Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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CHAPTER 125

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. Thus 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 nations. 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.

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 diagnosis will be made by a clinician or pathologist and the recognition of the event will be through geographic and epidemiologic anomalies. However, we have made important advances in environmental detection capability and point-of-care diagnostics for BT agents. Some diseases such as inhalational anthrax or smallpox may be relatively readily recognized by an alert clinician because of their distinctive presentations. However, the leading edge of a BT epidemic will arrive 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. 125.1***). Delay in recognizing the epidemic through reliance on surveillance or other surrogates will likely result in most 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. Viral hemorrhagic fevers (VHF) transmitted by aerosol[3] are underrepresented in naturally occurring case series, and a BT attack would provide an opportunity to answer questions about the underlying host factors and pathogenesis.

In October 2001, anthrax spores were distributed covertly in the US Postal Service, leading to 22 cases of human anthrax and billions of dollars spent on controlling a small inhalational anthrax epidemic.[4,5] Ever since the times of Greeks and Romans, humans have tried to inflict damage by the use of contagion on other populations.[6,7] Less than 4% of the groups responsible for terrorist attacks on human populations take responsibility for their actions.[8] 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).[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, A–C (***Table 125.1***).

EPIDEMIOLOGY OF A BIOTERROR ATTACK

All potential BT agents are capable of producing illness under natural circumstances. Therefore, the first challenge is to identify the etiologic agent, followed by a thorough epidemiologic and microbiologic analysis of the epidemic. 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 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, microbial forensics, sister to phylogenetics and "molecular epidemiology."

Differentiation between natural infections and a biological attack rests firstly on disease patterns given by epidemiological clues. They include presence of disease outbreaks of the same illness in noncontiguous areas, disease 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, VHF, inhalational anthrax), unusual strains of microorganisms when compared to conventional strains circulating in the same area, and genetically homogeneous organisms isolated from different locations.[9,10] These are a few

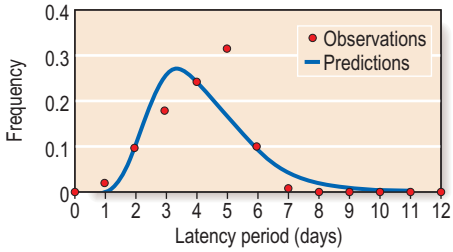

Figure 125.1 Log-normal distribution of incubation periods. (Reproduced from Sartwell P. The distribution and incubation periods of infectious diseases. Am J Hyg. 1950;51:310–318.)

Table 125.1 Potential Bioterror Agents

Category/Agent	Disease
Category A	
Viruses	
Variola major virus	Smallpox
Ebola, Marburg, CCHF, RVF, Lassa, Machupo, and Junin viruses	Viral hemorrhagic fevers
Bacteria	
Francisella tularensis	Tularemia
Yersinia pestis	Plague
Toxins	
Clostridium botulinum toxin	Botulism
Category B	
Viruses	
Alphaviruses (VEE, EEE, WEE)	Various encephalitides
Bacteria	
Rickettsia prowazekii	Epidemic typhus
Brucella spp.	Brucellosis
Coxiella burnetii	Q fever
Burkholderia mallei	Glanders
B. pseudomallei	Melioidosis
Toxins	
Ricin	
SEB	
Food- and waterborne viruses	
Shigella	
Salmonella	
Escherichia coli	
Vibrio cholerae	
Cryptosporidium	
Category C	
Emerging infectious agents such as Nipah virus and hantaviruses; *Mycobacterium tuberculosis*	

CCHF, Crimean-Congo hemorrhagic fever; RVF, Rift Valley fever; VEE, Venezuelan equine encephalitis; EEE, eastern equine encephalitis; WEE, western equine encephalitis; SEB, *Staphylococcus aureus* enterotoxin B.

guidelines that could prove helpful when investigating an outbreak; however, 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 by a covert attack planned by the Rajneeghee leadership was 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]

Biosurveillance

Due to the increased awareness of emerging infectious diseases and potential bioterrorist attacks, biosurveillance is now a health care priority. Approximately 70 pathogens have emerged between 1967 and 2009, and the majority of these microorganisms made the "jump" from their zoonotic host into the human species. Infectious disease surveillance has evolved dramatically during the last few years as part of the "molecular revolution" in life sciences. Infectious disease surveillance systems are generally divided into three categories: (1) laboratory-initiated infectious disease notification; (2) syndromic surveillance systems; and (3) genotyping-based surveillance of biothreats.[12] In any of these three complementary systems, the clinical laboratory plays a critical role.

