The role of nucleic acid-based assays in the public health response to bioterrorism and emerging infectious diseases

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Introduction

There have been a number of events over the past few years that have focused attention on the growing threat of bioterrorism in the United States (1). From the public health perspective, bioterrorism is defined as the deliberate release of pathogens or their toxins into a population for the purpose of causing illness or death. Although some authorities had initially felt that the threat of bioterrorism was exaggerated (2), the recent incident in which spores of *Bacillus anthracis* were sent through the United States mail has made bioterrorism a reality (3), and has focused attention on national preparedness should another crisis occur.

The Centers for Disease Control and Prevention (CDC) was designated by the Department of Health and Human Services to prepare the nation's public health system to respond to a bioterrorism event (4). Beginning in 1999, CDC began to fund cooperative agreements with every state, territory, and several large municipalities that focused on preparedness efforts (5). Five critical areas were emphasized during the first three years of this program: preparedness planning and readiness assessment; surveillance and epidemiology capacity; biological laboratory capacity; chemical laboratory capacity; and the health alert network and information technology (4). Based on lessons learned from the recent anthrax attack (3), additional resources and focus areas have been added: communicating health risks and health information dissemination; and, education and training.

Bioterrorist attacks can involve civilian populations, animals or plants. Possible routes of exposure are from aerosols, contaminated food, water, medicine, medical devices or blood, and infected arthropod vectors. Bioterrorist attacks can occur as one of two scenarios, i.e., covert or overt. Because we currently lack the ability to conduct real-time monitoring for the release of a biological agent in U. S. cities, an unannounced (i.e., covert) release of a biological agent would likely go unnoticed for some time, with those exposed leaving the area before the act of terrorism becomes evident. Due to an incubation period, the first signs that a biological agent has been released may not become apparent until days or weeks later, when individuals become ill and seek medical care. Thus, the "first responders" to a covert bioterrorism attack will likely be the astute clinician, laboratory, or public health worker who recognizes the index case or identifies the responsible agent. Because of their terrorism training, traditional "first responders" (e.g., firefighters, hazmat or law enforcement personnel) are the most likely to respond to an announced (i.e., overt) release of a biological agent or, more likely, to a hoax. In either scenario, the initial recognition of a bioterrorism event (or hoax) in the U.S., whether announced or unannounced, would be at the local and state level. A comprehensive public health response to bioterrorism (or for that matter, to any outbreak of infectious disease) will involved epidemiologic investigation, medical treatment and prophylaxis for affected persons, and the initiation of disease prevention activities. The success of these activities is dependent, to a large extent, upon the rapid and accurate identification of the threat agent.

The threat of bioterrorism has elevated the importance of rapid microbial identification and typing. Prior to the anthrax attack of 2001, it was necessary to identify a microbial agent so that control measures could be implemented (e.g., treatment) and, in some instances (e.g., food-borne outbreaks) to type them in order to identify their source. However, since the anthrax attack it has now become necessary to be able to more rapidly identify the infectious agent and, under certain circumstances, to confirm whether the infection is naturally occurring or due to bioterrorism, whether the agent has been genetically engineered, and where the agent came from (6).

Agents

Many biological agents can cause illness in humans, but not all are capable of impacting public health and medical infrastructures on a large scale (7). In order to bring focus to public health preparedness activities, CDC convened a meeting of national experts in 1999 to review the criteria for selecting the biological agents that posed the greatest threat to civilians and to help develop a prioritized list of agents (7). This list of "Critical Agents" (Table 1) was prioritized based on considerations such as: the ability of the agent to cause mass casualties; the ability of the agent to be widely disseminated either by aerosol or by other means; the ability of the agent to be transmitted from person to person; the public's perception, correctly or incorrectly, associated with the intentional release of the agent; and special public health preparedness needs (e.g., vaccines, therapeutics, enhanced surveillance, or diagnostics).

