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Distinguishing Tropical Infectious Diseases from Bioterrorism

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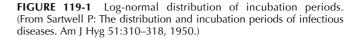## MICROORGANISMS AND TOXINS CAUSING TROPICAL DISEASES WITH POTENTIAL USE AS BIOTERROR AGENTS

Bioterrorism can be defined as the intentional use of infectious agents or microbial toxins with the purpose of causing illness and death leading to fear in human populations. The dissemination of infectious agents with the purpose of attacking livestock and agricultural resources has similar motives. Many of the agents that could potentially be used in bioterror (BT) attacks are also responsible for naturally occurring infectious diseases in the tropics. As such, naturally occurring outbreaks must be differentiated from BT attacks for public health, forensic, and security reasons. If a BT attack occurs in tropical underdeveloped countries, owing to their weak public health infrastructure, the public health implications would be even more dramatic than in developed countries. An outbreak of smallpox due to a BT attack would probably require vaccination and mandatory quarantine of millions of people in order to control the outbreak and quell global public unrest. This chapter will concentrate on selected infectious agents that have the potential to be used as bioterror agents in human populations.

The first step in managing the damage from a covert biological dissemination is recognition of the attack and the organism(s). As in most emerging infections, we predict that in bioterrorist attacks the etiological diagnosis will be made by a clinician or pathologist and the recognition of a bioterrorist event will be through geographical and epidemiological anomalies. We have very limited environmental detection capability at this time, and there are no comprehensive point-of-care diagnostics for most of the high-impact BT agents. Some diseases such as inhalational anthrax or smallpox may be relatively readily recognized by an alert clinician because of their very distinctive presentation in many cases. However, the leading edge of a BT epidemic may arrive on a pathologist's doorstep without prior suspicion. For example, individual cases of pneumonic plague as the earliest harbingers of an attack will presumably present as community-acquired pneumonia and probably die without clinical diagnosis. Given the short window available for successful treatment, the recognition of these earliest cases is paramount. Sartwell[1] has demonstrated empirically that incubation periods follow a log-normal distribution, which results in "front-loading" of cases (Fig. 119-1). Delay in recognizing the epidemic through reliance on syndromic surveillance or other surrogates will likely result in most of the cases of diseases such as plague and tularemia being well into their disease course and perhaps unsalvageable.[2]

Bioterrorist events will enlarge our knowledge of tropical diseases. For example, inhalational anthrax and several viral hemorrhagic fevers (VHF) thought to be transmitted mainly by aerosol[3] are under-represented in naturally occurring case series, and a BT attack would provide an opportunity to answer questions about the underlying host factors

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FIGURE 119-1 Log-normal distribution of incubation periods. (From Sartwell P: The distribution and incubation periods of infectious diseases. Am J Hyg 51:310–318, 1950.)

and pathogenesis. Indeed, the extension of the risk population to include children, the elderly, and the immunosuppressed is likely to provide considerable insight into these often-understudied groups. It is also likely that our lack of information about them will challenge our current diagnostic algorithms.

In October 2001, anthrax spores were distributed covertly in the U.S. postal service, leading to 22 cases of human anthrax and billions of dollars spent on controlling the potentially devastating effects of a small inhalational anthrax epidemic.[4,5] This attack was by no means the first intentional attempt to use infectious agents as weapons of terror. Ever since the times of the ancient Greeks and Romans, humans have tried to inflict damage by the use of contagion on other populations.[6,7] Less than 4% of the people or groups responsible for terrorist attacks on human populations take responsibility for their actions.[8] Therefore, the use of biological weapons is ideal to conduct covert attacks. In addition, it has been estimated that to kill the same number of human beings with biological weapons as compared to chemical or nuclear weapons, the cost is far less with biological weapons ($2/human casualty) compared with chemical ($2000/human casualty) and nuclear ($2,000,000/human casualty) weapons.[6] Hypothetical BT attacks would range from an overt attack of a large city with a bomb containing several kilograms of an agent (weaponized bacteria, viruses, or toxins) to discrete or covert intentional release of the infectious agent through a delivery system, such as spray devices, postal service, ventilation ducts, water supplies, and food supply.

Based on transmissibility, severity of morbidity and mortality, and likelihood of use (availability, stability, weaponization), potential BT agents are divided into three categories (Table 119-1). This chapter will concentrate on selected agents from categories A and B and on the diagnostic challenges posed by illnesses caused by such agents.

EPIDEMIOLOGY

All infectious agents described in Table 119-1 are capable of producing illness under natural circumstances. Therefore, the first challenge is to identify the infectious agent responsible for a certain disease correctly, followed by a thorough epidemiologic and microbiologic analysis of the epidemic or outbreak. In some circumstances, the identification of a BT attack would be obvious. A case of smallpox in any human population is an international emergency that would trigger a massive response of the public health systems around the world. Sophisticated epidemiological investigations would follow in order to characterize the outbreak, identify the source, and possibly label it "intentional." In other cases, the identification of the outbreak as secondary to intentional dissemination of an infectious agent will require the use of sophisticated epidemiological and molecular tools, especially for diseases endemic to the area where the outbreak occurs. The need to use genetic sequences as markers has spawned a new discipline referred to as microbial forensics, sister to phylogenetics and "molecular epidemiology."

Differentiation between natural infections and a biological warfare attack rests firstly on disease patterns given by several epidemiological clues. They include presence of disease outbreaks of the same illness in noncontiguous areas, disease

Table 119-1 *Potential Bioterror Agents*

Categories/Agent	Disease
Category A	
Viruses	
Smallpox	Variola major
Ebola, Marburg, CCHF, RVF, Lassa, Machupo, and Junin viruses	Viral hemorrhagic fevers
Bacteria	
Francisella tularensis	Tularemia
Yersinia pestis	Plague
Toxins	
C. botulinum toxins	Botulism
Category B	
Viruses	
Alphaviruses (VEE, EEE, WEE)	Various encephalitides
Bacteria	
Rickettsia prowazekii	Epidemic typhus
R. rickettsii	Rocky Mountain spotted fever
Brucella spp.	Brucellosis
Coxiella burnetii	Q fever
Burkholderia mallei	Glanders
Burkholderia pseudomallei	Melioidosis
Toxins	
Ricin	
SEB	

outbreaks with zoonotic impact, different attack rates in different environments (indoor versus outdoor), presence of large epidemics in small populations, increased number of unexplained deaths, unusually high severity of a disease for a particular pathogen, unusual clinical manifestations owing to route of transmission for a given pathogen, presence of a disease (vector-borne or not) in an area not endemic for that particular disease, multiple epidemics with different diseases in the same population, a case of a disease by an uncommon agent (smallpox, viral hemorrhagic fevers, inhalational anthrax), unusual strains of microorganisms when compared to conventional strains circulating in the same affected areas, and genetically homogenous organisms isolated from different locations.[9,10] These are a few guidelines that could prove helpful when investigating an outbreak, but it has to be kept in mind that the deduction will not be based on any single finding but rather the pattern seen in its totality. First and foremost, the possibility of an attack must be ever in mind, or differentiation of a covert BT attack and a natural outbreak of an infectious disease may not be made. In fact, the outbreak of salmonellosis in Oregon in 1984 was due to a covert attack planned by the Rajneeshee leadership and accompanied by distinctive epidemiological clues. It was not labeled as intentional until somebody came forward with the information leading to the responsible group; as in most of medicine, the unsuspected diagnosis is the easiest to miss.[11]