Laboratory-initiated infectious disease notification is mostly based on conventional microbiological techniques for pathogen identification and relies on clinical laboratories for reporting of notifiable diseases to public health authorities. This system is slow, insensitive, but more specific when compared to syndromic sureveillance.[12] The low sensitivity is due to the variable sensitivity of different diagnostic tests used in clinical laboratories to confirm infections and the fact that only a small fraction of human infections are actually confirmed by laboratory means. In essence, the overall sensitivity of the surveillance system depends on the coverage of the laboratory-based surveillance, characteristics of the laboratory tests, screening practices to detect asymptomatic individuals, and the actual reporting system.[12,13]

Syndromic surveillance systems, on the other hand, rely on health utilization patterns and the use of health-related data collected electronically for other purposes. Reporting sources include clinical laboratories, pharmacies, emergency rooms, primary care physicians, intensive care units, and hospital admission and discharge data. Monitoring occurs in real time, and whenever deviations from a "normal pattern" of patients reporting for doctor visits, utilization of laboratory tests or pharmaceuticals occurs, an alarm is triggered suggesting the possibility of an outbreak.[12,14–16] The biggest challenge is the establishment of a baseline above which the system should signal an alarm. Sensitivity and specificity range widely depending on the disease/syndrome and the threshold for triggering the alarm.[16] When syndromic surveillance is used in conjunction with laboratory-initiated surveillance, the low specificity and high sensitivity of syndromic surveillance are potentially compensated by the higher specificity of the laboratory-initiated surveillance system.[12]

Genomic-based surveillance evolved during the past decade with the advent of new molecular techniques and high-throughput diagnostic tests for detection of infectious agents, their markers of virulence, and resistance patterns to antimicrobials.[12] Molecular typing and subtyping have allowed national and international agencies to monitor and initiate early warnings to public health and clinical laboratories. In addition, powerful biosensors using molecular approaches have monitored selected environments to detect potential pathogens before onset of symptoms in exposed persons (BioWatch and Biological Aerosol Sentry and Information System,[12,17] and the Autonomous Pathogen Detection System).[17,18] The current challenge is to integrate all surveillance systems into a system that provides comprehensive information and timely alerts. The vast electronic data collected by syndromic and genomic surveillance require new technologies for data storing, mining, and temporal/spatial analysis of outbreaks. The ultimate goal is the creation of "global laboratories" or global real-time epidemiological surveillance in which all surveillance information is processed along with geographic information systems and a real-time output is rendered regarding infectious threats around the world.[12] Attempts to achieve this goal are represented by the World Health Organization (WHO) Global Outbreak Alert and Response Network and the International System for Total Early Disease Detection (InStedd).

Conventional epidemiological investigations are by no means obsolete. Confirmation of an outbreak 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 important tools to analyze epidemics and suggest the mode of transmission and propagation. A point source epidemic curve is classically log-normal in distribution[1] and suggests a common exposure of a population to an infectious agent.

CATEGORY A

Bacterial Agents

Bacillus anthracis (Anthrax)

B. anthracis (see Chapter 38) is 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 an ideal bioweapon.

Anthrax presents in humans as four different clinical syndromes, depending on the portal of entry: (1) cutaneous; (2) gastrointestinal; (3) oral/oropharyngeal; and (4) inhalational. In the event of a BT attack, either overt or covert, the clinical presentation of 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.[19–21] During those attacks, 50% of cases were cutaneous anthrax, presumably 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 a bioweapons program.[5,22–28]

Inhalational anthrax should be suspected clinically in any individual presenting with fever and a widened mediastinum on chest radiograph (due to hemorrhagic mediastinitis).[25,26] The incubation period is normally 3–5 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.[23] Based on research in rhesus monkeys, the LD_{50} is estimated to be 8000–10 000 spores.[27–29] However, as few as 1–3 spores may be capable of producing a fatal outcome in ~1% of those exposed to these quantities.[30] Mortality is 100% without antibiotic therapy.[26,31–33] Early diagnosis, aggressive treatment with antimicrobial agents to which the bacteria are susceptible, and aggressive supportive therapy decreased the mortality to 40% in 2001 attacks.[5] Pathologic studies performed on the Sverdlovsk victims revealed hemorrhagic tracheobronchial 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 led rapidly to central nervous system (CNS) manifestations terminating in coma and death.[22,34,35]