As currently defined, Category A, which includes some of the classic biowarfare agents, are high priority agents that are most likely to cause mass casualties if deliberately disseminated and require broad-based public health preparedness efforts. Natural infections caused by agents in Category A are uncommon in the U.S. or nonexistent. For example, prior to the bioterrorist attack with *B. anthracis* in 2001, the last case of inhalational anthrax in the U.S. was in 1976 (8). Furthermore, the World Health Organization (WHO) declared smallpox eradicated in 1977 (9). Category B agents also have some potential for large-scale dissemination, but generally cause less illness and death than those in category A. Many of these agents have been weaponized in the past, or have been considered as weapons by some state-sponsored programs (10). Some of the Category B agents could be used to contaminate food or water sources. In addition, many of these agents are relatively easy to obtain, and are thus more likely to be used in the setting of a biocrime (11). Biological agents that are not currently believed to present a high bioterrorism risk to public health, but which could emerge as future threats were placed in Category C. Some of these agents are associated with emerging infections or have characteristics that, in the future, could be exploited for deliberate dissemination. In light of the progress made in preparedness, the "Critical Agent" list has recently been reexamined and is currently being reprioritized.

In the U.S., both clinical and laboratory experience is limited with respect to the recognition and confirmation of Category A agents and for many of the agents in Category B. The low numbers of human infections in the U.S. caused by Category A agents (as well as for many of those in Category B) has been given as a reason why there has been a general lack of interest by the commercial sector in spending money for the development, manufacture, and FDA approval of diagnostic tests. This situation has created the need for the development and restricted distribution of biodetection assays and specialized reagents, which would not otherwise be available to support the public health infrastructure and national security interests of the U.S. (12).

Laboratory Response Network

An effective public health response to a bioterrorism event would have to be rapid since there is only a small window of opportunity during which prophylaxis or other control measures could be implemented to reduce the morbidity and mortality associated with such an event (13). In order to facilitate the rapid identification of threat agents, the Laboratory Response Network (LRN) was created. The LRN was initially designed to link state and local public health laboratories with advanced capacity clinical, military, veterinary, agricultural, water- and food-testing laboratories. It is a critical component of CDC's mission to lead the effort in strengthening the public health infrastructure, and consequently enhancing readiness to detect and respond to bioterrorism at the federal, state, and local levels. The LRN was developed by the CDC in concert with the Association of Public Health Laboratories (APHL) and with collaboration from the Federal Bureau of Investigation (FBI) and the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). It is the first example of a public health-law enforcement partnership. The LRN has a dual function in that it has the ability to detect and respond to outbreaks caused by agents released by bioterrorists as well as those that may occur naturally, including those considered to be emerging infectious diseases. This capacity is particularly important, since it will generally not be known at the time of detection whether the outbreak was intentional or natural.

The LRN collaborative partnership operates as a national network of laboratories comprised of sentinel laboratories, reference laboratories and national laboratories. Sentinel laboratories, for the most part, include hospital and other community clinical laboratories. In the aftermath of a covert bioterrorism attack,

patients will seek care at widely dispersed hospitals where such local laboratories will be called upon to conduct routine testing. Typically, these laboratories would participate in the LRN by ruling out or referring critical agents that they encounter to nearby LRN reference laboratories. To facilitate these efforts protocols and algorithms were developed for clinical laboratories to follow (14).

Reference laboratories that do confirmatory testing include primarily state and local public health laboratories, with biosafety level 2 facilities where biosafety level 3 practices are observed, and public health laboratories with biosafety level 3 facilities, or certified animal facilities, which are necessary for performing certain tests involving mice. Additional laboratories belonging to the Department of Defense, Food and Drug Administration, U.S. Department of Agriculture, and Environmental Protection Agency also serve as LRN reference laboratories. Some LRN reference laboratories can perform additional tests requiring biosafety level 3 containment (e.g., the handling of powders suspected of containing anthrax spores). There are more than 120 reference laboratories located in the U.S. and internationally.

Currently, two federal laboratories (CDC and USAMRIID), with biosafety level 4 capacity for handling viral agents such as Ebola and variola major, serve as national LRN laboratories. These federal laboratories also can perform all reference procedures. As part of their LRN responsibilities, they identify agents in specimens referred to them by reference laboratories and identify recombinant microorganisms that may not be recognizable by conventional isolation and identification methods. Federal laboratories also maintain extensive culture collections against which the isolate(s) from a bioterrorist event may be compared.