An increasing number of public health departments are now acquiring the technology necessary to perform syndromic surveillance. This new method of surveillance is based on syndromic disease rates such as respiratory, gastrointestinal, and neurological syndromes or analysis of other health-related activities such as laboratory test requests and results, purchasing

rates for certain pharmaceutical agents, unexplained death rates, and veterinary surveillance.[2,10,11] The purpose of syndromic surveillance is to detect a BT attack as early as possible by analyzing the previously mentioned variables by extracting and analyzing data through computer networks. The rationale behind syndromic surveillance is the nonspecific nature of early signs and symptoms of many of the illnesses caused by BT agents. Examples of proposed syndromes are as follows: gastroenteritis of any apparent infectious etiology, pneumonia with the sudden death of a previously healthy adult, widened mediastinum in a febrile patient, acute neurologic illness with fever, and advancing cranial nerve impairment with weakness.[12] A key component of this system is the continuous analysis of health-care variables to establish thresholds for all variables being analyzed. It is worth mentioning that one of the first flags raised by the human immunodeficiency virus (HIV) pandemic in its early stages in the United States was the increased number of orders for pentamidine from the Centers for Disease Control and Prevention (CDC) to treat several patients in California for *Pneumocystis carinii* pneumonia. As of May 2003, syndromic surveillance systems had been established in approximately 100 sites. One of the best known systems is the so-called Electronic Surveillance System for the Early Notification of Community-based Epidemics (ESSENCE II) being developed by the Johns Hopkins University Applied Biophysics Laboratory.[12] This project is sponsored by the Defense Advanced Research Projects Agency (DARPA) for use in the Department of Defense Global Emerging Infections System. The first system developed (ESSENCE I) is already in use at all U.S. military treatment facilities.[12] ESSENCE II uses the following syndromes for analysis: respiratory, gastrointestinal, fever, dermatological hemorrhagic, dermatological infectious, neurological, and coma.

Factors affecting syndromic surveillance include selection of data sources, definition of syndrome categories, selection of statistical detection thresholds, availability of resources for follow-up, recent experiences with false alarms, and criteria for initiating investigations. It must be emphasized that these systems are experimental and not yet of proven value in managing BT attacks. They are expensive, require follow-up confirmation, have unproven sensitivity and specificity, and ultimately depend on the clinician.[2] They may prove to be more useful in managing an event than in expeditiously detecting one.

Conventional epidemiological investigations are by no means obsolete with the availability of more sophisticated methods to study possible BT attacks. They include the confirmation of an outbreak once it is suspected. Confirmation is based in many cases on laboratory analysis of patients' samples or autopsy material. A case definition is constructed to increase objectivity of the data analyzed and to enable determination of the attack rate. Other variables are included in the analysis, such as time and place, and an epidemiological curve can be constructed.[10] Epidemiological curves are an important tool to analyze epidemics and suggest the mode of transmission and propagation. A point source epidemic curve is classically log-normal in distribution[1] and would suggest a common exposure of a population to an infectious agent. Of course, there can be variations depending on the presence of susceptible subpopulations (e.g., children, immunosuppressed, aged) and on varying doses of the agent. Propagative curves

are more characteristic of highly communicable agents such as smallpox.

A short description of selected category A and B agents follows. All these pathogens are addressed as naturally occurring disease agents in other chapters of this book.

CATEGORY A

Bacterial Agents

Bacillus anthracis (Anthrax)

B. anthracis (see Chapter 39) is without a doubt the microorganism that has received the most attention as a BT agent due to its high lethality (inhalational form), ease of propagation, and high environmental stability. Fortunately, the disease is not transmitted from person to person. However, the first three characteristics make it one of the ideal bioweapons.

Anthrax presents in humans as four different clinical syndromes, depending on the portal of entry: cutaneous (the most common form of the disease resulting from contact with infectious animal products), gastrointestinal and oral/oropharyngeal (both secondary to ingestion of contaminated meat), and inhalational (woolsorter's disease), secondary to inhalation of spores from the environment. In the event of a bioterror attack, either overt or covert, the clinical presentation of the patients affected by the attack would be that of inhalational anthrax. This form of anthrax is so rare that a single case of inhalational anthrax should raise immediate suspicion, as dramatically demonstrated during the BT attacks in the fall of 2001.[13–15] During those attacks, 50% of cases were cutaneous anthrax thought to be secondary to handling of anthrax-laced mail envelopes or environmental surface contamination in the presence of minor cutaneous lesions, providing a portal of entry for the spores.[5] An outbreak of inhalational anthrax also took place in Sverdlovsk (former Soviet Union) as a result of an accidental release into the air of *B. anthracis* spores from a facility producing anthrax for the bioweapons program in the USSR.[5,16–18]

Inhalational anthrax should be suspected clinically in any individual presenting with fever and a widened mediastinum on chest radiograph (due to hemorrhagic mediastinitis).[19,20] The incubation period is normally 3 to 5 days, but in some cases it can be as short as 2 days and as long as 60 days depending on inoculum and the time of germination of the spore.[17] Based on research performed on rhesus monkeys, the LD_{50} is estimated to be 8000 to 10,000 spores.[21–23] However, as few as 1 to 3 spores may be capable of producing a fatal outcome in approximately 1% of those exposed to these quantities.[24] The initial symptoms are nonspecific and consist of fever, malaise, anorexia, fatigue, and dry cough. These symptoms are followed in 3 to 4 days by an abrupt onset of respiratory insufficiency, stridor, diaphoresis, and cyanosis. The subsequent clinical course is rapid, and patients usually die within 24 to 36 hours after clinical deterioration. Mortality is 100% without antibiotic therapy.[20,25–27] Early diagnosis, aggressive treatment with antimicrobial agents to which the bacteria are susceptible, and aggressive supportive therapy decreased the mortality to 40% in the 2001 attacks.[5] Pathologic studies performed on the Sverdlovsk victims confirmed some of the findings in animal models of inhalational anthrax, such as hemorrhagic lymphadenitis and mediastinitis. However, many

patients also developed hematogenous hemorrhagic pneumonia. Pleural effusions were usually large and frequently led to severe lung atelectasis. In about half of cases, hemorrhagic meningitis developed, leading rapidly to central nervous system (CNS) manifestations terminating in coma and death.[16,28,29]

Yersinia pestis (Plague)

Y. pestis (see Chapter 42) is a gram-negative, aerobic, nonsporulating coccobacillus, member of the Enterobacteriaceae with a wide host range, including rodents, felines, and humans.[30] The most important reservoirs are urban rats, and its main vector is the rat flea. In rural epizootics, reservoirs include prairie dogs and squirrels in the United States.[31] Y. pestis has been responsible for some of the most devastating pandemics in human history in the preantibiotic era (6th, 14th, and 19th centuries).[32] Public health measures have made this disease a rarity in the United States (around 20 cases/year) and around the world, although approximately 1000 cases are reported to the World Health Organization (WHO) every year (countries reporting plague include Madagascar, Tanzania, and Peru, among others).