Yersinia pestis (Plague)

Y. pestis (see Chapter 41) is a Gram-negative, aerobic, nonsporulating coccobacillus, member of the Enterobacteriaceae, with a wide host range.[36] 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.[37] *Y. pestis* has been responsible for some of the most devastating pandemics in human history during the pre-antibiotic era (sixth, fourteenth, and nineteenth centuries).[38] 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 WHO every year (from Madagascar, Tanzania, and Peru, among others).

Clinical presentation in naturally acquired infections takes five forms: (1) bubonic; (2) septicemic; (3) pneumonic; (4) cutaneous; and (5) meningeal. The pneumonic form is the most likely presentation in a case of plague due to a BT attack. 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.[39,40]

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), followed by a rapid clinical course leading to respiratory failure and death if not treated with antibiotics early in the course.[36,37,41]

The factors that led to the severe Manchurian pneumonic plague outbreaks in the early twentieth century are unknown, but weather, hygiene, and crowding were important factors. More recent outbreaks worldwide and in the United States have been much smaller and readily controlled. Pneumonic cases occur 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 finds R_0 to be approximately 1 prior to any control measures.[42]

Francisella tularensis (Tularemia)

This was one of the most scientifically neglected microorganisms with BT potential. Tularemia is a zoonotic infection caused by a small strictly aerobic, Gram-negative, nonsporulating coccobacillus. *F. tularensis* subspecies *tularensis* (Jellison type A) is by far more virulent than *F. tularensis* subspecies *holarctica* (Jellison type B) and is present only in North America.[43] 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, including ticks, fleas, mosquitoes, and biting flies[43,44] – an impressive range for any human pathogen.

In natural infections the most common sources of infection are tick bite and handling infected animals such as wild rabbits. Six clinical syndromes have been described: (1) ulceroglandular; (2) glandular; (3) oculoglandular; (4) pharyngeal; (5) pneumonic; and (6) typhoidal. Marked overlap exists among all these forms, and for practical purposes two syndromes (ulceroglandular and typhoidal) have been proposed.[45–47] As a BT agent, *F. tularensis* would most likely cause disease with a primary pulmonary component and secondary dissemination (typhoidal/systemic). In natural infections both ulceroglandular and typhoidal forms can have a hematogenous pulmonary component that is more common in typhoidal tularemia. Case-fatality rate approaches 30% if not treated with appropriate antibiotics.[47]

Viral Agents

Smallpox Virus (Variola Major)

Smallpox (see Chapter 57) is the only disease so far eradicated from the face of 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,[48] and vaccination ceased around the world, rendering humankind vulnerable to reintroduction of the virus.[49–51] 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: Centers for Disease Control and Prevention (CDC), Atlanta, Georgia and the Institute for Polyomyelitis and Viral Encephalitides in Moscow, later moved to NPO VECTOR, Novosibirisk. 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).[52,53] 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 *Orthopoxvirus* genus capable of producing systemic disease in humans. Monkeypox is potentially indistinguishable from smallpox, where mortality rates in tropical Central Africa are around 10–15%. In May and June 2003, an outbreak of monkeypox occurred in the United States.[54] Thirty-seven infections were laboratory-documented and involved humans

exposed to infected prairie dogs which had become infected because of contact with infected Gambian rats and dormice shipped from Africa earlier that year. Cases included veterinarians, exotic pet dealers, and pet owners. The clinical spectrum in this outbreak ranged from asymptomatic seroconversions to febrile illness with papulovesicular rash, but no deaths. However, phylogenetic analysis of the virus placed it in the less virulent West Africa clade rather than the Central Africa clade, which carries a case-fatality rate of 10–15%.

