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# Forensic public health: epidemiological and microbiological investigations for biosecurity

*Ali S. Khan<sup>1</sup>, Philip S. Amara<sup>1</sup>, Stephen A. Morse<sup>2</sup>*

<sup>1</sup>College of Public Health, University of Nebraska Medical Center, Omaha, NE, United States;

<sup>2</sup>IHRC, Inc., Atlanta, GA, United States

## Introduction

Epidemiology is the study of how disease and injury is distributed in populations and of the factors that influence this distribution (Gordis, 1996). More broadly, it is the study of the distribution and determinants of health-related states or events in specified populations and the application of the results of this study to control health problems (Last, 1988). Epidemiology is based on the premise that disease, illness, and ill health are not distributed randomly in a population, and that individuals have certain characteristics (e.g., genetic, behavioral, social) that interact with the environment and predispose to, or protect against, a variety of different diseases. The specific objectives of epidemiology (Gordis, 1996) are to (i) determine the extent of disease present in the community; (ii) identify the etiology or cause of a disease and the factors that increase a person's risk for disease; (iii) study the natural history and prognosis of disease; (iv) evaluate new preventive and

therapeutic measures and new modes of health-care delivery; and (v) provide a foundation for developing public policy and regulations.

The field of microbial forensics emerged following the anthrax attack in the United States in 2001 to extend these epidemiologic principles to aid in the investigation of this and other bioterrorism incidents. Microbial forensics combines epidemiology with genomic and microbiologic methods, to identify, characterize, and ascribe the cause of an incident resulting from the intentional or unintentional release of a harmful pathogen (Rasko et al., 2011). Unlike routine epidemiologic investigations, microbial forensic investigations are undertaken when there is a potential crime due to the aforementioned release of a pathogen with disease-causing potential. The investigation is conducted to attribute cause to a source based on indisputable evidence and is used to support criminal charges against the perpetrator(s) (Sjödin et al., 2013). However, because bioterrorism may be unannounced, the initial

investigation will start the same as to any public health incident of concern.

This chapter discusses how epidemiology integrated with laboratory science can be used to identify the source of diseases caused by microorganisms or toxins—especially for attribution purposes.

### Dynamics of disease transmission

Disease has been classically described as the result of an epidemiological triad, where disease results from the interaction between a human host, an infectious agent or toxin, and the environment that promotes the exposure (Gordis, 1996). In some instances, an animal or an arthropod vector such as a mosquito or tick is involved in the maintenance or transmission of the pathogen. Among the assumptions necessary for this interaction to take place is that there is a susceptible host. The susceptibility of the host is influenced by a variety of factors, including genetic, nutritional, and immunological factors. Bacteria, viruses, prions, fungi, and parasites responsible for disease can be transmitted either directly or indirectly (Table 8.1). Different organisms spread in different ways, and the potential of a given organism to spread and produce outbreaks depends on the characteristics of the organism and the route by which it is transmitted from person to person.

Diseases can be defined as endemic, epidemic, and pandemic. The usual or expected level of a disease is determined through ongoing surveillance. *Endemic* can be defined as either the habitual presence of a disease within a given geographical area or as the usual occurrence of a given disease within such an area. *Epidemic* can be defined as the occurrence of a disease in a community or region, clearly in excess of what is normally expected, and generally derived from a common source or from a propagated source. *Epidemic* and *outbreak* are interchangeable linguistic choices used differentially to imply degrees of severity or concern. A cluster also implies an apparent excess of cases that may or may not be normal pending an epidemiological investigation or a circumscribed excess of cases when the expected number is near zero. *Pandemic* refers to a worldwide epidemic—often involving two or more continents—and usually infecting numerous people. The excess incidence of cases or their widespread distribution is not synonymous with severity. Many factors contribute to the emergence of infectious diseases, including human susceptibility to infection, international travel and trade, microbial adaptation and change, changing ecosystems, and intent to harm (Smolinski et al., 2003). The ability to exploit newly created biological conditions is both the hallmark and the challenge of emerging infections (Institute of Medicine, 1994). Using several

TABLE 8.1 Modes of agent transmission.