Clinical presentation in naturally acquired infections takes five forms, namely bubonic, septicemic, pneumonic, cutaneous, and meningeal. The pneumonic form is the most likely presentation in a case of plague due to a BT attack. It is worth mentioning that plague has already been used as a BT agent when Japan dropped thousands of Y. pestis–infected fleas over China leading to small outbreaks of bubonic plague in continental China during World War II.[33,34]

The incubation period for pneumonic plague is short, ranging from 2 to 3 days. It is the rarest form in natural infections (1% or less) but has the highest mortality, reaching 100% in untreated patients. The initial presentation is nonspecific and consists of cough, fever, and dyspnea. Cough may be productive (bloody, purulent, or watery in the initial phases). This is followed by a rapid clinical course leading to respiratory failure and the patient's demise if not treated with antibiotics early in the course of the disease.[30,31,35]

The factors that led to the severe Manchurian pneumonic plague outbreaks in the early 20th century are unknown, but weather, hygiene, and crowding were important factors. More recent outbreaks worldwide and particularly in the United States have been much smaller and readily controlled. Pneumonic cases are common in the United States, but secondary transmission has been rare in the last 50 years. Modeling of pneumonic transmission using eight small outbreaks to derive the parameters find average of secondary cases per primary case (Ro) to be approximately 1.3 prior to any control measures.[36]

Francisella tularensis (Tularemia)

This is one of the most scientifically neglected microorganisms with BT potential. Tularemia is a zoonotic infection caused by a strictly aerobic, gram-negative, nonsporulating small coccobacillus. Two subspecies are recognized, namely F. tularensis subspecies holarctica (Jellison type B) and F. tularensis subspecies tularensis (Jellison type A).[37] Type A is by far the more virulent and is present only in North America.

Of the bacteria with potential as BT agents, F. tularensis has by far the widest host range, including wild and domestic animals, humans, fish, reptiles, and birds. Vectors are also numerous and include ticks, fleas, mosquitoes, and biting flies.[37,38] This is an impressive range for any human pathogen.

In contrast to other diseases described in this chapter, tularemia does not have the remarkable history that some of the other pathogens have. In Europe, tularemia was first described in 1532; in the United States, it was first described in 1911 in California in the aftermath of the San Francisco earthquake.[38]

In natural infections, the most common source of infection is a tick bite and manipulation of infected animals such as wild rabbits. Six different clinical syndromes have been described as follows: ulceroglandular, glandular, oculoglandular, pharyngeal, pneumonic, and typhoidal. Marked overlap exists among all these forms, and for practical purposes two syndromes (ulceroglandular and typhoidal) have been proposed.[39–41] As a BT agent, F. tularensis will most likely cause a disease with a primary pulmonary component with secondary dissemination (typhoidal/systemic). In natural infections, both ulceroglandular and typhoidal forms can have a hematogenous pulmonary component, although it is more common in typhoidal forms. Pulmonary features include cough, pleural effusions, and multifocal bronchopneumonic infiltrates. If not treated promptly, patients usually develop adult respiratory distress syndrome leading to respiratory insufficiency and the patient's demise. Case-fatality rate approaches 30% if not treated with appropriate antibiotics.[41]

Viral Agents

Smallpox Virus (Variola Major)

Smallpox eradication remains the single most important victory in the war against infectious diseases. Smallpox (see Chapter 58) is the only disease so far eradicated from the face of the earth due to human intervention. The WHO declared smallpox eradicated in 1980 after the last case of natural disease was diagnosed in Somalia in 1977,[42] and vaccination ceased around the world, rendering humankind vulnerable to reintroduction of the virus.[43–45] A laboratory accident was responsible for two more cases in 1978 in England. This accident prompted the WHO to restrict the frozen virus to two places in the world: the CDC in Atlanta, Georgia, and the Institute for Polyomyelitis and Viral Encephalitis in Moscow, later moved to NPO VECTOR, Novosibirsk, Russia. However, it is suspected that secret military repositories exist after the fragmentation of the Soviet Union and the subsequent exodus of scientists involved in its bioweapons program (Biopreparat).[46,47] The agent responsible for this disease is an orthopox virus with no known animal reservoir, but high aerosol infectivity, stability, and mortality. Although not a category A agent, monkeypox is responsible for outbreaks in Africa and is the only other member of the orthopox genus capable of producing systemic disease in humans. The clinical disease is potentially indistinguishable from smallpox, where mortality rates in tropical Africa are around 10% to 15%. In May and June 2003, an outbreak of monkeypox occurred in the United States.[48] Thirty-seven infections were laboratory-documented and involved humans exposed to infected prairie dogs that had become infected because of

contact with infected Gambian rats and dormice, two animal species shipped from Africa earlier that year. Infected humans included veterinarians, exotic pet dealers, and pet owners. The clinical spectrum in this outbreak ranged from asymptomatic seroconversions to febrile illness with papulovesicular rash. No deaths were associated with this outbreak. However, phylogenetic analysis of the virus placed it in the West Africa clade as opposed to the Central Africa clade which carries the previously mentioned case-fatality rate of 10% to 15%.

A single case of smallpox would trigger a massive public health response in order to contain the outbreak. An outbreak in Germany in 1970 resulted in 19 cases with 100,000 people vaccinated to contain the infection. In 1972, Yugoslavia underwent an epidemic with a total of 175 cases (35 deaths) and a vaccination program that included 20 million people in order to contain the outbreak and obtain international confidence. Vaccination with the vaccinia virus (a related orthopox virus) is the most effective way to prevent the disease and can be administered up to 4 days after contact with ill patients. Strict quarantine with respiratory isolation for 17 days is also mandatory. The newer generation of antivirals that have been developed after the disease was eradicated has never been tested in human populations, but in vitro data and experiments in animal models of poxvirus disease suggest some antiviral activity for the acyclic nucleoside phosphonates such as cidofovir.[49] The only vaccine available in the United States is Dryvax, and sufficient doses have been manufactured to cover the entire U.S. population. However, newer vaccines that may have fewer side effects are being developed.