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 outbreak. 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 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 is also mandatory for 17 days. The newer generation of antivirals 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.[55] The only vaccine available in the United States is Dryvax, and sufficient doses have been manufactured to cover the entire US population. However, newer vaccines which 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, characterized by abrupt onset of fever, fatigue, malaise, and headaches. During this 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 (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 manifestations 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 2–3 weeks, leaving depigmented, scarred areas. This form of the disease, variola major, is fatal in up to 30% of unvaccinated patients and 3% of vaccinated individuals. Various hemorrhagic forms exist.[56]

Viral Hemorrhagic Fevers

This syndrome (see Chapter 67) is caused by a heterogeneous group of RNA viruses that belong to three different families: (1) filoviruses (Ebola and Marburg viruses); (2) arenaviruses (Lassa, Junin, Machupo, Guanarito, and Sabia viruses); and (3) bunyaviruses (Crimean-Congo hemorrhagic fever and Rift Valley fever viruses).[57–59]

The common denominator in these infections is the increased vascular permeability in the microcirculation leading to hemorrhagic diathesis and systemic manifestations such as pulmonary and cerebral edema related to leaky capillaries.[60] 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 (BSL)-4 laboratories for 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.

Diseases Caused by Toxins

Toxins in the context of BT agents are substances of biological 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 one-tenth or less lethal than anthrax on a weight basis. Other toxins such as ricin are less than one-thousandth as toxic as 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–G), all leading to the same clinical manifestations and lethality. The approximately 150-kDa toxins 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, with LD_{50} of 0.001 μg/g of body weight when administered parenterally.[31,61,62] The aerosol route decreases its lethality 80–100-fold. 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.[31,62]

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.

Viral Agents

Viral Encephalitides

These are caused by the genus *Alphavirus*, family *Togaviridae* (eastern, western, and Venezuelan equine encephalitis viruses; see Chapter 78). 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.[58,63]

Most of these viruses cause systemic illness characterized by fever, myalgias, and prostration.

Clinically apparent involvement of the CNS is present in some cases and varies among the different viruses. Eastern equine encephalitis is by far the most virulent, leading to case-fatality rates of 50–75%, and survivors usually have severe neurologic sequelae.[64,65] Venezuelan equine encephalitis, in contrast, leads to CNS manifestations in no more than 4% of cases, and almost all Venezuelan equine encephalitis infections are symptomatic even in the absence of CNS involvement.[66–68]

Bacterial Agents

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

Typhus (see Chapter 50) is another disease that has played a historic role in human populations.[69–72] Millions perished in World Wars I and 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, on the other hand, is transmitted by tick bites and occurs endemically in South, Central, and North America. Rickettsiae target the microvascular endothelium leading to leaky capillaries systemically.[73] The main causes of morbidity and mortality are noncardiogenic pulmonary edema and meningoencephalitis. Rickettsiae are remarkably underestimated biothreats as they are highly infectious by low-dose aerosol, possess a stable extracellular form, and are resistant to most empirically administered antibiotics, including β-lactams, aminoglycosides, and erythromycin, and infections are exacerbated by sulfonamides. Case-fatality rates can be as high as 40–50% without antibiotic therapy and 3–5% with adequate antibiotic coverage. Lethal cases are usually due to delayed diagnosis.[70,71,74]

These potent BT agents are often discounted because of their susceptibility to tetracycline and chloramphenicol. However, the severity of the illness, difficulty of clinical diagnosis, exhaustion of antibiotics in the face of a mass attack, and existence of antibiotic-resistant organisms suggest they are still formidable threats.

Coxiella burnetii (Q Fever)

This Gram-negative, obligately intracellular bacterium has a high degree of infectivity (1 organism by inhalation) and low lethality.[75–78] The distribution of Q fever is worldwide and results from exposure to animals such as sheep, cattle, goats, cats, rabbits, and others (see Chapter 53). *C. burnetii* can withstand harsh environmental conditions and be transported by wind. In natural infections, 60% are asymptomatic. In symptomatic cases, the presentation is nonspecific, including 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, Undulant Fever, Mediterranean Fever, Malta Fever)

Four species of these Gram-negative, aerobic, nonspore-forming coccobacilli are pathogenic to humans: *B. abortus, B. melitensis, B. suis,* and *B. canis* (see Chapter 40). Host ranges include goats and sheep (*B. melitensis*), swine and horses (*B. suis*), cattle, bison, elk and 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 is highly variable, even after inhalational exposure. 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.[75–81]

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 *Escherichia coli*, *Vibrio cholerae,* and *Cryptosporidium* spp.