Reference laboratories use standard protocols and reagents for the identification and confirmation of threat agents. Bioterrorism is a criminal act and specimens or cultures will be evidence in a criminal investigation. Thus, protocols also have information concerning chain of custody requirements. The protocols for the Category A and B agents (Table 1) were written by subject matter experts at CDC, USAMRIID and the FBI and reviewed for accuracy and ease of use by laboratories representing the LRN. The protocols, which are available to LRN members on a secure web site, contain the information for ordering the necessary reagents and control strains for performing the tests.

Prior to the enhancement of laboratory capacity, many U.S. public health laboratories were technologically behind those in the private sector. Thus, the initial protocols relied on techniques (e.g., culture and staining with fluoroscein-labeled antibodies [DFA]), which were already familiar to all public health laboratories, to identify many of the critical Category A agents. These protocols were revised and updated as new, rapid assays were developed and validated (15). The focal point for the advances in technology within the LRN was the Rapid Response and Advanced Technology (RRAT) laboratory at CDC, which was created in 1999. The RRAT laboratory is a vital component of CDC's bioterrorism preparedness effort and a partner to the LRN where it serves as a source of test methods, validation data, training, and proficiency testing for the member laboratories. The RRAT laboratory develops novel approaches to molecular screening for the Category A and B agents using real-time polymerase chain reaction (RT-PCR) assays. Because LRN member laboratories decide for themselves which brand of the commercially available instruments to purchase for performing RT-PCR, the RRAT laboratory has optimized each test protocol for use with a variety of such instruments including the LightCycler (Roche Diagnostics Corporation, Indianapolis, IN), Smart Cycler (Cepheid, Sunnyvale, CA), iCycler (Bio Rad, Hercules, CA), and the GeneAmp 5700, ABI PRISM 7000, and ABI PRISM 7700 Sequence Detection Systems (Applied Biosystems, Foster City, CA).

Many of the rapid nucleic acid amplification assays that are now available to LRN members were developed through a collaboration between the RRAT laboratory and Lawrence-Livermore National Laboratory. The assays use genomic and plasmid targets, which are highly specific. Extensive validation against large numbers of threat agents, which are representative of the geographical and temporal variation of each particular agent, indicated that panels of reagents comprised of several primers and probe sets would be necessary to identify all strains of a given agent. The assays were coupled with sample preparation protocols for the various sample types that were representative of those collected during a bioterrorism event. Some confirmatory assays can be completed within one hour using automated nucleic acid extractions and RT-PCR. The procedure used to develop and validate assays provides high confidence

assays for threat detection. After characterizing and sequencing the novel coronavirus associated with severe acute respiratory syndrome (SARS) (16), a similar procedure was employed to rapidly develop an assay for SARS and disseminate it to the LRN laboratories.

Early Detection

Some of the nucleic acid amplification tests that were developed for the LRN have also been used to screen environmental air samples for the presence of selected threat agents as part of a program called Biowatch. Biowatch is a Department of Homeland Security (DHS) program that monitors air samples collected in a number of cities in the U.S. for the presence of selected threat agents. It is hoped that an early detection of a biological agent release would result in a more rapid response, which would result in a reduction in the morbidity and mortality associated with an aerosol release (13). The assays are also used on a more limited basis in conjunction with air samples collected during the monitoring of special events such as the Winter Olympic Games and the Democratic and Republican National Conventions.

