a biological agent, however, often needs to be grounded in more information, especially given the consequences of an incorrect identification to both the military member as well as the military operation being conducted. In addition, identification of a biological agent based on nonmetabolic methods, in the absence of morbidity or mortality, always presents the possibility that the identification is detecting inactive materials.¹⁶

Biological materials, microbes and toxins, are fragile compared to nuclear or chemical agents. They can be inactivated during the course of dispersal (especially dissemination from munitions), through natural biocidal activity (sunlight both desiccates as well as inactivates through ultraviolet irradiation), ineffective weaponization processes, or myriad physical or chemical activities. The confidence in an identification of a biological attack is also affected by how it has been detected.^{7,8} Doctrinally, low, medium, and high confidence are part of the identification levels, yet the level of confidence an assay provides is also governed by factors that include the scientific quality and accuracy of the test methods, the target or purpose of the assay(s), experience and knowledge of testing personnel, and the environment in which the lab is operating.8 Detection by one biological detector system has a lower confidence level than if two detectors have made the detection. Theater validation identification (including two biomarkers) endorses and bolsters those automated detections, but confirmation should still be viewed with a level of suspicion resulting from inherent biological diversity. Until a full characterization of the agent can be undertaken, the term confirmed should be used with some level of reservation; and military commanders, responsible for both the mission and the welfare of service members, should proceed with the realization of the ambiguous nature that biological threats present.

Allies

US allies, including members of the North Atlantic Treaty Organization, have different doctrinal identification levels. Before 1995, the North Atlantic Treaty Organization recognized the need for common approaches for sampling and identification of biological and chemical warfare agents. Within its doctrine, three levels of identification also exist¹⁶:

- Provisional identification: A biological agent may be considered provisionally identified when one of three criteria is met (presence of a unique antigen, presence of a unique nucleic acid sequence, or positive culture or multi-metabolic assay);
- 2. Confirmed identification: The identification of a biological agent is confirmed when any two of the three criteria for provisional identification have been met in the presence of authentic reference standards (positive and negative controls) under identical experimental conditions; and
- Unambiguous identification: The unambiguous identification of a biological agent provides the highest level of certainty required for the development of strategic and political positions. Confirmed identification becomes unambiguous under four criteria: (1) positive response is obtained by a genetic identification method; (2) positive response is obtained by an immunological method; (3) positive match is obtained by in vitro culture or multimetabolic assay; and (4) the disease properties of the microbial agent are confirmed in an accepted animal model.

IDENTIFICATION APPROACHES

Specimen Collection and Processing

Clinical specimens can be divided into three different categories based on the ability to affect the disease course: (1) early postexposure, (2) clinical, and (3) convalescent/terminal/postmortem.^{5,17} Common specimens for biological warfare agents are similar to those collected for diagnosis of any infectious disease and typically correspond to clinical manifestations (Table 26-3). Specimens often include swabs, induced respiratory secretions, blood cultures, serum, sputum, urine, stool, skin scrapings, lesion aspirates, and biopsy materials.^{5,18} Nasal and facial swab samples should not be used for making decisions about individual medical care; however, they could support the rapid identification of a biological threat (postattack) and help direct force health protection efforts.^{19,20} Baseline serum samples (presymptomatic) should be collected on all potentially exposed personnel after an overt attack. These samples will help to both define the forces exposed but could also provide diagnostic information in the event that nontraditional agents are being used.

In cases of sudden or suspicious deaths, autopsy samples should be taken. Specimens and cultures containing possible highly infectious agents should be handled in accordance with established biosafety precautions. Specimens should be sent rapidly (within 24 hours) on wet ice (2°C–8°C) to an analytical laboratory capable of handling them. Blood cultures should be collected before the administration of antibiotics. If necessary, the blood cultures should be shipped to the laboratory within 24 hours at room temperature (21°C–23°C). Overseas laboratories should not attempt to ship clinical specimens to CONUS reference laboratories using only wet ice unless the provisions for reicing the samples are made with the carrier. Shipments requiring more than 24 hours should be frozen on dry ice or

liquid nitrogen if possible. Specific shipping guidance should be obtained from the supporting laboratory before shipment. Specimens should not be treated with permanent fixatives (ie, formalin or formaldehyde) unless that is the only way to ensure sample stability. Storage and shipping of samples at -20°C to 25°C is contraindicated.

Environmental samples, while not patient specific, are often highly useful to medical decision making. These samples include several different categories of materials such as buffers and filters from air sampling devices, powders, soil and vegetation, animals (including rodents and insects as potential vectors), food samples from both fresh and packaged materials if ingestion is suspected, and nearly everything else that is not a clinical sample. These samples, when taken before any overt disease onset, can help identify a causative agent and potentially lead to prophylactic treatment. Nonclinical samples represent the biggest challenge in the detection of biological agents because of the vast repertoire of sample types and microorganisms in the environment that cause false-positive and false-negative detection reactions in many laboratory assays.

A substantial amount of guidance exists—both military specific^{5,16} and general²¹—so details of taking and processing of environmental samples is beyond the scope of this chapter. Environmental samples will contain myriad physical and chemical agents that can potentially interfere with detection technologies and cause false negative results. Environmental samples include samples that are both highly stable as well as samples that will degrade with time similar to clinical samples. Guidelines for the submission of environmental samples. In general, environmental samples should be maintained at nearly the same state as when they were collected. Dry samples should be kept dry, moist or wet samples should be preserved from desiccation, and

TABLE 26-3

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SPECIMEN COLLECTION FOR SELECT BIOLOGICAL WARFARE AGENTS

			Postexposure ⁺		Clinical	Convales	cent/Terminal/Postmortem [‡]
Organism	Incubation Period*	Time	Samples	Time	Samples	Time	Samples
Bacillus anthracis	1–6 d; 3 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions	48–72 h	Serum for toxin assays; whole blood (blood cultures) and tissue smears for direct fluorescent antibody [§]	72 h–28 d	Serum for toxin assays; whole blood (blood cultures)
Brucella	5–60 d; 5 d	0–36 h	Nasal and throat swabs, and induced respiratory; <i>note</i> : notify laboratory for extended culture incubation protocol	72–168 h	Whole blood (blood cultures); <i>note</i> : notify laboratory for extended blood culture incubation protocol	7–28 d	Serum for immunoassays; whole blood (blood cultures); <i>note</i> : notify laboratory for extended blood culture incubation protocol
Burkholderia pseudomallei/ mallei	1–21 d; 3 d	0–48 h	Nasal and throat swabs, and induced respiratory secretions	24–96 h	Serum for capsular polysaccharide assays; whole blood (blood cultures)	7–28 d	Serum for capsular polysaccharide assays; whole blood (blood cultures)
Clostridium botulinum/ botulinum toxins A/B/E		0–24 h	Nasal and throat swabs, and induced respiratory secretions for toxin detection	24–72 h	Blood or serum for toxin detection	7–28 d	None; serum for IgM and IgG not really valid
Coxiella burnetii	7–41 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions (egg, tissue culture, or axenic media	3–14 d	Whole blood (blood cultures) and direct molecular detection [¥]	14–60 d	Serum for IgA, IgM, and IgG
Encephalitic viruses/ alpha viruses/VEE/etc	2–6 d	0–24 h	Nasal and throat swabs, and induced respiratory secretions	24–72 h	Throat swabs up to 5 days, then cerebro- spinal fluid and serum	6–21 d	Serum for IgM and IgG
Francisella tularensis	1–21 d; 3 d	0–24 h	Nasal and throat swabs, and induced respiratory secretions	24–72 h	Whole blood (blood cultures); direct fluorescent antibody [§]	6–21 d	Serum for IgM and IgG

Medical Aspects of Biological Warfare

(Table 26-3 continues)

Hemorrhagic fever viruses/Ebola/Marburg/ Dengue/etc	4–21 d	0–24 h	Nasal and throat swabs, and induced respiratory secretions	2–5 d	Serum	6–21 d	Serum or for IgM and IgG
Ricin	18–24 h	0–24 h	Nasal and throat swabs, and induced respiratory secretions	24–48 h	Serum/plasma for toxin assays; urine for ricinine is questionable	6–21 d	Serum for IgM and IgG
Staphylococcal enterotoxins A/B/C	3–12 h	0–4 h	Nasal and throat swabs, and induced respiratory secretions for toxin detection	2–6 h	Blood or serum		None; serum for IgM and IgG not really valid
Vesicular and pustular rash illnesses/Orthopox (Variola)	7–17 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions	2–5 d	Serum and lesions/ scrapings for microscopy and viral culture	6–21 d	Lesions/scrapings for microscopy, and viral culture; serum for IgM and IgG
Yersinia pestis	1–7 d; 2 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions	24–72 h	Whole blood (blood cultures); direct fluorescent antibody [§]	7–10 d	Wholeblood (blood cultures); serum for IgM and IgG*; typical period; initial presentation of a high- dose exposure; dependent on dose; aerosol route

*Typical period; initial presentation of a high-dose exposure; dependent on dose; aerosol route.

⁺Rapid molecular and immunoassays can be done, but none are FDA cleared for patient treatment.

¹Serology and other tests may not be FDA cleared for patient treatment, but convalescent/terminal/postmortem testing is rarely used to influence direct patient treatments. [§]Direct fluorescent antibody tests are not FDA approved, but accepted if done as a laboratory-developed test and validation data available.

^{*}FDA approved for direct patient treatment.

IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M; VEE: Venezuelan equine encephalitis

cold samples should be kept cool. One especially critical requirement for any environmental sample is the initiation and maintenance of chain of custody documentation,^{7,16,22} from the sample collection through to the analysis laboratory. Again, like shipping clinical samples, guidance should be obtained from the supporting laboratory before shipment.