The clinical presentation is characteristic. The incubation period ranges from 10 to 12 days. The initial phase is nonspecific, common to other viral syndromes, and is characterized by abrupt onset of fever, fatigue, malaise, and headaches. During this prodromal phase in 10% of patients with fair complexion, a discrete erythematous rash appears on the face, forearms, and hands. The typical smallpox rash has a centrifugal distribution (that is, more abundant on the face and extremities than on the trunk and abdomen). An enanthem also develops with presence of oral ulcerations by the time the exanthem appears. Systemic manisfestations begin to subside once the rash appears and can reappear with superinfection of skin lesions or superimposed bacterial bronchopneumonia. Progression of the lesions is synchronous (maculopapules, vesicles, pustules). After pustules rupture, scabs form and detach in 2 to 3 weeks, leaving depigmented, scarred areas. This form of the disease, called variola major, is fatal in up to 30% of unvaccinated patients and 3% of vaccinated individuals. Various hemorrhagic forms exist. In some cases, the rash progresses very slowly and hemorrhage develops into the base of the lesions, which remain flat and soft instead of tense, carrying a bad prognosis. In some other cases, the disease is hemorrhagic from the beginning, leading to death 5 to 7 days after the initial symptoms appear (case-fatality rate: 100%). Finally, in some cases, a severe and overwhelming illness is followed by dusky skin lesions; these patients have a large quantity of virus and are extremely dangerous epidemiologically. Previously vaccinated individuals usually develop a milder disease that consists of a mild pre-eruptive phase followed by few skin lesions that appear more superficial, evolve more rapidly, and are not as synchronous as the classical type.[50]

Viral Hemorrhagic Fevers

Viral hemorrhagic fever (VHF; see Chapter 65) is caused by a heterogenous group of RNA viruses that belong to several different families. The CDC identified filoviruses (Ebola and Marburg viruses), arenaviruses (Lassa, Junin, Machupo, Guanarito, and Sabia), and bunyaviruses (Crimean-Congo hemorrhagic fever [CCHF] and Rift Valley fever [RVF]).[51–53]

The common denominator in these infections is the increased vascular permeability in the microcirculation leading to hemorrhagic diathesis and systemic manifestations such as pulmonary edema and cerebral edema related to leaky capillaries.[54] These viruses usually have a very narrow geographic range determined by their natural reservoirs and vectors. Humans are accidental hosts. These diseases have caught great public attention due to their high mortality. This, combined with their aerosol infectivity, has led to the use of biosafety level 4 laboratories in their study.

Clinical presentation is usually nonspecific and consists of fever and malaise, followed by signs of increased vascular permeability and circulatory compromise. VHF usually terminates in shock, generalized mucocutaneous hemorrhages, and multiorgan failure. Differences exist among the clinical details and pathogenesis of the different viruses (see Chapter 65 for an overview and the individual chapters for details). For example, VHF due to filoviruses usually have prominent hemorrhagic manifestations and disseminated intravascular coagulation (DIC) as a terminal event. RVF virus leads to liver damage, DIC, and hemorrhagic manifestations in approximately 1% of patients with severe disease. CCHF also behaves like the filoviral infections with prominent hemorrhagic manifestations. Lassa fever has few neurologic or hemorrhagic manifestations. The South American arenaviral hemorrhagic fevers usually have hemorrhagic and neurologic components.

Diseases Caused by Toxins

Toxins in the context of BT agents are substances of biologic origin that are capable of producing human illness. Toxins are usually proteins synthesized by living bacteria, fungi, or plants. Toxins are generally less dangerous than infectious agents. The most potent biological toxin is that from *Clostridium botulinum* and it is 10-fold or more less lethal than anthrax on a weight basis. Other toxins such as ricin are more than a 1000-fold less toxic than botulinum toxin and sarin is 30-fold less toxic than ricin.

Clostridium botulinum Toxins (Botulism)

There are seven similar toxins produced by seven different serotypes of *C. botulinum* (A to G), all leading to the same clinical manifestations and with the same lethality. The toxins have a molecular weight of approximately 150 kDa and block neurotransmission at the presynaptic level in cholinergic neurons including the neuromuscular junction, leading to progressive palsies of cranial nerves and skeletal muscle. Botulinal toxins are among the most lethal substances known to mankind with LD_{50} of 0.001 μg/g of body weight when administered parenterally.[25,55,56] The aerosol route decreases its lethality 80 to 100 times. Both aerosol attacks and contamination of food supplies are potential BT scenarios.

Clinical manifestations consist of progressive bulbar and skeletal paralysis in the absence of fever, including diplopia, dysphagia, blurred vision, ptosis, dysarthria, dysphonia, mydriasis, dry mucosae, and descending paralysis.[25,56] The cause of death in lethal cases is respiratory insufficiency due to paralysis of respiratory muscles. Onset of symptoms is variable and depends on the inoculum, ranging from 24 hours to several days after exposure. Most cases of naturally occurring intoxication are related to consumption of improperly sterilized canned food or ingestion of preserved fish. Rare cases of inhalational botulism were documented in Germany in the early 1960s due to accidental laboratory exposure. The rapid absorption through the respiratory tract may offer a different pathogenesis and it is not known if antitoxin is useful in therapy, although animal models show efficacy in prophylaxis.

CATEGORY B AGENTS

All the agents in category A are generally recognized as serious threats for causing extensive casualties. Categories B and C are much more heterogeneous. They were considered to provide significant threat potential but there are continuing reassessments.

Viral Agents

Viral Encephalitides

These conditions are caused by the genus *Alphavirus*, family Togaviridae (eastern, western, and Venezuelan equine encephalitis [VEE] viruses; see Chapter 74). Natural infections are usually transmitted by mosquitoes, but aerosol transmission is the notorious cause of numerous laboratory infections and is the basis of its historic weaponization.[52,57] Most of these viruses cause systemic illness characterized by fever, myalgias, and prostration.

Clinically apparent involvement of the central nervous system is present in some cases and varies among the different viruses. Eastern equine encephalitis (EEE) is by far the most virulent, leading to case-fatality rates of 50% to 75%, and survivors usually have severe neurologic sequelae.[58,59] VEE, in contrast, leads to CNS manifestations in no more than 4% of cases and almost all VEE infections are symptomatic even in the absence of CNS involvement.[60–62]

Bacterial Agents

Rickettsia prowazekii (Epidemic Typhus) and *R. rickettsii* (Rocky Mountain Spotted Fever)