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Horizontal (transmission from one individual to another in the same generation)

Direct transmission

- Direct contact (touching, biting, sexual intercourse, etc.)
- Direct projections (large droplet spread, e.g., coughs to mucous membranes)
- Direct exposure (animals, soils)

Indirect transmission

- Vehicle borne (fomites, blood transfusion)
- Vector borne (mechanical or biological propagation)
- Airborne (droplet nuclei or dust)

Vertical (transmission from mother to offspring)

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*Modified from Gordis, L., 1996. Epidemiology, WB Saunders Co., Philadelphia, PA.*

different strategies and mechanisms, microorganisms are very efficient at infecting humans. These are exemplified both by the various strategies employed by the pathogen to survive before infecting a host, such as spore formation or survival in drought-resistant mosquito eggs, and by the various modes of transmission, such as direct contact (including large droplets) or indirect contact with fomites, or by insect vectors, and airborne via small particle droplets (Heymann, 2008). Natural outbreaks, however, have highlighted the true diversity in the abilities of microorganisms to infect humans and animals: multistate outbreaks of *Salmonella* linked to contaminated spring pasta salad (Centers for Disease Control, 2018) and to ice cream made from milk contaminated in a tanker that had previously contained raw eggs (Hennessy et al., 1996), legionellosis associated with grocery store misters (Mahoney et al., 1992), and pneumonic tularemia on Martha's Vineyard from mowing over a rabbit (Feldman et al., 2001). These few examples are a semblance of the seemingly endless list of novel ways that pathogens are spread.

Changes in technology can influence pathogen dynamics through the creation of new environments in which microbes thrive (e.g., legionellosis, a disease that emerged from the water distribution systems of large buildings including cooling towers). Climate change and human alteration of the ecosystem may contribute to the redistribution of pathogens or disease-causing vectors by impacting their life cycles, distribution, transmission, and survival (Wu et al., 2015). Pathogens have the ability to rapidly alter their genetic make-up, evolve, and develop new strains including antibiotic resistant strains. The influenza virus can vary the spikes or proteins on its outer envelope resulting in the emergence of new strains that can cause disease even in vaccinated individuals (Mostafa et al., 2016).

The outbreak of Ebola virus disease (EVD) in West Africa in 2014 demonstrated the impact of

global travel on the spread of infectious disease and the need to adopt a coordinated approach to the threat they pose. The first confirmed case (i.e., index case) of EVD diagnosed in the United States was a man who had traveled from West Africa to Dallas in September 2014. The index case died and two healthcare workers tested positive although both of them later recovered (<https://www.cdc.gov/vhf/ebola/history/2014-2016-outbreak/index.html>). Zika virus was first detected in rhesus macaques in 1947 in Africa. By 2007, it had reached the Pacific Ocean and by 2015 the virus spread to South America resulting in an outbreak in Brazil. Some cases of Zika virus were acquired in the United States in 2016. A yellow fever outbreak in Angola in December 2015 spread to the Democratic Republic of Congo, Uganda, Kenya, Ethiopia, and China. The first outbreak of chikungunya virus (endemic to Africa and Asia) was reported in Italy in 2007, followed by outbreaks in 2010 and 2014 in Southern France. In addition to travel-related cases, locally transmitted cases of the chikungunya virus were reported in Florida, Puerto Rico, American Samoa, and the U.S. Virgin Islands in 2014. Chikungunya virus became a nationally notifiable disease in the United States in 2015 (Rathore et al., 2017).

Many of the biological threat agents are also considered to be reemerging or emerging infectious pathogens. Viral hemorrhagic fever viruses are considered high-priority threat agents and are a concern as an emerging disease, as illustrated in the West African Ebola outbreak in 2014. Studies have shown that most human viruses are zoonotic pathogens. Of 586 mammalian viruses, 263 have been detected in humans of which 71.5% are zoonotic, i.e., detected at least once in humans and at least once in another mammalian species (Olival et al., 2017). For early detection and recognition of emerging infections, it is critical that proper epidemiologic investigations are integrated with laboratory surveillance (Feldman et al., 2001).

## Outbreak investigation

The occurrence of a disease at more than an endemic level may stimulate an investigation during which investigators may ask three questions (Gordis, 1996): *Who* has the disease? The answer to this question will help identify those characteristics of the human host that are closely related to disease risk (Last, 1988). *When* did the disease occur? Some diseases occur with a certain periodicity. This question is also addressed by examining trends of disease incidence over time (Rasko et al., 2011). *Where* did the cases arise? Answers to the previous questions lead to determining the *how* and *why* of an outbreak. As stated previously, disease is not distributed randomly in persons, time, and place. These questions are central to virtually all outbreak investigations. Investigation of an outbreak may be primarily deductive (i.e., reasoning from premises or propositions proved antecedently), inductive (i.e., reasoning from facts to a general conclusion), or a combination of both. Important considerations in the investigation of acute outbreaks of infectious disease include determining that an outbreak has in fact occurred; defining the population at risk; determining the method of spread and reservoir; and characterizing the agent. Steps used commonly for investigating an outbreak are shown in Table 8.2.