A multitude of international, domestic, and commercial regulations mandate the proper packing and documentation (including labeling) of biological materials (Table 26-4). Biological samples, infectious agents, and biological select agents and toxins all represent some level of dangerous goods that need special handling to protect the public, airline workers, couriers, and other persons who work for commercial shippers and who handle the dangerous goods within 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. Each of the regulations deals with specific shipping requirements, but in general, all define an infectious substance as a material known or reasonably expected to contain a pathogen (a microorganism that can cause disease in humans or animals). Universal examples of pathogens include bacteria, viruses, fungi, and other infectious agents. An infectious substance is assigned to one of the following three potential categories:

- 1. Category A: An infectious substance transported in a form capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy humans or animals when exposure occurs. Category A infectious substances are assigned the identification number UN 2814 or UN 2900, based on the known medical history or symptoms of the source patient or animal, endemic local conditions, or professional judgment concerning the individual circumstances of the source human or animal.
- 2. Category B: An infectious substance that does not meet the criteria for inclusion in Category A. Category B infectious substances bear the shipping term "Biological substance, Category B" and are assigned the identification number UN 3373.
- 3. Toxins from plant, animal, or bacterial sources that do not contain an infectious substance and are not contained in an infectious substance may be considered for classification as toxic substances; and they are assigned the identification number UN 3172.

In addition, other requirements may exist, including requirements for dry ice (dry ice is classified by the Department of Transportation and the International Air Transport Association as a "miscellaneous" hazard, class 9). The International Air Transport Association manual, *Dangerous Goods Regulations*, is the leading guide to shipping dangerous goods, including infectious agents by air, which generally includes most shipments from CONUS and outside of the continental United States (OCONUS). *Dangerous Goods Regulations* provided requirements for packaging a shipment to classify, mark, pack, label, and document dangerous goods to meet international requirements. Key issues in shipping biological materials include—at a minimum—the following:

- maintaining the sample integrity (especially metabolic viability);
- some identification of the sample if possible (determining appropriate Category A, Category B, or toxin);
- packaging requirements (packaging corresponding to category such as Category A must consist of three components: [1] a primary receptacle[s]; [2] a secondary packaging; and [3] a rigid outer packaging); and
- documentation (International Air Transport Association Shipper's Declaration for Dangerous Goods, DD Form 2890, DoD Multimodal Dangerous Goods Declaration, APHIS/CDC Form 2, Request to Transfer Select Agents and Toxins, and any import or export permits required).

Other considerations for shipping biological samples may exist^{5,23,24} and typically require personnel who have been trained and are certified to package hazardous materials for shipment (including but not limited to Transport of Biomedical Materials at https://phc.amedd.army.mil/Pages/CourseDetails.aspx?CourseID=89 [valid September 2016]). Specific specimen collection and handling guidelines for the bioterrorism agents are available from CDC and the American Society for Microbiology (see http://emergency.cdc.gov/bioterrorism/or http://www.asm.org/index.php/guidelines/sentinel-guidelines; both valid September 2016).

Culture-Based Microbiological Methods

Microbes that cause infectious disease are an example of a classic host–parasite relationship. Suspecting, or even having some evidence of a microbe's ability to produce disease, is still inferential science. Having

TABLE 26-4

INTERNATIONAL AND DOMESTIC STANDARDS FOR SHIPPING

International	Domestic
United Nations Economic Commission for Europe "Recommendations on the Transport of Dangerous Goods," also called the "Orange Book" 2009 (http://www.unece.org/trans/danger/publi/unrec/rev16/16files_e.html; valid February 2014)	49 CFR transportation (highway transportation regulations)
European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) (http://www.unece.org/trans/danger/publi/adr/ adr_e.html; valid February 2014)	42, 7, & 9 CFR for select agents
Final Governing Standards (each country; DoD Instruction 4715.5, Management of Environmental Compliance at Overseas Installations) (https://www.fedcenter.gov/Login/index.cfm?pge_id=3739& NotAuthorized=1&returnto=%2Fprograms%2Fcompliance%2Ffgs%2F index%2Ecfm%3F; valid February 2014)	9 and 21 CFR for biological products
International Air Transport Association Dangerous Goods Regulations (2014; 55th edition) (http://www.iata.org/publications/dgr/Pages/manuals.aspx; valid February 2014)	US Postal Service Domestic Mail Manual (10.17 Infectious Substances, Hazard Class 6, Division 6.2) and International Mail Manual (135.1 Infectious Substances)
International Civil Aviation Organization Regulations (http://www.icao.int /safety/DangerousGoods/Pages/default.aspx; valid February 2014)	DoD 4500.9-R, Defense Transportation Regulation; Part II, Chapter 204
International Maritime <i>Dangerous Goods Code</i> (http://www.imo.org/blast /mainframe.asp?topic_id=158; valid February 2014) (for maritime shipments)	Air Force Manual 24-204/(Interservice) TM 38-250 NAVSUP PUB/505 MCO P4030.19J DLAI 4145.3 (2012), Transportation; Preparing Hazardous Materials for Military Air Shipments
World Health Organization Guidelines Guidance on Regulations for the Transport of Infectious Substances 2007– 2008 (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS _EPR_2007_2cc.pdf valid February 2014) <i>Laboratory Biosafety Manual</i> - Third Edition (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR _LYO_2004_11/en/; valid February 2014)	Army Regulation 50-1, Nuclear and Chemical Weapons and Materiel Biological Surety (2008)
Globally Harmonized System of Classification and Labeling of Chemicals (https://www.osha.gov/dsg/hazcom/global.html; valid July 2016)	Army Regulation 190-17, Biological Select Agents and Toxins Security Program (2009)

CFR: Code of Federal Regulations; DLAI: Defense Logistics Agency Information; DoD: Department of Defense; MCO: Marine Corps Publications; NAVSUP PUB: Navy Supplement Publication; TM: Technical Manual

unequivocal proof of a specific etiological agent as the cause of an infectious disease requires the application of conventional microbial culture to validate Koch's postulates (the four standards of a logical chain of experimental evidence designed to establish a causal relationship between a causative microbe and a disease). Microorganisms can cause tissue damage (disease) by releasing a variety of toxins or destructive enzymes into the host. Although a number of ways exist to obtain indirect evidence of a microbe's effect on the host, propagating the causative microbial agent is still considered the gold standard for linking a specific microbial agent to the disease status.

Specific guidelines for identifying bioterrorism agents can be obtained from the CDC (http:\www. bt.cdc.gov) or the American Society for Microbiology (http://www.asm.org/index.php/guidelines/sentinelguidelines). Guidelines for identification of additional agents that cause other infectious diseases can be found in diagnostic microbiology textbooks. Although the ability to propagate infectious disease microbes in routine culture has been available for more than a century, many bioterrorism and infectious disease agents especially the viruses—are not always easily cultured. In addition, culturing a specific microbial agent from a clinical sample is often routine; culturing the same microbial agent from an environmental sample is manyfold more difficult. In either case, knowing which microbial agent(s) is needed will greatly help to create the right conditions for propagation. A physician's clinical observations or medical intelligence should help guide the analytical plan (see Table 26-3).^{18,25}

The bioterrorism and infectious disease agents are separated into aerobic and anaerobic bacterial agents and viruses. Fungal and parasitic microbial agents are not often encountered as bioterrorism and infectious disease agents targeted against humans. Most aerobic bacterial threat agents can be isolated by using three common clinical bacteriological media: (1) 5% sheep blood agar (SBA); (2) MacConkey agar; and (3) chocolate agar (CHOC). Cystine heart agar supplemented with 5% sheep blood has been suggested as a preferred medium for F tularensis, but CHOC agar usually suffices in clinical samples. Although Brucella agar was developed as a preferred medium for Brucella, improvements in SBA and CHOC agars support the growth of fastidious microorganisms such as Brucella. Nonselective SBA supports the growth of most bacterial agents, including B anthracis, Brucella, Burkholderia, and Y pestis. MacConkey agar, which is the preferred selective medium for gramnegative Enterobacteriaceae, supports Burkholderia and Y *pestis*. Liquid medium, such as trypticase soy broth, can also be used followed by subculturing to SBA or CHOC when solid medium initially fails to produce growth.

Anaerobic organisms (those organisms that do not require oxygen for growth; some of which may react negatively or even die if oxygen is present), such as *Clostridium* species, require the use of anaerobic media and methods. Anaerobic methods reduce the exposure of microorganisms to molecular oxygen through the use of anaerobic jars or anaerobic chambers, and use culture media that are especially designed to dissolve or deplete oxygen, allowing the anaerobes to propagate. The liquid medium thioglycollate readily supports anaerobic microorganisms and should be considered a routine medium if *Clostridium* species could be encountered.

The use of multiple bacteriological media is recommended both for redundancy as well as an aid to initial notification. Propagation of viruses is more complex and usually takes longer than those for bacteria. Since viruses are obligate intracellular parasites, propagation in various host systems is required. Most readily viruses are typically propagated in cultures of various cell lines, but laboratory animals and embryonated eggs are also used. Although no single cell culture is sensitive to all the viruses encountered, Vero (African green monkey kidney) cells are commonly used for many of the viruses (Table 26-5).

Cells used for propagating viruses require growth at an appropriate temperature and gas mixture (typically, 37°C, 5% CO₂ for mammalian cells) in an incubator. In addition, cell cultures also require special growth media that have stringent requirements for pH, glucose, antibiotics, growth factors, and other nutrients. Growth factors used to supplement media are often derived from the serum of animal blood, such as fetal bovine serum. Cell plating density (number of cells per volume of culture medium) and inoculation density of the virus are critical factors. Viruses manifest their presence in cell culture by different mechanisms including cellular degeneration (cytopathic effect), plaque formation, and metabolic inhibition testing. Some viruses require other means to demonstrate their presence in cell culture including fluorescent antibody testing or nucleic acid amplification methods.