Typhus (see Chapter 51) is another disease that has played a historic role in human populations.[63–66] Millions of people perished in World War I and World War II due to epidemic, louse-borne typhus. Large outbreaks of the disease still occur in tropical regions around the world in areas stricken by war, famine, and poverty. Rocky Mountain spotted fever (RMSF), on the other hand, is transmitted by tick bites and occurs endemically in South and Central America as well as North America. Rickettsiae target the microvascular endothelium leading to leaky capillaries systemically.[67] The main causes of morbidity and mortality are noncardiogenic pulmonary edema and cerebral edema leading to diffuse alveolar damage

and meningoencephalitis. Clinical manifestations are nonspecific and include fever, malaise, headache, myalgias/arthralgias, cough, nausea, vomiting, confusion, stupor, and coma in severe cases. Skin rash ranges from maculopapular to petechial, depending on the severity, and is observed in around 90% of patients with RMSF and 2% to 100% of cases of epidemic typhus, depending on the darkness of cutaneous pigmentation. Rickettsiae are remarkably underestimated biothreats as they are highly infectious by low-dose aerosol exposure, possess a stable extracellular form, and are resistant to most empirically administered antibiotics, including β-lactams, aminoglycosides, and macrolides, and are exacerbated by sulfonamides. Case-fatality rates can be as high as 40% to 50% without antibiotic therapy and 3% to 5% with adequate antibiotic coverage. Lethal cases are usually due to delayed diagnosis.[64,65,68]

These rickettsiae are highly infectious by aerosol and are potent BT agents. They are often discounted because of their susceptibility to tetracycline and chloramphenicol. However, the severity of the illness, the exhaustion of antibiotics in the face of a mass attack, and the existence of antibiotic-resistant organisms suggest they are still formidable players.

Coxiella burnetii (Q Fever)

This gram-negative, obligately intracellular bacterium has a high degree of infectivity (one organism is capable of causing infection by inhalation) and low lethality.[69–72] The distribution of Q fever is worldwide and results from exposure to animals such as sheep, cattle, goats, cats, rabbits, and others. *C. burnetii* has spore-like characteristics that can withstand harsh environmental conditions and be transported by wind to other places. In natural infections, 60% of cases are asymptomatic and are diagnosed by seroconversion. In symptomatic cases, the presentation is nonspecific and includes malaise, fever, myalgias, cough, chills, headaches, anorexia, weight loss, and in some cases pleuritic chest pain. Hepatomegaly and splenomegaly are sometimes observed, although not frequently.

Brucella spp. (Brucellosis; Other Names: Undulant Fever, Mediterranean Fever, Malta Fever)

Four species of these gram-negative, aerobic, non-spore-forming coccobacilli are pathogenic to humans: *B. abortus, B. melitensis, B. suis,* and *B. canis* (see Chapter 41). Host ranges include goats and sheep (*B. melitensis*), swine and horses (*B. suis*), cattle, bison, elk, horses (*B. abortus*), and dogs (*B. canis*).

Transmission occurs by exposure to infected animal products (meat, milk). Less common routes of infection are inhalational and cutaneous. The clinical presentation of brucellosis is highly variable, even after inhalational exposure. The clinical spectrum ranges from asymptomatic seroconversion to severe acute systemic disease. Intermediate forms include undulant fever or chronic disease, characterized by presence of *Brucella* in virtually any organ. Acute systemic disease is highly incapacitating with high fever, headache, nausea, vomiting, chills, severe sweating, and, in very severe cases, delirium, coma, and death. Undulant fever is characterized by relapses of fever, weakness, generalized aching, and headache. Chronic infections may have manifestations related to several organ systems such as the gastrointestinal and genitourinary tracts, CNS, joints, and bones.[73–75]

Food and Waterborne Pathogens

Developing countries with insufficient water treatment and food security are more vulnerable to enteric BT attack. These agents include *Shigella dysenteriae, Salmonella* spp., enterohemorrhagic *E. coli, Vibrio cholerae,* and *Cryptosporidium parvum.*

Shigella and *Salmonella* have in fact already been used as agents of biorevenge or biopolitics in small-scale attacks: one (*Shigella*) in an office setting by a disgruntled employee and one in Oregon by a religious sect that led to nearly 1000 cases of *Salmonella*-related gastroenteritis.[11,76] These agents are indeed ideal for small-scale attacks since large-scale attacks would require contamination of large water supplies which, because of enormous dilution factors and susceptibility of all these agents (except for *C. parvum*) to standard chlorinating procedures, would decrease the number of bacteria to below that required to infect large numbers of people.[69]

Occasional outbreaks of nontyphoidal *Salmonella* and *Shigella* infections occur in the United States. *Shigella* is a highly infectious organism that requires very low numbers (10^2–10^3 organisms) to provoke clinical disease. The illness caused by *Shigella* and enterohemorrhagic *E. coli* is explosive and starts with fever, vomiting, severe abdominal cramping, bloody diarrhea, and systemic manifestations such as hypotension, and circulatory collapse if not treated rapidly. Both microorganisms produce an exotoxin responsible for most of the systemic manifestations associated. A distinct complication, hemolytic uremic syndrome, occurs in a small percentage of cases, being more common in children younger than 10 years of age, leading to renal failure and hemolysis. *Salmonella* is less infectious and less explosive than *Shigella*, and leads to fever, vomiting, diarrhea, abdominal cramping, and in some cases to typhoidal manifestations. Imported cases of *V. cholerae* have been diagnosed in the United States in the past. However, the disease occurs in southern Asia and Latin America as large outbreaks. The clinical illness is characterized by explosive watery diarrhea that leads to rapid dehydration and circulatory collapse.

C. parvum infections are characterized by watery diarrhea and abdominal cramping for 2 to 3 weeks. The disease is self-limited except in patients with acquired immunodeficiency syndrome (AIDS) or other conditions of compromise, in whom illness can last for months or years if immune function is not restored. *C. parvum* is resistant to standard chlorine concentrations in water supplies.[77] The largest outbreak in this country occurred in Milwaukee in the early 1990s and was responsible for thousands of cases and increased mortality among those with AIDS.[69,78,79]

Category B Toxins

This section addresses other toxins considered of potential BT use, such as staphylococcal enterotoxin B (SEB) and ricin toxin (derived from castor beans, which in turn are the fruit of the *Ricinus communis* plant).

Ricin Toxin (Castor Beans from *Ricinis communis* Plants)

The ricin toxin is composed of two glycoproteins of approximately 66,000 kDa.[80] The toxin inhibits protein synthesis by blocking elongation factor 2 (EF2) at the ribosomal level. Ricin toxin is not a weapon of mass destruction since its lethal dose in humans is much higher than previously believed. However, the use of the toxin in small BT attacks is possible in the tropics because of its ready availability and relatively easy extraction from the beans. Clinical presentation depends on the route of administration as does the LD_{50}. In cases where large amounts of the toxin are ingested, the manifestations include nausea, vomiting, severe abdominal cramping, rectal hemorrhage, and diarrhea. As the clinical course progresses, anuria, mydriasis, severe headaches, and shock supervene leading to the patient's demise in 2 to 3 days. Clinical manifestations usually appear within 10 hours after ingestion of the toxin. Inhalational exposure leads to prominent pulmonary manifestations 8 to 28 hours after exposure and fever, dyspnea, progressive cough, cyanosis, and death. Histologically, there is widespread necrosis of pulmonary parenchyma and pulmonary edema. A single case of parenteral intoxication was documented. A defector from Bulgaria was injected with a pellet containing ricin from a weapon disguised in an umbrella, resulting in local necrosis, regional lymphadenopathy, gastrointestinal hemorrhage, liver necrosis, nephritis, and DIC.[81]

Staphylococcus aureus Enterotoxin B

Staphylococcus aureus enterotoxin B (SEB) is a 28-kDa, heat-stable exotoxin produced by certain strains of *S. aureus* and is responsible for food poisoning after ingestion of the preformed exotoxin in improperly handled food. In BT scenarios, exposure can occur either by inhalation or ingestion leading to SEB food poisoning or SEB respiratory syndrome. The toxin is highly incapacitating and not very lethal. The dose that causes symptoms in half of exposed persons and LD_{50} differ by a magnitude of 5 log scales for inhalational exposure.[82] Thus, it is thought of as an incapacitating agent.