Epidemiologic Clues to Bioterrorism

The largest deliberate use of a biological agent on a civilian population in the U.S. in the 20th century occurred in 1984, when members of the Rajneesh sect contaminated salad bars and other foods with *Salmonella* serotype Typhimurium at restaurants in The Dalles, Oregon to test their ability to affect voter turnout in a local election (17). More than 751 people became ill and several had to be hospitalized. Despite suspicions of the community, a rigorous epidemiological investigation failed to demonstrate that the outbreak was deliberately caused. More than one year later, a criminal investigation provided evidence that linked the religious commune with the outbreak. Today, we have been sensitized to the possibility of bioterrorism. Smallpox, which no longer exists as a naturally occurring disease, is the only disease that would obviously be the result of a deliberate (or perhaps accidental) release. Many of the Critical Agents of concern are endemic in the U.S. Thus, it may be necessary to determine epidemiologically whether an outbreak is of natural occurrence or intentional. Grunow and Finke (18) developed a scoring system based on selected criteria to either rule in or rule out the use of biological warfare in the event of an unusual outbreak of disease. Many of these criteria would be difficult to ascertain in a covert attack on civilian population. Treadwell et al. (19) generated a list of epidemiologic and laboratory clues suggestive of the deliberate dissemination of a biologic agent (Table 2). Identifying the cause of outbreaks as due to bioterrorism is now as important as excluding bioterrorism as the cause of an outbreak of unknown etiology. The clues in Table 2 focus on aberrations in the typical characterization of an outbreak by person, place, and time in addition to consideration of the microorganism. Some of the clues, such as a community-acquired case of smallpox, are quite specific for bioterrorism whereas others, such as similar genetic typing of an organism may simply denote a natural outbreak. A combination of clues, especially those that suggest suspicious point source outbreaks, will increase the probability that the event is likely due to bioterrorism. It is important to note that the epidemiologic clues can only be assessed in the context of a rapid and thorough investigation. The outbreak of West Nile encephalitis in New York highlighted the impact of concerns about bioterrorism even with a naturally occurring disease (20) as media reports suggested that the West Nile virus was deliberately released (21). Even the most specific of clues may signal a new natural outbreak. For example, the recent community outbreak of individuals with smallpox-like lesions in the mid-West may, on initial examination, have indicated a deliberate release of smallpox virus. However, a thorough integrated epidemiologic and laboratory investigation identified the disease as monkeypox, an exotic disease in the U.S., which in itself should suggest bioterrorism (22). The affected individuals were, in fact, infected by prairie dogs purchased as pets, which had acquired the infection while co-housed with infected Giant Gambian rats that had recently been imported from Ghana and not as a result of deliberate dissemination.

Molecular Strain Typing

The microbiology laboratory has made significant contributions to the epidemiology of infectious diseases. Strengthening the collaboration between laboratory and epidemiology practice has been a crucial component of bioterrorism preparedness. In dealing with an infection, it is often necessary to identify the species of infecting microorganism in order to prescribe effective therapy. Many of the techniques that have evolved for such purposes are both rapid and accurate but, in general, do not provide the kind of genetic discrimination necessary for addressing epidemiologic questions. Fortunately, typing methods for bacteria, fungi, protozoa and viruses have evolved to meet this challenge. Historically, the typing methods

that have been used in epidemiologic investigations fall into two broad categories: **phenotypic** methods and **genotypic** methods. Phenotypic methods (Table 3) are those that characterize the products of gene expression in order to differentiate strains. For example, the use of biochemical profiles to discriminate between genera and species of bacteria is used as a diagnostic method, but can also be used for biotyping. Other methods, such as phage typing, can be used to discriminate among groups within a bacterial species. Biotyping emerged as a useful technique for epidemiologic investigations in the 1960s, while phage typing of bacteria and serological typing of bacteria and viruses has been used for over 50 years. Today, the majority of these tests are considered inadequate for epidemiologic purposes. First, they do not provide enough unrelated parameters to obtain a good reflection of genotype. For example, serotyping of *Streptococcus pneumoniae* discriminates among only a limited number of groups. In addition, some virus species, e.g., human cytomegalovirus and measles virus, cannot be divided into different types by serology because significant antigenic differences do not exist. Second, the expression of many genes is affected by spontaneous mutations, environmental conditions and by developmental programs or reversible phenotypic changes, such as high frequency phenotypic switching. Because of this, many of the properties measured by phenotypic methods have a tendency to vary. Thus, most of these methods have been replaced by genotypic methods. The one major exception is multilocus enzyme electrophoresis (MLEE) (23), which is a robust phenotypic method that performs comparably with many of the most effective DNA based methods (24).

Extremely sensitive and specific molecular techniques have been developed over the past three decades to facilitate epidemiologic studies. Our ability to use these molecular techniques (genotypic methods), to detect and characterize the genetic variability of infectious agents is the foundation for the majority of molecular epidemiology studies. The application of appropriate molecular techniques has been an aid in the surveillance of infectious agents and in determining sources of infection. These molecular techniques can be used to study health and disease determinants in animal, including human, as well as plant populations. It requires choosing a molecular method(s) that is capable of discriminating genetic variants at different hierarchical levels coupled with the selection of a region of nucleic acid, which is appropriate to the questions being asked.