Automated Identification Systems

Many automated identification systems are commercially available that have some capability to identify the major bacterial biological threat agents (B anthracis, Brucella spp, Burkholderia mallei, Burkholderia pseudomallei, F tularensis, and Y pestis). These systems include the BioMérieux (Durham, NC) VITEK 2, Siemens (Tarrytown, NY) MicroScan, MIDI Sherlock Microbial Identification System (Newark, DE), Trek (Cleveland, OH) ARIS 2X, Biolog (Hayward, CA), and the Bruker (Billerica, MA) Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The Becton Dickinson (Franklin Lakes, NJ) Phoenix Automated Microbiology System does not appear to be capable of identification of the major bacterial biological threat agents listed. An advantage to the automated identification systems is that if a laboratory is routinely using one of these commercial systems, personnel are already trained and reagents are typically on-hand. The primary disadvantage is that often false-positives or false-negatives occur, including misidentifications as another organism (Table 26-6). Although some identifications on some systems are problematic, identification of some agents by the automated systems are very accurate and often highly discriminatory. The identification of *B* anthracis and F tularensis by the MIDI Sherlock Microbial Identification System is very specific and an accepted method.²⁶⁻²⁸ Blind acceptance of results from one of the automated commercial systems, however, needs to be avoided, and results need to be substantiated, or refuted, by other assay information.

TABLE 26-5

	Viral Hemorrhagic Fever						
	Virus	Endemic Area	Mortality	Cells and Incubation Time	Growth Characteristics		
Arenaviruses	Lassa virus	West Africa	1%-2%	Vero E6–Vero: 3–5 d	No CPE; requires 2nd assay; plaques		
	Junin	Argentinian pampas	30%	Vero: 3–5 d	No CPE; requires 2nd assay; plaques are difficult, but possible		
	Machupo	Bolivia	25%-35%	Vero E6: 3–5 d	No CPE; plaques fine		
Bunyaviruses	Crimean-Congo hemorrhagic fever virus	Africa, SE Europe, Central	30% <0.5%	SM 3–14 d; possible to passage in E6, SW13, or CER cells after initial isolation, but may require >1 blind passages	Plaque assays just as difficult		
	Rift Valley fever	Asia, India, Africa		Vero: 2–4 d	CPE/plaques		
	Hanta virus (Hantaan, Dobrava, Seoul, Puumala, Sin Nombre Andes)	Europe, Asia, South America (rare)	5% for HFRS	Vero E6: 10–14 d	No CPE; requires 2nd assay such as IFA or PCR; often requires blind serial passages to isolate; hard to plaque		
Filoviruses	Ebola virus	Africa, Philippines (Ebola Reston)	50%–90% for Sudan/ Zaire	Vero E6: 6–12 d	CPE/plaques		
	Marburg virus	Africa	23%-70%	Vero E6: 6–12 d	CPE/plaques		
Flavivirus	Yellow fever virus	Africa, South America	Overall 3% to 12%, 20% to 50% if severe second phase develops	MK2 cells (also BHK21): 3–6 d	Little to no CPE; requires 2nd assay such as PCR or IFA to confirm; plaques fine in Vero cells		
	Kyasanur Forest disease virus	Southern India	3%–5%	Vero/Vero E6 SM: 3–6 d	CPE/plaques		
	Omsk hemorrhagic fever virus	Siberia	0.2%-3%	Vero/Vero E6 SM: 3–6 d	CPE/plaques		

VIRAL HEMORRHAGIC FEVER CULTURE INFORMATION

CER: chicken embryo related; CPE cytopathic effect; HFRS: hemorrhagic fever with renal syndrome; IFA: immunofluorescence assay; PCR: polymerase chain reaction; SE: southeast; SM: suckling pig

Although not an automated identification system, identification of bacteria with sequence data of rRNA genes (16S or 23S) needs to be mentioned. Carl Woese pioneered this use of 16S rRNA in the late 1970s for use in phylogenetic studies.²⁹ 16S rRNA gene sequencing has become a standard reference method for identification of many microbes. Bacterial 16S rRNA gene sequences are available on public databases such as the National Center for Biotechnology Information and the

Michigan State University Ribosomal Database Project. Commercially, Applied Biosystems (Foster City, CA) sells 16S rDNA bacterial identification kits under the MicroSeq name that provide standardized reagents and protocols, but they are not yet FDA approved for direct patient care. Although implementation in a routine clinical microbiology laboratory has several drawbacks for microbial identification (time and cost predominately), the accuracy and practicality for many

TABLE 26-6

AUTOMATED IDENTIFICATION SYSTEMS FOR BIOLOGICAL THREAT AGENTS

		Siemens MicroScan		MIDI Sherlock Microbial Identification System	Trek ARIS 2X	Biolog	Bruker Biotyper*	
	bioMérieux/ VITEK 2	Rapid Neg ID/Type 3 Plate	Neg ID Type 2 Plate	Biodefense Library 3.0/BTR3 and RBTR3 Instant FAME	GNID Plate	Dangerous Pathogen Identification Database ⁺	Security-RelevantLibrary	
Bacillus anthracis	Yes – BCL card	_	_	Yes‡	_	Yes – GP plate	Yes	
Brucella spp	Yes – GN card§		Yes	Yes	Yes	Yes – GN plate	Yes	
Burkholderia mallei/ pseudomallei	Yes – GN card [¥]	_	—	Yes	_	Yes – GN plate	Yes¶	
Francisella tularensis	Yes – GN card ^{§, ¥}	_	_	Yes	Yes	Yes – GN plate	Yes	
Yersinia pestis	Yes – GN card	Yes	Yes [¥]	Yes	Yes	Yes – GN plate	Yes*	

*Another system, bioMérieux VITEK MS is similar.

*Another system, bioMérieux VITEK MS is similar.
*GENIII plate has been evaluated for all biological threat agents, but database is not commercially available.
*AOAC INTERNATIONAL cleared for *Bacillus anthracis* ID.
[§]Noted as a species that may be nonreactive.
*Known false results for this organism on this system.
[¶]Differentiation of *Burkholderia mallei* and *Burkholderia pseudomallei* may not be possible.
AOAC: Association of Official Analytical Chemists
BCL: *Bacillus* identification card

GN: Gram negative

GNID: Gram-negative ID

GP: Gram positive

ID: identification

Neg: negative

FAME: fatty acid methyl esterification

of the biological threat agents is useful. But like all systems, there are limitations to full implementation, predominately in that *B* anthracis, Brucella species, and *Y* pestis are often unable to be differentiated from near neighbors with sufficient resolution to make the system practical.

Antibiotic and Antimicrobial Susceptibility Testing

A principal reason for propagation of bioterrorism or infectious disease agents in culture is to screen the agent for antibiotic or antimicrobial agent resistance or susceptibility. Although most of the bacterial biological threat agents have well-characterized susceptibility to antibiotics (Table 26-7), it will be critical to distinguish those organisms that acquire natural or laboratory modifications to normal or traditional antimicrobial susceptibility.³⁰ Strains of B anthracis,^{31–33} Brucella abortus, Burkholderia spp,³⁰ F tularensis,^{34,35} and Y pestis^{36,37} have been reported to have natural antimicrobial drug resistance, including multiple drug resistances.^{38–43} The Clinical and Laboratory Standards Institute (www.clsi.org) has published standard protocols that include the biological threat agents to ensure accuracy and reproducibility of results. For the biological threat agents, classical minimum inhibitory concentration determinations are the preferred method.⁴⁴ Although commercial antibiotic susceptibility testing devices are available,^{45,46} they have not been standardized to ensure correspondence to the reference method. The CDC LRN does include the use of the Epsilometer test (E-test) for antimicrobial susceptibility testing of selected microorganisms. The E-test is a direct quantification agar dilution method⁴⁷ that has been adopted by many laboratories because of its ease of use and quantification capabilities. Molecular methods that screen for unique genetic markers of resistance have been developed^{37,48–54}; however, molecular analysis

approaches can be cumbersome when multiple loci are involved^{50,51} and do not always correlate with therapeutic effectiveness nor laboratory data.³⁰ DNA microarrays offer the potential for simultaneous testing for specific antibiotic resistance genes, loci, and markers,^{49,50,55} but are not sufficiently developed for routine use.

Microbial Culture Versus Rapid Methods

With the introduction of newer rapid methods for biological threat agent detection and the codification of the term biomarkers in the military doctrine, there has been avoidance on the discussion of classical microbiological culture in the detection of biological threat agents. Classical microbiology culture, whether for bacteria or viruses, has been stigmatized as archaic and overly time consuming. The concept of obtaining a result in less than an hour—and being able to do something with that result—has taken center stage. Although the newer rapid methods for biological threat agent detection have matured over the past decade, there are still problematic areas in the sole reliance on these newer methods.

Current concepts of operations for theater validation laboratories are for multiple technologies that do not necessarily include culture of the organism. Most often, the use of nucleic acid amplification (through PCR) and immunoassays are the predominant methods for rapid identification. Operation of a theater validation laboratory with PCR and immunoassay technologies does not require the containment of a BSL-3 facility.

To cause disease, microbial agents must be living or toxin agents must be biologically (metabolically) active. Unless an identification of a biological agent is based on some metabolic method, in the absence of morbidity or mortality, there is a possibility that the implicated agent has been inactivated.¹⁶ Inactivation of biological materials, especially in nonclinical

TABLE 26-7

Bacillus anthracis	Brucella spp	Burkholderia mallei/pseudomallei	Francisella tularensis	Yersinia pestis
Penicillin Doxycycline Tetracycline Ciprofloxacin	Gentamicin Streptomycin Doxycycline Tetracycline Trimethoprim/ sulfamethoxazole	Doxycycline Tetracycline Imipenem Amoxicillin-clavulanate Trimethoprim/ sulfamethoxazole	Gentamicin Streptomycin Doxycycline Tetracycline Ciprofloxacin Levofloxacin Chloramphenicol	Gentamicin Streptomycin Doxycycline Tetracycline Ciprofloxacin Chloramphenicol Trimethoprim/

STANDARD ANTIBIOTIC SUSCEPTIBILITY TESTING FOR BIOLOGICAL THREAT AGENTS

samples, readily occurs and culture (or multimetabolic assays for toxins) is the only way to ensure that an implicated biological agent is actually capable of causing disease.