Incubation time after ingestion is short (4–12 hours) followed by explosive vomiting that persists for several hours. Weaponization of the toxin as an aerosol is possible due to its high stability. Manifestations after inhalation of the SEB are related to the respiratory system and consist of fever, cough, chills, myalgias, chest pain, and pulmonary insufficiency due to alveolar edema. General symptoms and signs are universal and consist of multiorgan failure secondary to a cytokine storm.[25] These toxins are superantigens due to their ability to bind to major histocompatibility complex (MHC) class II molecules on large numbers of lymphocytes and macrophages, leading to a hyperactivation of the immune system and massive cytokine release including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL-6), and other mediators such as leukotrienes and histamine.[82]

DIAGNOSIS

The role of the clinical laboratory in the diagnosis of possible cases related to a BT attack is of utmost importance.[83,84] On the one hand, standard clinical microbiology laboratories will be receiving specimens for diagnostic purposes, and communication with clinicians regarding their suspicions is critical. Certain isolates in the laboratory are not pursued further (*Bacillus* spp. is a classic example) unless specifically requested due to the frequent isolation

of contaminants with similar characteristics. In addition, handling of certain specimens will require added biosafety level requirements due to their infectivity (Table 119-2). Certain samples will have to be shipped to highly specialized laboratories for initial or further work-up. Environmental testing is challenging due to the complexity of the samples to be analyzed.[85,86] This type of testing takes place in highly specialized laboratories and is not undertaken by the standard clinical microbiology laboratory.

Conventional and Molecular Diagnosis of Potential BT Agents

General Principles

The bacterial diseases caused by the BT agents outlined in this chapter, with the exception of *C. burnetii* and *Rickettsia* spp., can be diagnosed by standard isolation techniques in clinical microbiology laboratories. Isolation of rickettsiae and the BT viruses requires specialized laboratories with BSL-3 or BSL-4 biocontainment.[87] Serological assays are available for detection of antibodies against all BT agents. However, for many organisms serological assays require the presence of rising antibody titers, and therefore the serologic diagnosis is usually retrospective in nature. For some viral diseases, a reliable diagnosis can be established based on elevation of immunoglobulin M (IgM) titers in the acute phase of the disease.

With the advent of molecular techniques, rapid and sensitive diagnostic tests are becoming available for BT agents during the acute phase of the disease.[88–90] This is of utmost importance in a BT event since identification of the first cases would be critical for a rapid and effective public health response. In addition, treatment and prophylactic measures can also be initiated as quickly as possible. Molecular diagnostic techniques can be applied to potential BT agents in an additional setting: as part of the epidemiological and forensic investigations that a BT attack would immediately trigger. Postmortem diagnosis is also possible by analysis of frozen or paraffin-embedded tissues by immunohistology or nucleic acid–based amplification techniques.

Rapid diagnosis of the initial case (cases) in a BT event requires a high degree of clinical suspicion from the physicians having contact with such patients in the emergency room or outpatient setting. The clinical laboratories would then play a critical role in detecting the suspected agent and/or referring the appropriate specimens to higher level laboratories for specialized testing (Table 119-3).[83,85,91]

Several of the agents discussed in this chapter are zoonotic diseases. Therefore, diagnosis of certain zoonotic diseases in animals may be important in identifying some BT attacks. In such situations, animals could be seen as either direct victims of the attack or as sentinel events in a human outbreak. There are currently efforts to establish a network of laboratories dedicated to diagnosis of veterinary agents.[85]

Table 119-2   Biosafety in Microbiologic and Biomedical Laboratories

Level	Definition	Examples
BSL-1	Suitable for work involving well-characterized agents not known to cause disease in healthy adult humans and of minimal potential hazard to laboratory personnel and the environment.	*Bacillus subtilis* *Naegleria gruberi* Canine hepatitis virus
BSL-2	Suitable for work involving agents of moderate potential hazard to personnel and the environment. Laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists; access to the laboratory is limited when work is being conducted; extreme precautions are taken with contaminated sharp items; and certain procedures in which infectious aerosols or splashes may be created are conducted in biological safety cabinets or other physical containment equipment.	Measles virus *Salmonella* spp. Toxoplasma spp. Hepatitis B virus
BSL-3	Suitable for work with infectious agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route. In addition to the requirements described for work in BSL-2 environment, all procedures are conducted within biological safety cabinets, or other physical containment devices, and by personnel wearing appropriate personal protective clothing and equipment. Laboratory should be located in a separate building or an isolated zone within a building. Laboratories are equipped with double door entry, directional inward flow, and single-pass air.	*Coxiella burnetii* *Rickettsia* spp. *M. tuberculosis* Alphaviruses
BSL-4	Required for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease. Members of the laboratory staff have specific and thorough training in handling extremely hazardous infectious agents. They are supervised by competent scientists who are trained and experienced in working with these agents. Access to the laboratory is strictly controlled by the laboratory director. The facility is either in a separate building or in a controlled area within a building, which is completely isolated from all other areas of the building. All activities are confined to Class III biological safety cabinets, or Class II biological safety cabinets used with one-piece positive pressure personnel suits ventilated by a life support system. The Biosafety Level 4 laboratory has special engineering and design features to prevent microorganisms from being disseminated into the environment.	Filoviruses Arenaviruses

Table 119-3 *Laboratory Response Network for Bioterrorist Attacks*

Level	Functions
A	Community level laboratories that should recognize the clues of a possible bioterrorist agent and be able to package samples and ship them for confirmation at the upper-level laboratories.
B	State and county public health laboratories with capacity to work with BSL-2 and some with BSL-3 agents. Capable of isolation of some of the agents, presumptive level testing, and antibiotic susceptibility profiles.
C	Greater BSL-3 capabilities than level B and molecular testing capabilities for rapid identification.
D	Highest level of containment (BSL-4) for isolation and identification of highly pathogenic viruses.