## Deliberate introduction of a biological agent

Deliberate dissemination of a biological agent via several different routes, including air, water, food, and infected vectors, presents the latest challenge to global public health security. The deliberate nature of such dissemination may be obvious, as in the case of multiple mailed letters containing spores of *Bacillus anthracis*. However, some forms of bioterrorism may be more covert,

for example, the deliberate contamination of salad bars with *Salmonella typhimurium* in The Dalles, Oregon, in 1984, by a religious cult to test their ability to incapacitate the local population before an upcoming election (Torok et al., 1997). This outbreak, which sickened more than 750 persons, was specifically excluded as bioterrorism during the initial investigation and was only recognized as such following a tip from an informant (Torok et al., 1997; Carus et al., 2002). Given the natural ability of infectious agents to emerge, the Oregon outbreak serves to highlight difficulties in determining a characteristic signature for an infectious disease outbreak resulting from covert but intentional introduction.

These difficulties in identifying a covert dissemination of a biological agent serve as a caution for public health practitioners, because in the aforementioned investigation of a food-borne outbreak, there was a very unusual pattern with a rare strain of *S. typhimurium* (Torok et al., 1997). Although the possibility of intentional contamination was considered early in the investigation, it was specifically excluded for the following reasons: (i) such an event had never been reported previously; (ii) no one claimed responsibility; (iii) no disgruntled employee was identified; (iv) no motive was apparent; (v) the epidemic curve suggested multiple exposures, which was presumed to be unlikely behavior for a saboteur; (vi) law enforcement officials failed to establish a recognizable pattern of unusual behavior; (vii) a few employees had onset of illness before the patrons, suggesting a possible inside source of infection; (viii) the outbreak was biologically plausible—even if highly unlikely; and (ix) it is not unusual to be unable to find a source in even highly investigated outbreaks. Although one of the initial reasons to exclude terrorism (i.e., no prior incidents) is no longer applicable, based on similar actions since 1984, determining if an unusual outbreak is biologically plausible will remain a challenge. In this context, it is

**TABLE 8.2** Commonly used steps in investigation of infectious disease outbreak.

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Step 1. Verify the diagnosis
Step 2. Establish a case definition (person, place, and time)
Step 3. Identify cases
Step 4. Verify you have an epidemic (descriptive epidemiology)
Time: Look for temporal clustering and time–place interactions
Place: Look for geographic clustering
Person: Examine the risk in subgroups of affected population according to personal characteristics: sex, age, residence, occupation, social groups, etc.
Look for combination (interactions) of relevant variables
Step 5. Develop hypotheses based on the following:
Existing knowledge (if any) of the disease
Analogy to diseases of known etiology
Step 6. Test hypotheses
Further analyze existing data (e.g., case–control studies)
Collect additional data, environmental samples, animal/vectors
Step 7. Recommend and implement control and prevention measures
Control of present outbreak
Prevention of future similar outbreaks
Step 8. Communicate findings

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important to remember that the first case of inhalation anthrax identified in Florida in 2001 was initially thought to be natural exposure. It is clear from the two documented cases of bioterrorism in the United States—the 1984 Oregon *Salmonella* outbreak and the 2001 anthrax attack—that a terrorist will not necessarily announce his/her intentions or take credit for such an attack (Torok et al., 1997; Jernigan et al., 2001).

Research with highly transmissible and virulent pathogens has come under increasing scrutiny due to concerns about biosafety and biosecurity. Discomposure about the potential for accidental or deliberate escape of pathogens with lethal or pandemic potential from at least one of the several laboratories engaged in research with such agents is not unwarranted.

For example, in 2014, CDC reported two incidents at its main campus in Atlanta, Georgia: (i) the unintentional release of potentially viable anthrax spores and (ii) the potential exposure of one of its lab staff to noninactivated Ebola virus (Centers for Disease Control and Prevention, 2014). Another incident was reported in 2015 when a private company that received regular shipments of specimens from the Department of Defense (DoD) notified the CDC that supposedly “inactivated” *B. anthracis* spores in its possession were still viable. CDC investigation revealed that the samples came from a DoD facility in Utah. Furthermore, investigators found that over the past decade, 86 facilities in the United States and 7 other countries had received samples of “inactivated” *B. anthracis*

spores that also contained low numbers of viable spores from the same facility (Department of Defense, 2015). Although none of these incidents were a threat to public health, it made sense to worry that some accidental releases could pose a significant threat especially since there was precedent in the accidental release of variola virus, SARS coronavirus, and the 1977 Influenza A/H1N1. Based on an assessment of historical data on lab accidents, Klotz and Sylvester (2012) estimated that the probability of an accidental laboratory release of a pathogen with pandemic potential was 0.3% per laboratory per year. With approximately 42 laboratories worldwide working with pathogens such as SARS and H5N1 bird flu, they calculated that there was an 80% likelihood of at least one accidental release occurring in one of these labs over a 13-year period (Klotz and Sylvester, 2012).