CDC LRN reference laboratories typically include the use of BSL-3 facilities because the CDC LRN identification requires identification based on culture of the organism(s). Current DoD doctrine, at the theater validation level, does not include culture as a requirement. The CDC LRN, however, does not include viral diagnostics/detection capabilities other than the inclusion of smallpox and other orthopoxviruses. In some areas of operations, consideration for viral threat agents is just as high, if not higher, than for the more traditional bacterial agents. Although deployed assets for the diagnostic/detection of viruses are not robust for practical reasons, consideration for those agents must be included in operations planning. Bacterial culturing can be done in BSL-2 facilities for the majority of the biological threat agents (Department of the Army Pamphlet [DA PAM] 385-69)⁵⁶; however, it invokes enhanced requirements on facilities engaged in culturing any organisms, even those less than BSL-3. Any laboratory doing culture work will have to comply with all the provisions of that reference. Laboratories not doing culture work do not invoke the requirements of DA PAM 385-69.

Another consideration for inclusion of microbial culturing technologies includes the ability to provide sufficient samples for forensic science analysis and attribution. Without the propagation of the causative agents, the ability to conclusively confirm the agent as well as the ability to share samples among attribution laboratories will be greatly hindered.

Integration of In Vivo and In Vitro Diagnostic Tests

Integrated diagnostics, or orthogonal testing, is a recommended testing strategy for both clinical as well as environmental samples. Orthogonal diagnostic testing is the key to improving the reliability of rapid diagnostic technologies. Orthogonal testing refers to tests that are statistically independent or nonoverlapping but—in combination—provide a higher degree of certainty of the final result. Although orthogonal testing is not a standard perspective in the clinical diagnostic industry, the concept and its application are paramount when investigating some infectious agents. Any single detection technology has a set of limits with regard to sensitivity and, most importantly, specificity. Orthogonal testing seeks to overcome the inherent limitations of individual test results with the strength of data combinations.¹⁸ The application of orthogonal diagnostic testing uses an integrated testing strategy where more than one technology, technique, or biomarker is used to produce diagnostic results, which are then interpreted collectively (Figure 26-1).

Immunodiagnostic Methods

An integrated approach to agent detection and identification, using both immunological and nucleic acid-detection, will provide the most reliable laboratory data and is essential for a complete and accurate disease diagnosis.¹⁸ Understanding the strengths and weaknesses of each assay is paramount in the interpretation of results. Nucleic acid-detection assays are exquisitely sensitive and specific; this is the strength of the assay, but it can also be a weakness in particular situations. Immunodiagnostic assays are comparatively less sensitive, but have broader specificity; this is a weakness of the assay, but it can also be a strength in certain situations.

In an orthogonal system, the advantages of the nucleic acid and immunological assays will offset the disadvantages. Detection of an endemic pathogen will rely on the high sensitivity of the nucleic acid-detection assay; however, for a newly emerging genetic variant the specificity of the nucleic acid-detection assay may result in a false negative. A detection system that incorporates immunodiagnostic assays will detect the variant with the broader specificity of antibodies. This can be illustrated with the detection of the newest ebolavirus, Bundibugyo. Initially, PCR-based assays failed to detect the virus because of the genetic variation. Only when the less sensitive but more broadly reactive antigen detection and capture immunoglobulin M ELISAs were used was the virus detected and identified as an ebolavirus.⁵⁷ Clearly, both immunodiagnostic and nucleic acid-detection assays are vital when detecting pathogens that exhibit genetic variation whether natural or intentionally engineered.

Immunodiagnostic techniques diagnose disease by detection of agent-specific antigens and/or antibodies present in clinical samples. The most significant problem associated with development of an integrated diagnostic system is the inability of immunodiagnostic 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, and they could therefore actually complicate medical decisions. However, continued 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. Detection of specific proteins 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 for diagnosing the diseases they cause. Nearly all methods for detecting antigens and antibodies rely on production of complexes made of one or more receptor molecules and the entity being detected (Figure 26-5).

Diagnosing disease using immunodiagnostic technologies is a multistep process involving formation of complexes bound to a solid substrate. This process is like making a sandwich in which detecting the biological agent or antibody depends on incorporation of all of the sandwich components. The assays are relatively simple and robust, but elimination of any one part of the sandwich results in a failure and a negative response. Primary ligands used in most immunoassays are polyclonal or monoclonal antibodies or antibody fragments. Generally, the first step in an immunodiagnostic assay is binding one or more antibodies for the target of interest onto a solid support. Immunoassays are 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.

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.

Once the test sample is reacted with the capture element, the final step in any immunoassay is 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, reactants may be detected visually, electronically, chemically, or physically. A single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction; therefore, these labels are effective at amplifying assay signals. Most common



Figure 26-5. Representation of common enzyme-linked immunosorbent assay formats. The assay can be configured to detect antigen or antibodies. The target of interest (direct and indirect assays) or a capture antibody (sandwich assay) is immobilized by direct adsorption to a solid support such as a 96-well plate or magnetic bead. Detection of the target is accomplished using an enzyme-conjugated primary antibody (direct assay) or a matched set of unlabeled primary and conjugated secondary antibodies (indirect and sandwich assays).

E: enzyme; 1°: first degree; 2°: second degree

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 resulting from intrinsic fluorescent 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 can be visualized by the naked eye or by instruments and are stable under a variety of environmental conditions. However, these labels are essentially inert and therefore do not produce an amplified signal. Signal amplification is useful and desirable because it results in increased assay sensitivity.

Advances in the fields of 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. An overview of technologies currently being used for identification of biological agents and either being used for diagnosing or being developed for use in diagnosing the diseases they cause will be presented.

Enzyme-Linked Immunosorbent Assay

Since the 1970s, ELISA has remained a core technology for diagnosing disease caused by a wide variety of infectious and noninfectious agents. As a result, ELISA is perhaps the most widely used and best understood immunoassay technology. Assays, which have been developed in many formats, can be designed to detect either antigens associated with the agents themselves or antibodies produced in response to infection. ELISAs that detect biological agents or agent-specific antibodies are heterogeneous assays that capture agent-specific antigen or host-derived antibody onto a plastic multi-well plate by an antibody or antigen previously bound to the plate surface (capture element). Complexed antigen or antibody is then detected using a secondary antibody (detector antibody). The detector antibody can be directly labeled with a signal-generating molecule such as in a direct ELISA, or it can be detected with another antibody that is labeled with an enzyme such as in an indirect or capture (sandwich) ELISA formats. These enzymes catalyze a chemical reaction with substrate that results in a colorimetric change. Intensity of this color can be measured by a modified spectrophotometer that determines the optical density of the reaction 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 advantages of ELISAs are their ability to be configured for a variety of uses and applications. ELISAs can be used in field laboratory settings, but they require power for temperature-controlled incubators and refrigerators 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 quantitation difficult, and are relatively slow.

At the US Army Medical Research Institute of Infectious Diseases, antigen-detection ELISAs have been developed for nearly 40 different biological agents, and antibody-detection ELISAs have been developed for nearly 90 different agents. All of these assays were developed to use the same solid phase, buffers and other reagents, with similar incubation periods, incubation temperatures, and general procedures (Table 26-8). Although significant variation exists in assay limits of detection, ELISAs typically are capable of detecting as little as 1 ng of antigen per ml of sample.

Electrochemiluminescence

Immunodiagnostic technologies based on ECL detection are of continued military interest. ECL technology, commercially developed by BioVeris (Gaithersburg, MD), was incorporated into a field ready immunodiagnostic system, the M1M. The assay formats are similar to those of ELISA; however, magnetic beads serve as the solid support and magnets are used to concentrate target agents. The detection of target uses a chemiluminescent label (ruthenium, Ru). The small size of Ru (1,057 kDa) makes it easily conjugated to any protein ligand (antigen or antibody) using standard chemistries without affecting immunoreactivity or solubility of the protein. The heart of the M1M ECL analyzer is an electrochemical

TABLE 26-8

	ELICA	ECI	Luminov		шца
	ELISA	ECL	Lummex		IIIA
Antibody Requirements					
Purity	None	Required	Required		Required
Labeling	None	Biotin/ruthenium	Biotin/bea	ds	Beads
Assay Parameter					
Coating time	12 h	0	0		0
Incubation time	3.5 h	15 m	30 m		15 m
Read time	1 sec/well	1 m/tube	20–120 sec	z/well	30 sec
No. of steps	5	1	1		1
No. of buffers required	3	1	1		1
Specialized reagents	Conjugate	Assay buffer	Sheath flu	id	Sample buffer
	Substrate	Cell cleaner			1
Solid phase used	Microtiter well	Magnetic bead	Colored latex bead		Nitrocellulose
Reaction	Bound	In solution	In solution		Bound
Detector label used	HRP	Ru	PE		Gold
Detection method	Colorimetric	Chemiluminescence	Fluorescence		Visual
Amount of sample per test	100 ml	50 ml	50 ml		200 ml
Prozone	No	Yes	Yes		No
Sample matrix effects	No	Yes	Yes		Yes
Multiplexing	No	No	Yes		Potential
Intraassay variation (%)	15%-20%	2%-12%	10%-25%		Undetermined
Potential for PCR analysis	Yes	Yes	Yes		Yes
Limit of Detection (per ml)			Single	Multiplexed	
V nactic F1 (CFU)	250,000	500	62 500	125 000	ND
Stanbylococcal enterotoxin B (ng)	2.50,000	0.05	3 13	6 25	ND
Vonozuolan aquina anconhalitic	1.05×10^7	1.0×10^7	3.13×10^8	6.25×10^8	1×10^8
virus (PFU)	1.23 X 10	1.0 X 10	3.13 X 10°	0.23 X 10°	1 X 10

COMPARISON OF IMMUNODIAGNOSTIC METHODS

CFU: colony-forming unit; ECL: enhanced chemiluminescence; ELISA: enzyme-linked immunosorbent assay; HHA: hand held assay; HRP: horseradish peroxidase; ND: not detected; PCR: polymerase chain reaction; PE: phycoerythrin; PFU: plaque-forming unit; Ru; ruthenium

flow cell with a photo-detector placed just above the electrode. A magnet positioned just below the electrode captures the magnetic bead-Ru-tagged immune complex and holds it against the electrode. 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 by a charge-coupled device camera. The system's strengths come from its speed, sensitivity, accuracy, and precision over a wide dynamic range. Magnetic beads provide a greater surface area than conventional surface-binding assays like ELISA. The reaction does not suffer 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 are possible with a linear dynamic range that can span six orders of magnitude.58,59

Assay configurations can be identical to ELISA, direct, indirect, or sandwich assays. For antigen detection assays, the beads are coated with capture antibody, whereas for antibody detection assays the beads are coated with antigen or capture antibody. The coated paramagnetic beads, in the presence of biological agent (target), form immune complexes that are detected by the Ru-conjugated detector antibody. 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 minute per sample cleaning and reading time). Conveniently, the reagents can be lyophilized. The system uses 96-well plates that allow high sample throughput.