Genotypic methods are those that are based on an analysis of the genetic structure of an organism. Over the past decade a number of genotypic methods have been used to fingerprint pathogenic microorganisms (Table 4) (25,26). The data generated by these typing methods are most effective when they are collected, analyzed, and integrated into the results of an epidemiological investigation. Microbial fingerprinting should supplement, and not replace, a carefully conducted investigation (27). In some cases, typing data can effectively rule out an outbreak and thus avoid the need for an extensive epidemiologic investigation. In other cases, these data may reveal the presence of outbreaks caused by more than one strain (28) or even reasons why infections did not occur after release of a biological agent (29). Data interpretation is facilitated greatly by an appreciation of the molecular basis of genetic variability of the organism being typed and the technical factors that can affect results. It is important to remember that with the exception of whole genome sequencing, both the gene- or genome-based molecular methods analyze only a small portion of the organisms' genetic complement. Thus, isolates that give identical results are classified as "indistinguishable" not "identical". Theoretically, a more detailed analysis should uncover differences among the isolates that appeared to give identical patterns, but that were epidemiologically unrelated. This is unlikely to occur when a set of epidemiologically linked isolates are analyzed (30). For this reason, only whole genome sequencing would provide the unequivocal data required for attribution (6, 31).

Molecular Techniques in Surveillance and Epidemiological Investigations

The power of molecular techniques in epidemiological investigations is well exemplified by PulseNet. PulseNet, the national molecular subtyping network for food-borne disease surveillance, was established by CDC and several state health departments in 1996 to facilitate subtyping of bacterial food-borne pathogens for epidemiologic purposes (32). Twenty years ago, most food-borne outbreaks were local problems that typically resulted from improper food handling practices. Outbreaks were often associated with individual restaurants or social events and often came to the attention of local public health officials through calls from affected persons. Today, food-borne disease outbreaks commonly involve widely distributed food

products that are contaminated before distribution, resulting in cases that are spread over several states or countries. Such outbreaks may be unintentional or intentional (bioterrorism).

The PulseNet network, which began with 10 laboratories typing a single pathogen (*Escherichia coli* O157:H7), has grown and now includes 46 state and 2 local public health laboratories and the food safety laboratories of the U.S. Food and Drug Administration and the U.S. Department of Agriculture (32). The vast majority of the PulseNet laboratories are also members of the LRN. Currently, four food-borne pathogens (*E. coli* O157:H7, nontyphoidal *Salmonella* serotypes, *Listeria monocytogenes*, and *Shigella*) are being subtyped by pulsed field gel electrophoresis (PFGE) as part of routine surveillance for food-borne disease. The laboratories follow a standardized protocol using similar equipment so that results are highly reproducible and DNA patterns generated at different laboratories can be compared electronically. Isolates are subtyped on a routine basis and the data analyzed promptly at the local level. Clusters can often be detected locally that could not have been identified by traditional epidemiologic methods alone. PFGE patterns are also shared between laboratories electronically. This can serve to link apparently unrelated outbreaks and facilitates the identification of a common vehicle (33). The theoretical impact of PulseNet can be estimated using the data from the 1993 *E.coli* O157:H7 outbreak in the western U.S. in which 726 cases and 4 deaths were associated with consumption of ground beef at a fast food restaurant chain. Using conventional methodology, the outbreak was first recognized after 40 days of illnesses. If the outbreak had occurred in 1998, it would likely have been detected about 1 week earlier and the number of cases would have been reduced by 68%. In another example, in May 1998, PulseNet facilitated the investigation of two simultaneous clusters of *E. coli* O157:H7 infections in the northeastern U.S. PFGE fingerprinting of the *E. coli* O157:H7 isolates by PulseNet laboratories in that region revealed two simultaneous clusters (32 isolates in four of five states with one PFGE pattern and 25 isolates in all five states with a second pattern). One of the clusters could be traced to two supermarkets that received ground beef from the same distributor. Without molecular typing, epidemiologists would have found it difficult to identify cases associated with each cluster. On the other hand, the use of PFGE subtyping as part of routine surveillance has benefits beyond outbreak detection. For example, the temporal association of unrelated cases is not uncommon, and without molecular typing, valuable public health resources would be wasted investigating pseudo-outbreaks. Everything that was said above applies to the use of molecular methods for the identification of potential bioterrorism events.