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 almost 1000 cases of *Salmonella*-related gastroenteritis.[11,82] 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 *Cryptosporidium* spp.) to standard chlorinating procedures, would decrease the number of bacteria to below that required to infect large numbers of people.[75]

Occasional outbreaks of nontyphoidal *Salmonella* and *Shigella* infections occur in the United States. *Shigella* and *Crystosporidium* are highly infectious organisms that require very low numbers (10^2–10^3 organisms) to provoke clinical disease. 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.

Cryptosporidium spp. infections are characterized by watery diarrhea and abdominal cramping for 2–3 weeks. The disease is self-limited except in patients with acquired immunodeficiency syndrome (AIDS) or other conditions of compromise; in such cases illness can last for months or years if immune function is not restored. *Cryptosporidium* spp. are resistant to standard chlorine concentrations in water supplies.[83] The main human pathogen is *C. hominis,* followed by *C. parvum*. Other less common pathogens include *C. meleagridis, C. muris*, *C. felis,* and *C. canis*. The largest outbreak in the United States was in Milwaukee in the early 1990s, and was responsible for thousands of cases and increased mortality among AIDS patients.[75,84,85]

Category B Toxins

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

The toxin composed of two glycoproteins of approximately 66 kDa[86] inhibits protein synthesis by blocking elongation factor 2 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}. When large amounts of toxin are ingested, the manifestations include nausea, vomiting, severe abdominal cramping, rectal hemorrhage, and diarrhea. As the course progresses, anuria, mydriasis, severe headaches, and shock supervene, leading to death in 2–3 days. Clinical manifestations usually appear within 10 hours after ingestion of the toxin. Inhalational exposure leads to prominent pulmonary manifestations 8–28 hours after exposure with fever, dyspnea, cough, cyanosis, and death. Histologically, there is widespread necrosis of pulmonary parenchyma and pulmonary edema. A single case of parenteral intoxication is 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 disseminated intravascular coagulation.[87]

Staphylococcus aureus Enterotoxin B

Staphylococcus aureus Enterotoxin B (SEB), a 28-kDa, heat-stable exotoxin produced by certain strains of *S. aureus,* causes food poisoning after ingestion in improperly handled food. In BT scenarios exposure can occur by either inhalation or ingestion, leading to SEB food poisoning or SEB respiratory syndrome. The toxin is highly incapacitating and not very lethal. The doses that cause symptoms in half of exposed persons and LD_{50} differ by a magnitude of 5 log scales for inhalational exposure.[88]

The incubation period 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 SEB are respiratory and consist of fever, cough, chills, myalgias, chest pain, and pulmonary insufficiency due to alveolar edema. General manifestations consist of multiorgan failure secondary to cytokine storm.[31] These toxins are superantigens that bind to major histocompatibility complex class II molecules on large numbers of lymphocytes and macrophages, leading to hyperactivation of the immune system and massive release of cytokines, including interferon-γ, tumor necrosis factor-α, interleukin-6 and other mediators, such as leukotrienes and histamine.[88]

DIAGNOSIS

The role of the clinical laboratory in the diagnosis of possible cases related to a BT attack is of utmost importance.[89,90] 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 (BSL) requirements due to their infectivity (***Table 125.2***). 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.[91,92] This type of testing takes place in highly specialized laboratories and is not undertaken by the standard clinical microbiology laboratory.

Table 125.2 Laboratory Response Network for Bioterror Attacks

Level	Functions
A	Community-level laboratories that should recognize the clues of a possible bioterror 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

BSL, biosafety level.

Table 125.3 Molecular Diagnostic Techniques

Type	Techniques
Nonamplification techniques	Labeled-nucleic acid probes Hybridization protection assay (HPA) *In situ* hybridization
Amplification techniques	
Signal amplification	Branched DNA (bDNA)
	Hybrid capture assays
Target amplification	Polymerase chain reaction (PCR), reverse transcription (RT)-PCR, real-time PCR
	Transcription-based amplification Transcription-mediated amplification (TAM) Nucleic acid sequence-based amplification (NASBA)
	Strand displacement amplification (SDA)
Probe amplification	Cleavase-invader technology
Detection systems	Gel analysis
	Colorimetric microtiter plates
	Hybridization
	Direct sequencing
	Quantitative methods: Diagnostic chips, microarrays Bead-based flow cytometric analysis Gas chromatography/mass spectrometry (GCMS)

Conventional and Molecular Diagnosis of Potential Bioterror Agents

General Principles

The bacterial diseases caused by the BT agents in this chapter, with the exception of *Coxiella 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.[93] 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 IgM titers during the acute stage of the disease.