The ECL system effectively can detect staphylococcal enterotoxin B, ricin toxin, botulinum toxin, F *tularensis*, Y *pestis* F1 antigen, B *anthracis* protective antigen (PA) and capsule, and Venezuelan equine encephalitis virus.^{18,60-63} The system, which had been demonstrated in field settings, was used as one part of an integrated diagnostic system in several deployable and/or deployed laboratories. In 2007, Roche (Basel, Switzerland) acquired BioVeris to expand its ECLbased Elecsys Systems, which ultimately led to the demise of the M1M platform and its use by the DoD. The platform remains in use, but Roche is no longer producing reagents and the system will be forced into obsolescence when supplies are no longer available. Critical assay performance characteristics and detection limits from three typical ECL agent-detection assays are shown in Table 26-8.

Meso Scale Diagnostics (Rockville, MD) has developed a line of immunodiagnostic instruments based on the ECL technology. Unlike the M1M that was single plex, analyzing a single sample for a single target, the MSD instrument is capable of multiplex analysis, analyzing for multiple targets on a single sample. The Meso Scale Diagnostics MULTI-ARRAY technology uses ECL to detect binding events on patterned arrays. In multiwell microplates, capture antibodies are bound to carbon electrodes integrated into the bottom of the plate. The plates can have up to 10 electrodes per well, with each electrode coated with a different capture antibody. Similar to the sandwich ELISA, the target of interest is captured on the electrode and detected by the target-specific Ru-conjugated detector antibody. As in the M1M system, electrochemical stimulation results in the Ru label emitting light at the surface of the electrodes, from which the concentration of target associated with the particular electrode can be determined.

Evaluation of the technology at USAMRIID found sample testing in simple matrices, like the high volume air handler buffer, worked well, but the assays suffered from increased backgrounds in more complex matrices, like blood or serum. The ECL analyzer PR2 is available in a manual configuration, Model 1800, and a fully automated configuration, Model 1900, each of which is capable of high-throughput analysis. For environmental testing, the Model 1500 is designed for automated aerosol sample testing.

This multiplexed immunoassay platform has more than 400 assays commercially available for use in clinical, environmental, and research applications, with kits that are specifically designed for biodefense. MSD assays can be customized; however, antibody printing onto the electrodes must be done by the company, rendering laboratory derived tests less flexible, more complicated, and most likely more expensive. The NGDS acquisition program has identified the MSD PR2 instruments for possible inclusion as the immunodiagnostic component in its portable human diagnostic system. Dependence on any single company for both instrument and assays increases the risk to the DoD diagnostic and detection programs, which is reminiscent of the BioVeris experience.⁶⁰

Flow Cytometry

Flow cytometry, the measurement of physical and chemical characteristics of small particles, has many current applications in research and healthcare 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 works by 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. Light is scattered, and the distribution and intensity of scattered light is characteristic of the sample passing through. The wavelength of light is selected such that it causes the dye-bound to the particle of interest-to fluoresce. A computer counts and/or analyzes the fluorescent sample as it passes through the laser beam. Using the same excitation source, fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and signals interpreted by specialized software. Multiplexed flow cytometry assays have been demonstrated for a variety of cytokine targets.⁶⁴ Particles can also be sorted from the stream and diverted into separate containers by applying a charge to the particles of interest.

The Luminex xMAP technology (Austin, TX) has resulted in significant improvements in multiplex flow cytometry-based diagnostics. The xMAP technology is based on polystyrene bead sets encoded with different intensities of red and infrared dyes (unique address to a bead set) and coated with a specific-capture antibody against one of the analytes of interest. Interrogation of the beads by two lasers identifies the spectral property of the bead (address) and hence the associated analyte, in addition to the phycoerythrin labeled secondary antibody against the specific analyte.

The Luminex 100/200 (Austin, TX) and the FLEX-MAP 3D systems are flow cytometry-based instruments that can rapidly perform up to 100 tests simultaneously on a single sample. They incorporate three familiar technologies: (1) bioassays, (2) microspheres, and (3) fluorescence. Assays occur in solution; thus, reaction kinetics are rapid and incubation times are shorter. 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; that is, it detects antigen in a typical antigen-detection assay. The instruments use two lasers: one for detection of the microsphere itself, and the other for the detector. Microspheres 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, dynamic range of 3 to 4 orders of magnitude, and claims results are highly consistent and reproducible.⁶⁵ 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 use tubes as well as 96- and 384-well plates, can be automated. In addition to the Luminex instrument, a plate shaker and liquid handling devices are required to complete assays. As with most technologies, many different formats can be used. Many multiplexed assay kits are commercially available from different manufacturers for various cytokines, phosphoproteins, and hormones.

The FLEXMAP 3D instrument is capable of high throughput and can be automated, which makes it better suited for a large clinical laboratory. No fieldready versions of the Luminex 100/200 are available, which limits the practical use of this instrument in deployment situations. No commercial or DoD sources for biological threat agent assays are available for this platform.

MAGPIX

Flow cytometry-based systems can be accommodated in large diagnostic laboratories where environmental conditions are controlled and qualified technicians perform preventative maintenance to ensure the flow cells and lasers are clean, aligned, and functioning properly. Recently, the MAGPIX instrument based on the Luminex xMAP technology was introduced. The instrument, which eliminates some of the shortcomings of the flow cytometry-based instruments, has tremendous potential for forward laboratory applications in such resource-limited environments. MAGPIX uses magnetic color-coded microspheres to perform multiplexed assays. Fifty different individually addressable bead sets can be used on an instrument. Instead of interrogating individual microspheres sequentially through flow cytometry, MAGPIX uses magnetic force to move the microspheres to a stage and then images all the magnetic microspheres from that sample at once using a charge-coupled device camera. Three images, each taken with a different filter, are used to discriminate bead sets and determine assay signals. Two images are used to identify the unique bead address and the third image measures the presence of tracer fluorophore, indicating the presence of target analyte. The MAGPIX carries sufficient drive fluid onboard (650 mL) to analyze eight full microtiter plates (768 samples) and has a throughput rate of approximately 96 samples per hour, or 1.6 samples per minute. The system is fully compatible with all magnetic bead-based assays currently performed on the Luminex flow cytometers; all assay, sample, and reagent preparation protocols for both systems are analogous. The sensitivity of the MAGPIX system is similar or identical to the Luminex 100/200 instrument, which can detect ricin in the pg/mL range.

Sensitivities of bead-based assays are typically in the same range as - or in some cases superior to - those obtained in ELISAs.^{66,67} Previous limitations in fieldability for the Luminex flow cytometric instruments (large size, susceptibility of the laser alignment to shock or vibration) have also been largely overcome in the new MAGPIX instrument; this latter platform is smaller and more rugged. Per instrument cost has also been significantly decreased, which may also make it more affordable for widespread deployment in forward facilities. Featuring a flexible, open-architecture design, xMAP technology can be configured to perform a wide variety of bioassays quickly, cost effectively, and accurately. Six assays are commercially available for biodefense toxin targets: botulinum toxins A, B, E, F, staphylococcal enterotoxin B (SEB), and ricin.

Hand Held Assays

HHAs are immunodiagnostic assays that are ideally suited for field-based diagnostics. Commonly found on the commercial market, they are simple enough to use and interpret that some types are even approved for over-the-counter use by the FDA; the best known one is the home pregnancy test. HHAs 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 some visible marker element is placed on a sample application pad. As sample flows across the membrane, antigen or antibody present in the sample binds to labeled antibody and is captured as the complex passes the bound antibody or antigen (Figure 26-6). 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.

HHAs are advantageous because they are relatively inexpensive, simple, and require little training to use, and results can be obtained in only 5 to 15 minutes. One of the greatest advantages of HHAs is the lack of reliance on instrumentation and logistical needs associated with those instruments. However, this lack of instrumentation decreases the utility of the tests because results cannot be quantified. To respond to this deficiency, several technologies are available to make these assays more quantitative and have the added benefit of increasing their sensitivity. One technology, produced by Response Biomedical Corporation (Vancouver, British Columbia, Canada), allows for quantitative interpretation of the HHA.^{68–71} The Rapid Analyte Measurement Platform (RAMP) cartridges for biodefense can detect B anthracis, ricin, botulinum toxin, and smallpox virus. Another method for quantitative detection of antibody/antigen complex formation in HHAs is use of up-converting phosphors.^{72,73} Paramagnetic particles have similarly been used in



Figure 26-6. Illustration of a typical hand held immunoassay. Photograph: Courtesy of US National Aeronautics and Space Administration.

assays: instruments capable of detecting changes in magnetic flux within the capture zone (Quantum Design, San Diego, CA) have proven useful by improving sensitivity by as much as several orders of magnitude over more traditional HHAs.