There are several of caveats concerning the use of molecular typing methods. For example, the marker that is selected must be stable. The method chosen must have the ability to type the vast majority of isolates of a given species and must be able to discriminate between epidemiologically related and unrelated strains. The method must be reproducible between laboratories as well as give reproducible results using the same strain. Not all methods can be used for typing all of the different pathogenic microorganisms. While the greatest discrimination can be obtained through genome sequencing, its utility in a specific situation has to be weighed against its cost. Lastly, microorganisms can undergo genomic variation over time as a result of different storage and culture conditions. These differences can be used in an attempt to identify the source of an isolate as was recently done with *B. anthracis* (31).

Emerging Infectious Diseases

An Institute of Medicine Report (34) highlighted the observation that during the past three decades, scientists have identified a number of apparently “new” infectious diseases (e.g., Lyme disease) that affect more and more people every year. Selected examples of emerging infectious diseases are shown in Table 5. Scientists have also shown that a number of widely occurring diseases, whose exact cause had until recently remained a mystery, are probably the result of a microbial infection (e.g., peptic ulcer). Over the past several decades, the incidence of a number of known infectious diseases has increased, including some that were thought to be under control (e.g., tuberculosis). A number of factors have been identified that are responsible for the emergence or reemergence of infectious diseases. These include: global travel; globalization of the food supply and the centralized processing of food; population growth and increased urbanization and crowding; population movements; alterations in the habitats of disease-carrying insects and animals; behavior; increased use of antimicrobial agents; and the increased contact with reservoirs of infection.

Nucleic acid-based technologies have played an important role in the recognition of newly emerging infectious diseases. For example, in late 2002, cases of a life-threatening respiratory disease with no identifiable cause were reported from Guangdong Province, China. This was followed by additional reports of cases from Vietnam, Canada, and Hong Kong of a severe febrile respiratory illness that could be spread to household members and health acre workers. In March 2003, this syndrome was designated "severe acute respiratory syndrome" or SARS and global efforts to determine the cause of this illness and prevent its spread were begun. In the laboratory, inoculation of specimens into cell culture produced a cytopathic effect on the cell monolayers; electron microscopy of these cell cultures revealed the presence of coronavirus-like particles (35). This observation was the impetus for deciding to amplify a 405-bp segment of the coronavirus polymerase gene from the isolation material using RT-PCR. The amplicon was sequenced and shown to be highly similar to a similar region of the polymerase genes of group II coronaviruses. The identical nucleotide sequence was identified in 12 patients from several locations (35). The genome of the virus (SARS-CoV) was subsequently sequenced and shown to be distinct from all other coronaviruses (16). Once the sequence was available, unique regions were identified and used to develop a nucleic acid amplification test that was rapidly validated and disseminated to members of the LRN.

Summary and Conclusions

The microbiology laboratory through the development and use of nucleic acid-based assays and molecular typing methods has made significant contributions to the detection, response, and epidemiologic investigation of bioterrorism and other emerging infectious threats. Highly sensitive and specific nucleic acid-based assays have been developed and disseminated to a network of >120 laboratories in the U.S. and abroad, which can be used to identify threat agents as well as those responsible for emerging infectious diseases. Using newer technologies such as automated nucleic acid extraction and real-time PCR, many of these assays can provide a result in less than one hour. Genotypic methods have also been developed to "fingerprint" pathogenic microorganisms. This information is most useful when collected, analyzed, and integrated into the results of an epidemiological investigation. An example of how these methods have enhanced epidemiological investigations is PulseNet, a national molecular subtyping network for food-borne disease surveillance, which will also help identify food-related bioterrorism events.

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Table 1. Critical biological agents for public health preparedness (modified from reference 5)

Category A[a]

Variola major (smallpox)
Bacillus anthracis (anthrax)
Yersinia pestis (plague)
Clostridium botulinum neurotoxins (botulism)
Francisella tularensis (tularemia)
Filoviruses (e.g., Ebola and Marburg) and arenaviruses (e.g., Lassa and Junin) (hemorrhagic fever)

Category B[b]

Coxiella burnetti (Q-fever)
Brucella spp. (brucellosis)
Burkholderia mallei (glanders)
Alphaviruses (i.e., Venezuelan, Eastern, and Western equine encephalitis viruses) (encephalitis)
Ricin from *Ricinus communis* (ricin intoxication)
Episilon toxin of *Clostridium perfringens*
Staphylococcal enterotoxin B
Food and waterborne agents (e.g., *Salmonella* spp., *Shigella dysenteriae*, *Escherichia coli* O157:H7, *Vibrio cholerae*, *Cryptosporidium parvum*)