Advancements in molecular biology make it possible to genetically modify, edit, or disrupt the genome of pathogens. Gene editing may result in a loss of function through knock-out, a change of function through gene replacement, or a gain of function through knock-in techniques (Zhang et al., 2017). Genome editing has important therapeutic benefits and holds enormous potential for improving public health (Naldini, 2015). For example, gain-of-function research (i.e., research intended to increase the transmissibility and/or virulence of pathogens) can actually improve our understanding of how pathogens interact with their hosts, help us assess the potential of pathogens to cause pandemics, and aid in the development of medical countermeasures and public health preparedness (Selgelid, 2016). The CPISPR Cas9 gene editing tool has been successfully used to create a gene driver—a genetic system use to hijack a population through the propagation of a gene through multiple generations—to control the spread of malaria that, in time, could be used to edit the DNA of any living organism (Selgelid, 2016). Nevertheless, the publication of the results of a successful attempt by researchers to genetically

modify influenza A/H5N1 virus so that it was transmissible by the airborne route in ferrets raised serious biosecurity concerns. By demonstrating that avian A/H5N1 influenza virus could be transmitted by the airborne route between mammals, the researchers highlighted its pandemic potential for humans (Herfst et al., 2012). Critics questioned the potential benefits of the results when compared with the biosafety and potential dual-use risks. Concerns have been raised not only over the potential misuse of the results of gain-of-function research but also on research in human germline editing and gene drives and the potential for misuse of emerging gene editing technologies. The risk of intentional or unintentional release of a gene drive—modified organism during research and development, transfer between labs, or due to inadequate containment procedures is small but not zero. Newly developed gene editing tools such as zinc-finger nucleases, transcription activator-like effector nucleases, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas) systems are publicly available (DiEuliis and Giordano, 2018; Maeder and Gersbach, 2016). The most popular and widely used of these tools are the CPISPR systems (CRISPR-Cas9, CRISPR-Cpf1, and CRISPR-C2c1) (Zhang et al., 2017). CRISPR tools for editing prokaryotic and eukaryotic genomes are readily available online at an affordable cost, including user friendly instructions (Sneed, 2017). In the United States, biohacker boot camps teach basic gene editing skills, and interest in gene editing is growing. Just recently, it was demonstrated that mail-order DNA could be used to create horse pox virus de novo (Kupferschmidt, 2017; Noyce et al., 2018). Mass casualties could occur if modified strains of A/H5N1 influenza virus with increased virulence and human-to-human transmissibility are produced, aerosolized, and intentionally released.

Without ignoring the current limitations of gene editing technology for nefarious purposes, a 2016 US Intelligence Community Assessment

stated that genome editing research on pathogens with pandemic potential may pose a national security risk if not regulated. The Director of National Intelligence in testimony to the US Congress in February 2016 warned that “Given the broad distribution, low cost, and accelerated pace of development of this dual-use technology, its deliberate or unintentional misuse might lead to far-reaching economic and national security implications” (Clapper, 2016). The European Academies Science Advisory Council working group on gene editing acknowledged the potential for misuse but recommended regulating specific applications rather than gene editing itself as a new technology (EASAC, 2017).

Fortunately, a number of epidemiological clues, alone or in combination, may suggest that an outbreak is deliberate. Divining motives behind an attack should be abandoned as a public health tool to assess whether an outbreak is natural or deliberate in nature. It is essential to make this determination not only from a law enforcement standpoint to prevent future such actions but to protect the public health. There is a very short “window of opportunity” in which to implement postexposure prophylaxis for many of the agents likely to be used for bioterrorism (Khan et al., 2000). Therefore, it is critical that all outbreaks be rapidly investigated and assessed for whether they are of deliberate origin.

A set of epidemiological clues (Table 8.3) has been proposed by the CDC in collaboration with the Federal Bureau of Investigation (Treadwell et al., 2003). They are based on distinctive epidemiological and laboratory clues of varying specificity to evaluate whether an outbreak may be of deliberate origin. The clues focus on aberrations in the typical characterization of an outbreak by person, place, and time in addition to consideration of the causative agent. Some of the clues, such as a community-acquired case of smallpox, are quite specific for bioterrorism, whereas others, such as a similar genetic type 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. Although these clues are an important set of criteria to help evaluate outbreaks, no list will replace sound epidemiology to assess an outbreak.