DoD commonly uses HHAs to detect biological threat agents. The DoD Medical Countermeasure Systems, Critical Reagent Program, a repository for DoD diagnostic reagents, offers lateral flow assays for this purpose. In addition, several commercial 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 HHAs is their inability to incorporate the capability to run a full spectrum of control assays on a single strip assay. Recently, FDA approved two lateral flow assays for the detection of B anthracis for use in clinical settings.⁷⁴ As with any diagnostic test, understanding its strengths and weaknesses will aid in proper interpretation of the results. HHAs are useful in initial screening of samples for biological threat agents, but results should be followed with confirmatory testing using an orthogonal system.

Future Perspectives

Traditionally, assays for detecting proteins and other nonnucleic 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 nonantibody molecules, including aptamers, peptides, and engineered antibody fragments, are now being used in affinity-based detection technologies.^{75–83}

Since an immunodiagnostic assay is directly related to the characteristics of the antibody components used, improved antibodies or antibody-like elements have the potential to significantly improve the sensitivity, specificity, and robustness of the assays. Naturally occurring single domain antibodies (sdAbs) derived from camelids and sharks possess unique properties that could improve present day immunodiagnostics. Through convergent evolutionary processes, both camelid and shark immune systems naturally possess nonconventional antibody subsets composed only of heavy chain homodimers and a single variable domain.^{84,85} The variable (V) domains of these antibodies represent the smallest naturally occurring antigen binding domains known. These extremely small (12–15 kDa) sdAbs can target enzyme clefts and cryptic antigens that conventional antibodies cannot. Unique structural characteristics provide them a high temperature (>60–90°C), proteolytic and pH stability,^{86–93} high solubility,⁹⁴ and efficient and economical expression in a variety of microorganisms (including *Escherichia coli*).⁹⁵ The unique features of these naturally occurring molecules could vastly improve the utility of any immunodiagnostic assay.

Antibody-based biosensors provide the most reliable detection capability across the broadest range of biowarfare agents. They are, therefore, the preferred platform for DoD biosensor applications. However, the fragility of the antibody molecule together with the short shelf life (typically 2 weeks or less) of antibodybased biosensors severely complicates their use outside of a clinical laboratory environment. In addition, the variability in affinity across various antibody systems has precluded the development of multiplexing antibody arrays for biosensor applications. The Defense Advanced Research Projects Agency sponsored the Antibody Technology Program to develop and demonstrate approaches for achieving revolutionary improvements in the stability of antibodies while simultaneously demonstrating the ability to control antibody affinity for use in immunological detection.⁹⁶⁻⁹⁸ Each performer was supplied with the same starting material, single chain fragments (scFvs), and was asked to improve the antibodies by engineering them for improved stability and affinity. The desired metrics for improvements were decreasing the affinity of the antibody by at least 100-fold and increasing the stability of the supplied antibody such that it maintained its activity at 70°C for 1 hour.

Initially, the performers achieved these requirements in separate proteins before attempting to meet both requirements in one protein. Each group



Figure 26-7. Generic overview of PLA reactants and assay. (**a**) In addition to PCR reagents, PLA consists of antibodies to two different epitopes, each labeled with a unique oligonucleotide (proximity probe) and a connector oligonucleotide complementary to the free ends of each proximity probe. Unique to our design is the inclusion of magnetic beads coated with antigen-specific antibodies. (**b**) After formation of a bead/antigen/proximity probe complex, the free 5' and 3' ends of the antibody-bound oligonucleotides that are in close proximity to each other hybridize onto the connector oligonucleotide and are covalently joined by DNA ligase. Once joined, these provide a template for PCR amplification. Ab: antibody; Comp: complementary; Hyb: hybridization; PCR: polymerase chain reaction; PLA: proximity ligation assay

approached the solution differently, but produced antibodies with greater binding to the target ligand and improved thermostability. The Antibody Technology Program increased antibody affinity by a factor of 400. Temperature stability of antibody molecules was improved by a factor of 36, which translated into an increased shelf life at room temperature from about 1 month to 3 years. Similarly, antibody survival at 70°C increased from 5 to 10 minutes to 48 hours. By creating these stable antibodies, it was postulated that different variable regions could be grafted onto the developed backbone to increase the stability of antibodies in general, without altering the affinity. These improvements would translate into improved immunodiagnostic assays that would function well in more austere environments, as well as decreasing the cold chain needs for these reagents.

Often the Achilles heel of immunodiagnostic assays is the lower sensitivity when compared to PCRbased assays. Advances in antibody development or engineering can improve antibody characteristics and therefore the resulting assays, but other advancements combine antibody detection with PCR to achieve sensitivity levels equivalent to PCR. Immuno-PCR assays are similar to ELISAs, but substitute the detector antibodies conjugated to enzymes with antibodies that are labeled with DNA.⁹⁹ Using label-specific PCR primers, the DNA label is amplified and can result in increased sensitivity of 105-fold. These assays that relied on a single DNA-labeled antibody exhibited high background signals that frequently resulted in false-positive results.^{100,101} The proximity ligation assay eliminated the background limitations of immune-PCR by requiring the binding of antibodies to at least two different epitopes on the target antigen.¹⁰² Each antibody is labeled with a specific oligonucleotide containing a PCR primer site and having either a free 5' or 3' end (Figure 26-7).

When the antibodies bind the target, the DNA labels are brought into proximity and the two complementary ends hybridize to a connector oligonucleotide with compatible ends. The hybridized strands are joined by DNA ligase and serve as a template for amplification and fluorescent probe detection. The amplified DNA is a surrogate marker for the target protein of interest. The 5' or 3' oligonucleotide ends that fail to hybridize completely with connectors cannot be amplified and reduce the background and the possibility of false positives. Proximity ligation assay detection of viruses and bacterium has proven to be more sensitive than ELISA and as sensitive as real-time PCR.¹⁰³ In addition, the assays work in a wide variety of biological matrices, serum, plasma, cerebrospinal fluid, cell culture media, and lysates of cells and tissues.^{102,104} Improvements in technology and the components of immunodiagnostic assays continue to close the gap in sensitivity between protein detection and nucleic-acid detection making an orthogonal system ever more powerful.

Molecular Detection Methods

PCR is the predominant methodology for detection of molecular signatures. Originally conceived in 1983 by Kary Mullis,¹⁰⁵ the first published application of PCR was by Saiki et al amplifying beta-globin genomic sequences and thus hallmarking the advent of the molecular biology field.¹⁰⁶ In its simplest form, PCR consists of target genomic material, two oligonucleotide primers that flank the target sequence, a heatstable DNA polymerase, a defined solution of salts, and an equimolar mixture of deoxyribonucleotide triphosphates. This mixture is subjected to repeated cycles of defined temperature changes that facilitate denaturation of the template, annealing of the primers to the target, and extension of the primers so that the target sequence is amplifying. With each cycle, a theoretical doubling of the target sequence occurs. 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. Under ideal conditions, a single copy of a nucleic acid target can be amplified over a billionfold after 30 cycles, thus allowing amplification from targeted genomic signature with potential detection of etiologic agents down to a single copy.^{107–109} Genomic material, DNA or RNA (in the form of cDNA), can be targeted by this method of amplification. Rapid detection methods typically rely on real-time PCR where targeted genomic signatures are amplified via primers and detection accomplished through oligonucleotide probe hybridization. To this end, numerous PCR-based technologies are currently implemented in the clinical setting for diagnosis of infectious agents.

Real-Time Polymerase Chain Reaction

The most important development in rapid identification of biological agents is real-time PCR methods. Although traditional PCR is a powerful analytical tool that launched a revolution in molecular biology, it is difficult to use in clinical and field laboratories. As originally conceived, gene amplification assays can require 5 to 6 hours to complete, not including the sample processing required to remove PCR inhibitors.¹¹⁰ The improvement of assay time-to-answer came with the development of assay chemistries that allowed the PCR reaction to be monitored during the exponential amplification phase, that is, real-time (Figure 26-8). In this context, Lee et al and Livak et al developed real-time assays for detection and quantification of fluorescent reporters where fluorescence increase was directly proportional to the amount of PCR product generated in the reaction.^{111,112} In this scenario, higher starting copy numbers of the nucleic acid target resulted in earlier amplification where significant increase in fluorescence is observed.

Three main probe-based fluorescence-monitoring systems exist for DNA amplification: (1) hydrolysis probes; (2) hybridization probes; and (3) DNA-binding agents. Hydrolysis probes, most exemplified by TaqMan (Applied Biosystems, Foster City, CA) chemistries, have been the most successful for rapidly identifying biological threats.¹⁰⁸ Numerous assays have been developed against biological threat and infectious

agents using these approaches by the DoD, the CDC, and the US Department of Energy.^{108,109}

The JBAIDS is the current DoD fielded platform for molecular diagnostic/real-time PCR detection in reference laboratory, combat support hospital, and forward operating settings. This system supports assays primarily in the identification of several biological threat agents for clinical diagnostic application while also supporting assays for biosurveillance screening of biological threats as well as some infectious diseases. FDA-cleared assays for clinical diagnostics include *B anthracis*, *F tularensis*, *Y pestis*, *C burnetii*, and several forms of influenza (H5N1, A, B and A subtyping). Other assays for biosurveillance purposes cover additional biological threat targets, toxins, and foodborne pathogens. These assays can be run in approximately 30 minutes with up to 32 samples per run. With this



Figure 26-8. Overview of real-time PCR reactants and reaction conditions, generic. (**a**) Real-time PCR reactions (TaqMan probes depicted) consist of the canonical PCR reactants, such as forward and reverse primers as well as a DNA template. In addition to these reactants, real-time PCR contains either a fluorescently labeled probe or intercalating dye that is used to monitor amplicon quantities. In the depicted scenario, a sequence of DNA complementary to target sequence separates a fluorophore (F) and a quencher (Q). Fluorescence from the fluorophore in proximity to the quencher is greatly diminished compared to absence or distal fluorescence. (**b**) Similar to conventional PCR, real-time PCR reactions begin with a denaturing of the DNA template. Reducing the temperature allows amplicon-specific primers to anneal to the target sequence and amplification to begin. In some type of real-time reactions, amplified double-stranded DNA is directly quantified through measurement of DNA intercalating dyes such as SYBR green, which only fluoresces when intercalated. In the instance depicted, the probe anneals to the DNA template in similar fashion to the primers. When DNA polymerase encounters the probe, the enzyme's exonuclease function cleaves the probe liberating the fluorophore. No longer in proximity to the quencher, fluorophore fluorescence can be monitored and then correlated to target sequence concentration. Subsequent cycling and amplification yield progressively more DNA template and, consequently, more fluorophore fluorescence. PCR: polymerase chain reaction

system, a presumptive identification of most biological agents can be completed in 3 hours or less. Although it is an excellent system for detecting biological threat agents, this system suffers from lack of use in the field setting because of the lack of assays for more commonly acquired pathogens that are more routinely seen in the clinical setting. To mitigate this issue, future generations of molecular detection instruments should have regulatory cleared assays for common infectious diseases to make use and maintenance worthwhile.