With the advent of molecular techniques, rapid and sensitive diagnostic tests are becoming available for BT agents during the acute disease.[94–96] 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 also be applied to potential BT agents as epidemiological and forensic investigations. Postmortem diagnosis is also possible by analysis of frozen or paraffin-embedded tissues by immunohistology or nucleic acid-based amplification techniques. Several molecular techniques have been developed for nucleic acid amplification and detection (***Table 125.3***).

Rapid diagnosis of the initial 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 125.2***).[89,91,97]

Several of the agents cause 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 sentinel events in a human outbreak. There are currently efforts to establish a network of laboratories dedicated to diagnosis of veterinary agents.[91]

DIAGNOSIS OF SPECIFIC BIOTERROR AGENTS

Category A 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*.[98–102]

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).[99] 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.[96]

Serological tests available for clinical diagnosis are based on detection of antibodies directed against protective antigen. 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}).[103,104] Liposome PCR, liposomal technology combined with real-time PCR (for a DNA reporter sequence), has also been developed for cholera and botulinum toxins, with analytical sensitivity in the attomolar to zeptomolar (10^{-18} to 10^{-21}) range.[105] The specificity of this assay is determined by the toxin-capturing antibody.

Nucleic acid amplification techniques (PCR) are also available in both standard and real-time formats. 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.[96] Real-time PCR tests have been developed by Applied Biosystems (TaqMan 5′ nuclease assay) and Roche Applied Science (LightCycler).[106–108] The analytical sensitivity of both techniques is extremely high, and testing times have been decreased to 1–2 hours.

Portable PCR instruments are being developed for rapid deployment to the field.[109] Examples include the rugged advanced pathogen identification device (RAPID),[106] the Smartcycler (Cepheid, CA),[107] and the miniature analytical thermal cycler instrument (MATCI), developed by the Department of Energy's Lawrence Livermore National Laboratory.[110] This instrument later evolved into the advanced nucleic acid analyzer (ANAA) and handheld advanced nucleic acid analyzer (HANAA).[111] The only commercially available test approved by the Food and Drug Administration (FDA) is the JBAIDS anthrax detection kit (Idaho Technology Inc., Salt Lake City, UT).

Molecular subtyping of *B. anthracis* is also possible by using the 16S rRNA subunit gene, multiple-locus variable number tandem repeat analysis of eight genetic loci, and amplified fragment length polymorphism techniques.[112,113]

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).[114,115]

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 is based on demonstration of the bacillus in blood or sputa. Standard diagnostic techniques include visualization of Gram-negative coccobacilli, which by Giemsa, Wright, or Wayson stains reveal a "safety pin" appearance. Isolation is performed on blood and McConkey agar plates where colonies appear as nonlactose fermenters. The organisms are identified preliminarily by direct immunofluorescence assay with *Y. pestis*-specific antibodies, with final identification based on biochemical profiles.[116]

Molecular diagnostic techniques based on real-time PCR became 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.[117–119] Assays 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 (***Fig. 125.1***) 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 by demonstration of the microorganisms in secretions (sputa, exudates) by direct immunofluorescence or immunohistochemically in biopsy specimens. Isolation may be achieved on 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 is based on the biochemical profile.[120]

Molecular diagnostic techniques are based on PCR detection of *F. tularensis* using primers for genes such as outer-membrane protein (*Fop*) or *tul4* and real-time detection systems.[96,121,122] A commercial FDA-approved PCR kit is available (JBAIDS Tularemia Detection Kit, Idaho Technology, Inc., Salt Lake City, UT).

Smallpox Virus

Diagnosis of variola major is suggested by its clinical presentation and visualization of Guarnieri bodies in skin biopsy samples. Preliminary confirmation requires identification of 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 smallpox is performed only under BSL-4 containment facilities at CDC.[53]

Molecular techniques are based on PCR amplification using real-time or standard technology, followed by sequencing or use of restriction fragment length polymorphism for identification.[123] Techniques developed for smallpox molecular testing include Taqman- and LightCycler-based assays with primers designed for the hemagglutinin gene and A-type inclusion body proteins.[124–127]

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.[96]

Viral Hemorrhagic Fevers

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

Initial diagnosis of these diseases is suspected on clinical and epidemiological 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.[128–132]

Molecular diagnostic techniques have also improved dramatically. Serum or blood is the most common specimen for reverse transcription-PCR amplification of viral nucleic acids.