Diagnosis of Specific BT Agents

Bacillus anthracis

The diagnosis of inhalational anthrax is based on isolation and identification of *B. anthracis* from a clinical specimen collected from an ill patient. In cases of inhalational anthrax, samples of sputum, blood, or cerebrospinal fluid (CSF) may yield growth of the agent. Demonstration of *B. anthracis* from nasal swabs has more epidemiological and prophylactic implications than clinical importance. Standard diagnostic techniques are based on visualization and isolation in the clinical microbiology laboratory and serological demonstration of antibodies against *B. anthracis*.[92–96]

Visualization of *B. anthracis* from clinical specimens (blood cultures, CSF, and cutaneous lesions) by Gram stains is not difficult. *B. anthracis* appears as large gram-positive, spore-forming rods with a bamboo appearance. Isolation is achieved by inoculating standard sheep blood agar plates, and colonies appear as small, gray-white, nonhemolytic colonies. A selective medium (polymyxin-lysozyme-EDTA-thallous acetate agar) is available mostly for environmental samples and inhibits the growth of other *Bacillus* spp., such as *B. cereus*. Growth is rapid (24–48 hours).[93] Confirmatory tests include γ-phage lysis, detection of specific cell wall and capsular antigens, and polymerase chain reaction (PCR) amplification of DNA followed by sequencing.[90]

Serological tests available for clinical diagnosis are based on detection of antibodies directed against protective antigen (PA). Cross-reactive antibodies decrease the specificity of this test. Assays based on toxin detection are available in specialized centers and are based on capture of anthrax toxins by using antibodies. Antibody-coated immunomagnetic beads are then analyzed by electrochemiluminescence technology. The analytical sensitivity of this technique for detection of anthrax toxin is at the picogram to femtogram level (10^{-12} to 10^{-15}).[97,98] Immunoliposomal technology combined with real-time PCR (for a DNA reporter sequence) is also in the early stages of development for several toxins (ricin, cholera, and botulinum) and appears promising with analytical sensitivity in the attomolar to zeptomolar (10^{-18} to 10^{-21}) range for cholera toxin.[99] The specificity of this assay is given by the toxin-capturing antibody.

Nucleic acid amplification techniques (PCR) are also available both in standard format and real-time format. Extraction of DNA from spores is challenging and requires modification of DNA extraction protocols in order to facilitate release of DNA from spores or induction of germination prior to DNA extraction.[90] Real-time PCR tests have been developed by Applied Biosystems (TaqMan 5' nuclease assay) and Roche Applied Science (LightCycler).[100–102] The analytical sensitivity of both techniques is extremely high, and testing times have been decreased to 1 to 2 hours. Portable PCR instruments are being developed for rapid deployment to the field.[103] Examples include the rugged advanced pathogen identification device (RAPID),[100] the Smartcycler (Cepheid, CA),[101] and the miniature analytical thermal cycler instrument (MATCI) developed by the Department of Energy's Lawrence Livermore National Laboratory.[104] This instrument later evolved into the advanced nucleic acid analyzer (ANAA) and handheld advanced nucleic acid analyzer (HANAA).[105]

Molecular subtyping of *B. anthracis* is also possible by using the 16S ribosomal RNA (rRNA) subunit gene, multiple-locus vari-able number tandem repeat analysis of eight genetic loci, and amplified fragment length polymorphism (AFLP) techniques.[106,107]

Environmental testing also plays a role in the investigation of a BT event. In this setting, detection of *B. anthracis* relies heavily on molecular techniques for confirmation of potentially contaminated samples (e.g., surfaces, air).[108,109]

Postmortem diagnosis is also possible by using Gram stains on paraffin-based tissues or immunohistochemical procedures using polyclonal or monoclonal antibodies against various anthrax antigens.

Yersinia pestis

Diagnosis of *Y. pestis* is based on demonstration of the bacillus in blood or sputa from patients. Standard diagnostic techniques in the laboratory include visualization of gram-negative coccobacilli, which by Giemsa, Wright, or Wayson stains reveal a "safety pin" appearance. Isolation is performed in blood and McConkey agar plates on which colonies appear as nonlactose fermentors. The organisms are identified preliminarily by direct immunofluorescent assay with *Y. pestis*–specific antibodies, with final identification based on biochemical profiles in clinical microbiology laboratories.[110]

Molecular diagnostic techniques based on real-time PCR have become available in recent years and involve detection of *Y. pestis* genes such as plasminogen activator (*pla*), genes coding for the Yop proteins and the capsular F1 antigen, and the 23S rRNA gene, which allows distinction from other *Yersinia* spp.[111–113] Assays have been developed to detect resistance to particular antibiotics. The importance of these diagnostic techniques in a disease such as plague is evident. The log-normal epidemic curve with a narrow dispersion of the incubation periods (see Fig. 119-1) and the short interval for successful antibiotic therapy mandate

recognition of the earliest cases if the bulk of the exposed are to be saved. Molecular subtyping of *Y. pestis* is also possible by analyzing polymorphic sites in order to identify the origin of strains in the event of a BT attack.

Francisella tularensis

Diagnosis is made in the clinical laboratory by demonstration of the microorganisms in secretions (sputa, exudates) by direct immunofluorescence or immunohistochemically in biopsy specimens. Isolation in the clinical laboratory may be achieved by using regular blood agar plates, posing a risk to laboratory personnel not employing BSL-3 facilities and procedures.

The procedure for isolation of *F. tularensis* in the laboratory is very similar to that described for *Y. pestis*. Final identification in the clinical laboratory is based on the biochemical profile.[114] Molecular diagnostic techniques are based on PCR detection of *F. tularensis* by using primers for different genes such as outer membrane protein (*Fop*) or *tul4* and real-time detection systems.[90,115,116]

Smallpox Virus

Diagnosis of variola major is suggested by its clinical presentation and the visualization of Guarnieri bodies in skin biopsy samples. Preliminary confirmation requires visualization of the typical brick-shaped orthopox virus by electron microscopy, followed by isolation from clinical specimens and accurate molecular identification to differentiate it from the morphologically (and sometimes clinically) similar monkeypox virus. Confirmation of this diagnosis is performed only under BSL-4 containment facilities at the CDC.[47]

Molecular techniques are based on PCR amplification using real-time or standard technology followed by sequencing or use of restriction fragment length polymorphism (RFLP) for accurate identification.[117] Technologies so far developed for smallpox molecular testing include Taqman- and LightCycler-based assays with primers designed for the hemagglutinin gene and A-type inclusion body proteins.[118–121]

Sequencing of the smallpox genome has been completed for some Asian strains of variola major and one of variola minor. Other strains are being sequenced and will provide more information for probe design and treatment targets.[90]

Viral Hemorrhagic Fevers

Diagnosis of these diseases is performed in highly specialized centers in the United States because special isolation procedures and highly contained laboratories are required.