Category C[c]

Nipah virus
Hantaviruses
Tickborne hemorrhagic fever viruses
Tickborne encephalitis virus
Yellow fever virus
Multi-drug-resistant *Mycobacterium tuberculosis*

a. Category A includes many well-recognized biowarfare agents, which are likely to cause mass casualties and require broad-based public health preparedness.
b. Category B agents also have some potential for large-scale dissemination, but generally cause less-severe illness than those in Category A. Many of these agents have been or are being weaponized. Moreover, Category B agents could be used to contaminate food or water sources, and many of them are relatively easy to obtain.
c. Category C agents are those that are not currently believed to present a high bioterrorism risk to public health, but which could emerge as future threats. Some of these agents are associated with emerging infections or are those with characteristics that could be exploited for disease dissemination.

Table 2. Epidemiologic clues, which may signal a biologic attack (modified from reference 19)[a]

1. Single case of disease caused by an uncommon agent (e.g., glanders, smallpox, viral hemorrhagic fever, inhalation or cutaneous anthrax) without adequate epidemiologic explanation.
2. Unusual, atypical, genetically engineered, or antiquated strain of an agent (or antibiotic resistance pattern).
3. Higher morbidity and mortality in association with a common disease or syndrome or failure of such patients to respond to usual therapy.
4. Unusual disease presentation (e.g., inhalation anthrax or pneumonic plague)
5. Disease with an unusual geographic or seasonal distribution (e.g., plague in a non-endemic area, influenza during the summer months in the U.S.)
6. Stable endemic disease with an unexplained increase in incidence (e.g., tularemia, plague).
7. Atypical disease transmission through aerosols, food, or water, in a mode suggesting sabotage (i.e., no other possible explanation)
8. No illness in persons who are not exposed to common ventilation systems (have separate closed ventilation system) when illness is seen in persons in close proximity who have a common ventilation system.
9. Several unusual or unexplained diseases coexisting in the same patient without any other explanation.
10. Unusual illness that affects a large, disparate population (e.g., respiratory disease in a large heterogeneous population may suggest exposure to an inhaled pathogen)
11. Illness that is unusual (or atypical) for a given population or age group (e.g., outbreak of measles-like rash in adults)
12. Unusual pattern of death or illness among animals (which may be unexplained or attributed to an agent of bioterrorism) that precedes or accompanies illness or death in humans.
13. Unusual pattern of death or illness in humans that precedes or accompanies illness or death in animals (which may be unexplained or attributed to an agent of bioterrorism).
14. Ill persons who seek treatment at about the same time (point source with compressed epidemic curve).
15. Similar genetic type among agents isolated from temporally or spatially distinct sources.
16. Simultaneous clusters of similar illness in noncontiguous areas, domestic or foreign.
17. Large numbers of cases of unexplained diseases or deaths.

a. Clues are listed from “most specific” to “least specific” for bioterrorism.

Table 3. Phenotypic typing methods

Biotyping
Antibiogram typing
Serotyping
Phage typing
Bacteriocin typing
Multilocus enzyme electrophoresis

Table 4. Genotypic typing methods. From (26).

1st generation	Analysis of plasmid content Plasmid DNA restriction digests
2nd generation	Total chromosomal restriction digests Analysis of RFLPs by hybridization with probes -Ribotyping
3rd generation	Pulsed-field gel electrophoresis (PFGE) PCR-based amplification methods -RAPD -REP-PCR -AFLP -PCR-ribotyping
4th generation	Multilocus sequence typing (MLST) DNA sequencing

Table 5. Examples of emerging infectious diseases

Lyme disease (*Borellia burgdorferi*)
Toxic shock syndrome
Escherichia coli O157:H7
Multi-drug-resistant *Mycobacterium tuberculosis*
Bovine spongiform encephalopathy agent (BSE)
Legionnaires' disease *(Legionella pneumophila)*
Hantaviruses
Human immunodeficiency viruses (HIV-1, HIV-2)
Hepatitis C virus
Human papilloma virus
West Nile virus
SARS (SARS-CoV virus)