Design of primers for this heterogeneous 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.[133,134] 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 fluorescence applications.[134–136] Multiplex has evolved during the past several years, and molecular test combinations are now possible. Both low target number multiplexing (3–6) and highly multiplexed assays (>10 targets) have been developed. The Luminex xTag RVP assay is capable of detecting 16 targets using universal bead array sorting technology. The use of microchips and microarrays 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 severe acute respiratory syndrome (SARS) outbreak in 2003 are excellent examples of the power of hybridization-based microchips. Chips and microarrays vary in complexity. Some are designed for influenza typing and some others, such as the GreeneChipResp, can identify 15 or more virus families.

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 periods in order to demonstrate seroconversion or rising titers. The diagnosis is therefore retrospective.[74,137] 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 genes, *ompA* and *ompB*.[137–141] The clinical sensitivity and specificity of standard or real-time PCR techniques have not been determined. 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 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 pre- or postmortem.[142–144]

Salmonella spp, *Shigella dysenteriae*, *Escherichia coli* O157:H7 and *Cryptosporidium* spp. (Acute Enteric Syndromes)

Diagnosis of *Salmonella*, *Shigella,* and *Vibrio* infections is based on isolation of the offending agent on standard microbiological media followed by specialized confirmatory tests to identify the specific serotype.[145] Diagnosis of *Cryptosporidium* spp. is based on visual identification of the protozoan in fecal specimens using modified trichrome stain.[145] Other methods include antigen detection in fecal specimens using enzyme immunoassay or fluorescence detection methods.

Coxiella burnetii (Q Fever)

The diagnosis rests on serological demonstration of antibodies by immunofluorescence antibody analysis 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.[94]

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 4–6 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.[96] Serologic assays for demonstration of rising antibody titers are retrospective. PCR detection is promising, but is not standardized.[146–148]

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.[149–151]

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 due to ingestion of contaminated food is achieved by examination of gastric samples, feces, blood, and urine. However, detection of minute amounts of toxin would be difficult by current immunoassay systems such as ELISA platforms.[151] Detection techniques based on electrochemiluminescence and immunoliposomes are currently under development.[105,152] PCR assays can be performed in cases of ingestion of bacterially contaminated food in order to detect *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 based on clinical presentation and requires a high index of suspicion due to the nonspecific signs and symptoms. Laboratory diagnosis rests on detection of the toxin in body fluids by immunoassays (capture ELISA and IgG ELISA).[151] A new generation of tests using more sensitive detection methods is under development (see liposome PCR description, above).

Staphylococcal Enterotoxin B

Diagnosis is suspected on clinical grounds and confirmed by demonstration of the toxin in nasal swabs early in the disease, feces, and, in fatal cases, kidneys and lung tissue. Serum can be analyzed by ELISA, and PCR can be performed for detection of toxin genes of *S. aureus* if present.[151]

Development of Multiplex Detection Systems

The creation of an automated, easily deployable instrument capable of detecting all possible potential BT agents by highly sensitive techniques such as electrochemoluminescence or PCR would be ideal. The nonspecific nature of presenting symptoms is a major problem for several agents. An early diagnosis of the epidemic must be established, particularly for organisms such as *Y. pestis* in which there is only a short window for successful treatment. An example is the automated biological agent testing system (ABATS), that combines the above techniques.[92] The system integrates several commercially available technologies into one single automated, 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, electrochemiluminescence detectors for immunobased assays, sequencers, and software programs for sequence analysis. Another powerful system is the Ibis T5000 Universal BW sensor based on broad-range PCR and high-performance mass spectrometry.[153,154] This system is based on technology developed for the Department of Defense and known as TIGER (triangulation identification for the genetic evaluation of risks). Mass spectrometry of amplified products derives the identity of the agent based on the composition of amplicons. This technology has been used successfully to amplify nucleic acids of bacteria, viruses, fungi, and protozoans. Several pairs of broad-range primers are used in a multiplex format, and amplified products are analyzed by mass spectrometry followed by species/strain identification using sophisticated software systems. Liquid-based microassay bead systems (Luminex) are also providing an outstanding platform for development of multiplex detection systems.

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