Next Generation Molecular Diagnostics

The JBAIDS device is currently fielded in DoD medical laboratories, and several of the aforementioned problems exist with this system including the lack of routine usage resulting from limited assay availability and the limited capability to run independent or replicate samples (32 samples per run). To address some of these issues, the Joint Program Executive Office, the office that fielded the JBAIDS, acquired the Biofire FilmArray platform for the NGDS. While the FilmArray (Biofire Diagnostics, Salt Lake City, UT) was chosen as the NGDS device, several other viable diagnostics were considered within source selection, including the Liat Analyzer (IQuum, Marlborough, MA) and the 3M Focus Integrated Cycler (Focus Diagnostics, Cypress, CA). Overall, the FilmArray was chosen based on ease of use, sensitivity, and available FDA-cleared assays for respiratory or other commonly acquired infectious diseases.

FilmArray is an integrated sample prep and multiplex PCR diagnostic platform capable of detecting bacteria and viruses in a single reaction. This system can run FDA-cleared assays for common respiratory organisms or assays for biological threat detection in a pouch-based array, thus providing a routine application for the instrument in a clinical setting. In addition to the respiratory pouch, several other pouches have been evaluated, to include the blood culture and biological threat pouches verifying performance characteristics.¹¹³⁻¹¹⁶ Up to 48 independent reactions can be run in a single run; however, only a single sample can be run per pouch thereby limiting the throughput of this device. Overall, the system is a simple use instrument using syringe and closed pouch-based system to bead-beat and extracts nucleic acid with downstream application to an array-based set of real-time PCR reactions. Given the low complexity of operation, FDA is evaluating it for a CLIA-waver; however, currently, it is considered a moderate complexity device.

Overall, this system provides an incremental step forward in technology compared to the JBAIDS that it will replace. Assay versatility will be sacrificed for integrated sample processing and clinically applicable assays upon deployment. While these additions to the DoD portfolio will augment current biosurveillance and biological threat detection capabilities, further development is required to truly advance the frontline military diagnostic applications. The current forerunner for filling this capability is next-generation sequencing (NGS) applications.

The Horizon–Agnostic Diagnostic Applications

The current endpoint and desired capability for diagnostics in the DoD is an agnostic molecular platform. All the aforementioned technologies require some a priori knowledge of the organism; for instance, realtime PCR requires sequence information regarding the target of interest to design primers and probe. In addition, in the application of real-time PCR, guidance from medical intelligence, symptomology, or endemic diseases is required because there are limitations to the number of discrete targets and samples that can be queried in a single run. These limitations could be overcome by application of agnostic diagnostic approaches such as NGS pathogen detection strategies.

NGS has many potential benefits over current molecular diagnostic approaches. In terms of agnostic detection, NGS has the capability to sequence an entire genome of an organism, thus obviating the need for specific a priori knowledge of the pathogen. For example, the detection of novel filovirus variants such as Lujo virus was accomplished via NGS discovery.¹¹⁷ While numerous methodologies have come and gone throughout technology development, current field leaders are Illumina's sequence-by-synthesis (Illumina Inc, San Diego, CA) and PacBio's (Pacific Biosciences, Menlo Park, CA) single molecule real-time sequencing. Each system has advantages and disadvantages. Illumina is the current leader with shorter sequence reads (72–250 bp), but generating significantly more sequence data (>10 GBp). PacBio, however, generates much longer reads (1–10 kb), but has significantly higher error rates. As the field progresses, newer nanopore technologies, such as the MinION (Oxford Nanopore, Oxford, UK), may supplant these current leaders in the near future.

Combinatory approaches between these two technologies have been applied to mitigate independent disadvantages while retaining platform-specific advantages.¹¹⁸ Numerous lab-derived tests and even 510(k) submissions have cleared the FDA for use in detecting cancer. However, several steps and obstacles require mitigation before these technologies can be applied to regulatory compliant detection of a pathogenic organism. Principal in these issues include mitigation of high amounts of background host-derived nucleic acid, lack of specificity resulting from agnostic nature, and sensitivity issues. Current efforts within academia and DoD show promise toward mitigating these issues and bringing NGS into the diagnostic toolbox.

BIOSURVEILLANCE AND EMERGING THREATS

The emergence of new biological threats is a particular challenge for the military clinical or field laboratory. In the past, the biological defense research program for diagnostics has focused on agentspecific identification using collections of biological threats in the biological weapons programs of the United States (ended in 1969) and the former Soviet Union.^{119,120} However, several critical events have broadened the scope of the biological threat over the past 3 decades. The maturation and proliferation of biotechnology have resulted in several laboratory demonstrations of genetically engineered threats with new, potentially lethal characteristics.¹²¹⁻¹²⁵ Jackson et al demonstrated that the virulence of orthopoxviruses was enhanced by the insertion of immunoregulatory genes, such as interleukin-4.124 In other work, Athamna et al demonstrated the intentional selection of antibiotic-resistant B anthracis.¹²¹ Borzenkov, Pomerantsev, and Ashmarin modified Francisella, Brucella, and Yersinia species by inserting beta-endorphin genes.^{122,123}

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. Secondly, the emergence of more virulent and/or infectious strains of naturally occurring infectious diseases has posed significant public health challenges to civilian and military populations. The emergence of the H5N1 and H1N1 variants of influenza is a recent example of the challenge that naturally occurring infectious diseases can present, the latter resulting in a pandemic from 2009 to 2010. These new threats will require the development of identification and diagnostic systems that can be used flexibly to allow early recognition of a unique biological threat, representing one of the next major research and development challenges for the DoD, DHHS, and DHS. The ability to identify and characterize genetically engineered threats or naturally emerging infectious diseases before they negatively affect military and public health is the focus of new initiatives in biosurveillance.

A national effort on biosurveillance was formally initiated on October 18, 2007 in Homeland Security Presidential Directive-21,¹²⁶ which defines biosurveillance as the process of active data gathering with appropriate analysis and interpretation of biosphere data that might relate to disease activity and threats to human or animal health—whether infectious, toxic, metabolic, or otherwise, and regardless of intentional or natural origin-to achieve early warning of health threats, early detection of health events, and overall situational awareness of disease activity. The DoD community has accepted biosurveillance as defined above as a working definition, and as synonymous with health surveillance as defined in DoD Directive 6490.02E, Comprehensive Health Surveillance, which establishes policies and assigns responsibility for routine, comprehensive health surveillance of all military service members.¹²⁷ The DoD has an extensive health surveillance program for all military personnel, and the Armed Forces Health Surveillance Center executes this effort.¹²⁸ However, in addition to human health surveillance, biosurveillance encompasses active data gathering and interpretation of data from the entire biosphere, including animal health surveillance, vector surveillance, and environmental surveillance.¹²⁹

The challenge 7 years removed from the Homeland Security Presidential Directive-21 is accessing, collecting, and interpreting all of the surveillance data that are available in a way that provides actionable information to affect public health. Specific challenges that must be addressed include information sharing, information technology tools to assimilate and analyze data, and algorithms to interpret and report the subset of data that affects public health. Within the confines of biosurveillance, diagnostic testing results are a very small percentage of the health surveillance data, and an even smaller percentage of the biosurveillance data. Therefore, care must be exercised to ensure that diagnostic testing data feed into biosurveillance without allowing the biosurveillance mission to become the critical requirements for diagnostic assay and platform development. Diagnostics must continue to focus on assisting clinicians in making correct medical decisions about the treatment and prognosis of individual patients. The ultimate goal and the significant challenge for the biosurveillance enterprise is translating the identification of a potential public health threat through biosurveillance to a medical countermeasure, such as an in vitro diagnostic test. Doing so in a timely manner will be critical to maintain military readiness and minimize public health impacts.

Ultimately, the success of biosurveillance depends on the tools and technologies available to survey the biological space that affects human health. These tools must move away from agent specific identification, which is currently the foundation of most FDA-cleared in vitro diagnostic tests, to a more agnostic approach. Unlike diagnostic tests, which are typically chosen based on clinical suspicion of a particular disease, biosurveillance platforms must attempt to identify all agent(s) in a particular sample. This identification can be approached through the use of multiple complementary identification technologies or agent agnostic platforms. The service lab component of the NGDS acquisition program will deliver several complementary platforms to OCONUS research laboratories to enhance the DoD's biosurveillance capability. The instruments will include the Applied Biosystems (Foster City, CA) 7500 Fast Dx Real-Time PCR Instrument, the Luminex (Austin, TX) MAGPIX, and the Illumina (San Diego, CA) MiSeq instrument. The 7500 Fast Dx is an FDA-cleared molecular diagnostic device for the detection of nucleic acids by real-time PCR, whereas the MAGPIX is a highly multiplexed combined immunoassay/molecular assay platform for the detection of proteins or nucleic acids. Combined, these instruments could potentially cover the nucleic acid and protein biological space to include identification of viruses, bacteria, and toxins. The critical challenge for these two instruments will be the availability of assays that are capable of extensively surveying the infectious disease space.