Initial diagnosis of these diseases is suspected on clinical and epidemiologic grounds. Laboratory diagnosis involves isolation, electron microscopy, and serological assays. Immunohistochemical detection of hemorrhagic fever viral antigens in paraffin-embedded tissues is also performed in highly specialized centers such as the CDC.[122–126]

Molecular diagnostic techniques have also improved dramatically during the last few years. Serum or blood is the most common specimen used for reverse transcriptase–PCR amplification of viral nucleic acids. Both standard and real-time techniques are available.

Design of primers for this heterogenous group of RNA viruses that are highly variable is one of the limitations.[90] Therefore, multiplex PCR techniques are required to detect as many targets as possible in a single assay.[127,128] Real-time PCR based on detection of the target sequence using fluorescent probes therefore limits the number of targets that can be identified because of the limited wavelength range for fluorescent applications (usually only four different wavelengths can be detected at the same time).[128–130] The use of microchips containing several thousands of oligonucleotides from all viruses known to be pathogenic to humans is an encouraging development. In fact, the rapid identification and characterization of the novel human coronavirus responsible for the SARS outbreak in 2003 is an excellent example of the power of hybridization-based microchips.

The creation of an automated and easily deployable instrument capable of detecting all possible potential BT agents based on highly sensitive techniques such as electrochemoluminescence (ECL) or PCR would be ideal. The nonspecific nature of presenting symptoms is a major problem with several of the agents. The rapid recruitment of cases into the infected cohort requires that an early diagnosis of the epidemic be established, particularly for organisms such as *Y. pestis* in which there is only a short window for successful treatment. In fact, such projects are already in the making. An example of this system is the Automated Biological Agent Testing System (ABATS) that combines the techniques mentioned previously.[86] The system is the result of integrating several commercially available technologies into a single automated and robotized instrument for detection of viruses, bacteria, and parasites considered potential BT agents. The technologies incorporated into this "super system" include automated specimen preparation (both nucleic acid–based and protein-based such as immunodiagnostics), thermocyclers for PCR detection, chemiluminescent detectors for immunobased assays, sequencers, and software programs for sequence analysis.

Category B Agents

Rickettsia prowazekii (Epidemic Typhus) and R. rickettsii (Rocky Mountain Spotted Fever)

Diagnosis of these infections in the clinical microbiology laboratory currently rests on the identification of antibodies in serum during the acute and convalescent period in order to demonstrate seroconversion or rising titers. The diagnosis is therefore retrospective.[131,132] Detection of rickettsial DNA from blood or skin samples during the acute phase of the disease is possible via PCR assays. However, these assays are not standardized and are not commercially available. Primers have been designed for amplification of several rickettsial genes including citrate synthase, 17-kDa protein gene, OmpA, and OmpB.[132–136] The clinical sensitivity and specificity of standard or real-time PCR techniques have not been determined. Most likely real-time PCR is superior due to the higher analytical sensitivity of this technique and low risk of sample contamination with DNA amplicons when compared to standard PCR amplification methods.

Isolation of rickettsiae from clinical specimens is performed in very few specialized laboratories in the nation

and requires the use of cell monolayers, embryonated eggs, or animals. Detection of rickettsial antigens or whole bacteria in blood specimens is theoretically possible by using ultrasensitive methods, but such assays are currently only in the early phases of development. Immunohistochemical detection of rickettsiae in paraffin-embedded tissue has also been applied to tissue samples obtained pre- or postmortem.[137–139]

Salmonella spp., Shigella dysenteriae, Vibrio cholerae, and Cryptosporidium parvum (Acute Enteric Syndromes)

Diagnosis of *Salmonella*, *Shigella,* and *Vibrio* infections is based on isolation of the offending agent on standard microbiological media in the clinical laboratory, followed by specialized confirmatory tests to identify the specific serotype involved.[140] Diagnosis of *C. parvum* is based on visual identification of the protozoan in fecal specimens by using modified trichrome stain.[140]

Coxiella burnetii (Q Fever)

The diagnosis rests on serological demonstration of antibodies by immunofluorescent assay (IFA) or enzyme-linked immunosorbent assay (ELISA). Antibodies remain elevated for years after the acute infection, and therefore a fourfold rise in titers is the gold standard for diagnosis. PCR detection of *C. burnetii* DNA from blood or tissues also yields a diagnosis of Q fever.[88]

Brucella spp.

Diagnosis of brucellosis requires a high degree of clinical suspicion due to the protean manifestations related to this disease. Laboratory diagnosis is based on isolation of the microorganism from blood, bone marrow, or other tissue samples. Isolation is not easy due to the slow-growth of *Brucella* spp. Colonies usually appear after 4 to 6 weeks, and therefore communication with the clinical laboratory is important so that appropriate media will be used and the cultures will be held long enough for colonies to be detected.[90] Serologic assays for demonstration of rising antibody titers are available, although the diagnosis is retrospective. PCR detection is promising, but it is not standardized.[141–143]

Alphaviruses (Encephalitic Syndromes: Venezuelan, Eastern, and Western Equine Encephalomyelitis)

Diagnosis is based on isolation of the virus from serum or brain (postmortem specimens) in a BSL-3 environment. PCR detection of viral sequences is also possible. Serologic diagnosis is based on demonstration of antibodies in acute and convalescent sera.[144–146]

Botulinum Toxins

The diagnosis of botulism relies heavily on clinical parameters. An afebrile patient with signs and symptoms of progressive bulbar palsies and descending neuromuscular paralysis is highly suspected of having botulism. Demonstration of the toxin in cases of botulism due to ingestion of contaminated food is made from gastric samples, feces, blood, and urine. However, detection of minute amounts of toxin (and contacts with samples from cases may prove fatal due to the toxin's potency) would be difficult by current immunoassay systems such as ELISA platforms.[146] Detection techniques based on electrochemiluminescence and immunoliposomes are currently under development.[99,147] PCR assays can be performed in cases of ingestion of contaminated food in order to detect the genetic material present in *C. botulinum*. If weaponized toxin is used in the absence of *C. botulinum* organisms, detection of the genetic material would be difficult and would rely on the presence of residual DNA after toxin purification procedures. If inhalational botulism is suspected, respiratory secretions and nasal swabs should be obtained as early as possible. Postmortem samples of liver and spleen can be used for detection of botulinum toxins.

Ricin Toxin

Diagnosis is also based on clinical presentation and requires a high index of suspicion due to the nonspecific nature of the signs and symptoms. Laboratory diagnosis rests on detection of the toxin in body fluids by immunoassays (capture ELISA and IgG ELISA).[146] A new generation of tests using more sensitive detection methods is under development (see preceding discussion).

Staphylococcal Enterotoxin B

Diagnosis is also suspected on clinical grounds and confirmed by demonstration of the toxin in nasal swabs early in the disease process, feces, and, in fatal cases, from kidney and lung tissue. Serum can be analyzed by ELISA, and PCR can be performed for detection of toxin genes of *S. aureus* if present.[146]

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