In addition, the Illumina MiSeq instrument is a nucleic acid sequencing instrument that may potentially be used as an agnostic approach to agent identification.^{130,131} Metagenomic sequencing has become a favored approach to identify all biological components in clinical and environmental samples, and significant investments have been made to stand up genomic sequencing centers within the DoD. The roll out of MiSeq instruments in overseas laboratories is the DoD's attempt to take this capability beyond reference laboratories. Although sequencing has advanced significantly in the past decade, it has proven most useful in samples where the amount of organisms is not limiting, which is often not the case in clinical samples where the concentration of organism is extremely low in relation to the host nucleic acid in the sample. Teasing out the sequences that are significant for biosurveillance and public health purposes is the critical biochemical and bioinformatic challenge for metagenomic sequencing approaches.¹³²

Although sequencing provides a wealth of information, sequence data alone does not substitute for the need to propagate and maintain the viable organisms necessary for medical countermeasure development efforts. This capability is critical, especially for unknown or emerging threats, as all vaccine, therapeutic, and diagnostic development will require enough purified agent material to perform the necessary investigations. Overall, a rapid response capability from agent identification to therapeutic delivery to the warfighter requires integration across program areas with logical transition from one capability area to another within DoD. A comprehensive biosurveillance plan will include sample acquisition, identification, and characterization capability that allows for rapid development of medical countermeasures. Transition of the deliverables from biosurveillance should bridge pathogen discovery with diagnostics, animal model development, and vaccine and therapeutic evaluation, thereby shortening the timeline between agent identification and fielding of medical countermeasures. Ultimately, data from biosurveillance efforts must lead to actionable information to respond rapidly with medical countermeasures such as vaccines, therapeutics, and diagnostics.

Ultimately, the information provided by biosurveillance needs to translate into products that can be used in an emergency situation to enhance military readiness and public health. The nation's ability to react to a biological event to minimize casualties and impacts, or biopreparedness, is critical during an emerging outbreak or intentional release of a biological threat agent. The emergence of H1N1 and H5N1 strains of influenza was a valuable lesson for the US government to provide medical countermeasures in a response that included the availability of in vitro diagnostic tests. In 2004, the Project BioShield Act amended the Federal Food and Drug Cosmetic Act (21 USC 360bbb-3; sec 564) to include a process by which non-FDA approved products or off-label uses of approved products could be rapidly fielded in declared emergency situations. Only the Secretary of Defense, Secretary of Homeland Security, or Secretary of Health and Human Services can determine whether an emergency situation meets the criteria established in the act. Once this occurs, the US Secretary of Health and Human Services issues a declaration allowing EUA submissions to the FDA for consideration and potential use. Declared emergencies are not limited to ongoing emergencies, but also include situations that may present a heightened risk for potential attacks or events.

Åny potential situation that would pose a significant risk to the public or to US military forces, or has the potential to adversely affect national security could be declared an emergency situation. This process was activated, refined, and used for in vitro diagnostics during the H1N1 pandemic in 2009–2010.¹³³ The typical process to use an in vitro diagnostic test during an emergency involves the declaration of emergency, the submission of performance data to the FDA, FDA review, and FDA authorization to use the test under the EUA. One outcome of the H1N1 EUA process for diagnostics was the development of a pre-EUA process to streamline this process. Based on the FDA's H1N1 guidance document,¹³³ the DoD and FDA worked together to define a process for prepositioning performance data for in vitro diagnostic tests that were not yet FDA cleared but could be invaluable during a declared emergency. By allowing pre-EUA submissions for diagnostic tests, the FDA can review data, request additional data, and make preliminary decisions on utility before an emergency is declared, greatly reducing the time between the declaration of an emergency and the authorization to use the test. Pre-EUA approval does not grant permission to use or market the product under nonemergency conditions, but greatly enhances biopreparedness should a biological threat event occur. The DoD submitted 73 assays for pre-EUA consideration to the FDA in July 2010, and eight assays have been accepted after providing additional performance data on the JBAIDS and Applied Biosystems 7500 FAST DX real-time PCR platforms. The pre-EUA process continues to expand the immediate availability of in vitro diagnostics during a declared emergency, and it adds previously unavailable biopreparedness capability for the DoD and the nation.

Success in responding to emerging or genetically engineered biological threats is dependent on identifying, characterizing, and reducing the health impacts of the threat, which requires a continuum from identification of the threat at the point of presentation (clinically or environmentally) through rapid medical countermeasure deployment. Doing so quickly requires the assimilation of all available biological data, determination of which data are meaningful, and identification of actionable information signifying a threat to public health. These are the underlying goals of biosurveillance. However, the collection of samples, characterization of the threat agent, development or identification of the appropriate countermeasures, and deployment of those countermeasures to be used under regulatory compliance are necessary to achieve the desired end state, thus minimizing the public health and military readiness impacts of emerging and engineered threats.

FUTURE APPROACHES

Early Recognition of the Host Response

Early recognition is critical for the diagnosis and treatment of biological threat agents because of their disease progression, persistence, and lethality (Table 26-9). 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.

Host gene responses to biological threat insults can manifest in multiple ways. Studies suggest that the 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.^{134,135} Poxviruses may possess several mechanisms to inhibit innate immunity.¹³⁶ Gibb, Norwood, Woollen, and Henchal 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.¹³⁷ Others have shown that Ebola virus infections are characterized by dysregulation of normal host immune responses.¹³⁸ 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.^{139,140} A complicated picture is emerging that goes beyond dysregulation of genes related to innate immunity. Relman suggested that there are genome-wide responses to pathogenic agents.141 Mendis identified cDNA fragments that were differentially expressed after 16 hours of in vitro exposure of human peripheral blood mononuclear cells to staphylococcal enterotoxin B.142 By using custom cDNA microarrays and real-time 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: 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

TABLE 26-9

BIOLOGICAL WARFARE AGENT DISEASE CHARACTERISTICS

Disease	Human-to-Human Transmission	Infective Dose (Aerosol)	Incubation Period	Duration of Illness	Lethality	Persistence of Organism
A1	N	0.000 1.000	1 ()		TT: 1	
Anthrax	No	8,000–1,000 spores	1–6 d	3–5 d (fatal if untreated)	High	High
Brucellosis	No	10–100 cells	5–60 d; usually 30–60 d	Weeks to months	Low	High
Glanders	Low	5,000–10,000 cells (NHP)	10–14 d	7–10 d (fatal if untreated)	Moderate to high; >50%	High
Melioidosis	Low	50–80 cells (NHP)	1–21 d; up to years	2–3 d (fatal if untreated)	Moderate	High
Plague	Moderate	500–15,000 cells	1–7 d; usually 2–3 d	1–6 d (fatal if untreated)	High	High
Tularemia	No	10–50 cells	1–21 d; usually 3–6 d	Fatal if untreated	Moderate	High
Q fever	Rare	1–10 cells	7–41 d	2–14 d or longer if not treated	Low	High
Smallpox	High	10–100 organisms	7–17 d; average 12 d	4 weeks	High	High
VEE	Low	10–100 organisms	2–6 d	Days to weeks	Low	Low
Viral hemorrhagic fevers	Moderate	1–10 organisms	4–12 d	Death between 7–16 d	Moderate to high	Low
Botulism	No	0.003 µg/kg for type A	12 h–5 d	Death in 24–72 h; lasts for months if not lethal	High	Low (weeks)
SEB	No	0.0004 μg/kg	3–12 h	Hours	Low	Low
Ricin	No	3–5 μ g/kg (mouse LD ₅₀)	18–24 h	Days	High	High

LD: lethal dose

NHP: nonhuman primate SEB: staphylococcal enterotoxin B VEE: Venezuelan equine encephalitis

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.

SUMMARY

Military clinical and field laboratories play a critical role in the early recognition of biological threats, serving as unique sentinels in CONUS and OCONUS areas for biological threats and emerging infectious diseases. While performing regulatory compliant patient diagnostics for biological threats is difficult in a theater of operation, the fielding of the JBAIDS real-time PCR platform has had some success. The NGDS acquisition program will incrementally improve this capability by providing a highly multiplexed "sample in/answer out" capability for molecular biological threat identification. Although these fielded platforms provide a diagnostic capability in theater, they are not definitive means of identification and are based on targets that are currently well understood. Definitive identification requires orthogonal testing to improve the reliability of rapid diagnostic technologies and reduce risk.

The integration of culture as well as nucleic acid and immunological biomarkers for the identification of biological threat agents is critical to elevate the level of confidence in identifying these high consequence infectious diseases. The network of laboratories available for confirmatory and definitive testing is strong and has improved significantly within the past 5 years. Future technologies will further increase the orthogonal capabilities of diagnostic platforms and strive toward agent agnostic agent identification. The integration of molecular and immunological identification on a single platform using common analytical chemistries may be realized within the next 5 to 10 years, and whole genome metagenomic sequencing holds the promise of identifying all infectious agents in a given sample. These approaches will be critical to accommodate the identification of emerging as well as genetically engineered agents.

Although indications show that these future approaches are making progress, regulatory challenges will occur for diagnostic use of highly multiplexed and sequencing technologies. Fortunately, the FDA has been forward thinking and is currently engaged in identifying the key standards required for both highly multiplexed and whole sample sequencing based approaches for clearance of diagnostics. Biosurveillance initiatives may provide a means to evaluate and improve future platforms that could ultimately transition to diagnostic devices if costs permit. In the meantime, medical diagnostics for biological threat agents will rely on proven technologies that incorporate incremental improvements to simplify and improve the reliability and robustness of diagnostic devices for use throughout the military clinical and field laboratories.

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