It is important to note that forensic investigations are conducted in the context of a rapid and thorough epidemiological investigation. Not surprisingly, ongoing surveillance to identify increases in disease incidence is both the first step and the cornerstone of bioterrorism epidemiology. Most of the clues described in Table 8.3 simply suggest an unusual cluster of cases. They have been reorganized by specificity to trigger increasingly broader investigations by state and federal public health officials and to alert law enforcement authorities (Tables 8.4 and 8.5). However, even the most specific of clues may signal a new natural disease outbreak. An epidemiological investigation should consider all potential sources and routes of both natural and potential deliberate exposure. For example, the community outbreak of individuals with smallpox-like lesions in the Midwest in 2003 may, on first blush, have indicated the deliberate release of smallpox virus. However, a thorough integrated epidemiological and laboratory investigation identified the disease as monkeypox, an exotic disease in the United States, which in itself could suggest bioterrorism (Centers for Disease Control, 2003). Instead, affected individuals were sickened by infected prairie dogs purchased as pets, which had acquired their infection while cohoused with infected giant Gambian rats that had been imported from Ghana, and not from deliberate dissemination. In 2005, four US soldiers acquired hemorrhagic fever with renal syndrome in the Republic of South Korea near the demilitarized zone (Pasteur et al., 1998). Despite initial suspicions of deliberate infection, epidemiological and laboratory analysis ultimately linked exposure to rodent hosts at training sites visited by the soldiers (Pasteur et al., 1998).



**TABLE 8.3** Epidemiological clues that may signal a biological or chemical terrorist attack.

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 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 nonendemic area, influenza in the summer)
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 physical explanation)
8. No illness in persons who are not exposed to common ventilation systems (have separate closed ventilation systems) 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 or chemical agent)
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 or unexplained diseases or deaths

*Modified from Treadwell, T.A., Koo, D., Kuker, K., Khan, A.S., 2003. Epidemiologic clues to bioterrorism. Public Health Rep. 118, 92–98.*

**TABLE 8.4** Recommendations for level of public health involvement for investigation of potential biologic or chemical terrorism.

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#### Initial investigation at local level

- a. Higher morbidity and mortality than expected, associated with a common disease or syndrome
- b. Disease with an unusual geographic or seasonal distribution
- c. Multiple unusual or unexplained disease entities coexisting in the same patient
- d. Unusual illness in a population (e.g., renal disease in a large population, which may be suggestive of toxic exposure to an agent such as mercury)
- e. Ill persons seeking treatment at about the same time
- f. Illness in persons suggesting a common exposure (e.g., same office building, meal, sporting event, or social event)

#### Continued investigation with involvement of state health department and/or Centers for disease control and prevention

- a. At least a single, definitively diagnosed case(s) with one of the following:
    - Uncommon agent or disease
    - Illness due to genetically altered organism
  - b. Unusual, atypical, or antiquated strain of agent
  - c. Disease with unusual geographic, seasonal, or “typical patient” distribution
  - d. Endemic disease with unexplained increase in incidence
  - e. No illness in persons not exposed to common ventilation systems
  - f. Simultaneous clusters of similar illness in noncontiguous areas, domestic or foreign
  - g. Cluster of patients with similar genetic type among agents isolated from temporally or spatially distinct sources
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*Modified from Treadwell, T.A., Koo, D., Kuker, K., Khan, A.S., 2003. Epidemiologic clues to bioterrorism. Public Health Rep. 118, 92–98.*

**TABLE 8.5** Considerations for notifying law enforcement of possible biologic or chemical terrorism initial investigation at local level.

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Immediate notification of the FBI when

- a. Notification is received from individual or group that a terrorist attack has occurred or will occur
- b. A potential dispersal/delivery device such as munition or sprayer or questionable material is found

Notification of the FBI as soon as possible after investigation confirms the following:

- a. Illness due to unexplained aerosol, vector, food, or water transmission
- b. At least a single, definitively diagnosed case(s) with one of the following:
  - Uncommon agent or disease occurring in a person with no other explanation
  - Illness due to a genetically altered organism

Notification of FBI after investigation confirms the following (with no plausible natural explanation):

- a. Disease with an unusual geographic, seasonal, or “typical patient” distribution
  - b. Unusual, atypical, or antiquated strain of agent
  - c. Simultaneous clusters of similar illness in noncontiguous areas, domestic or foreign
  - d. Clusters of patients presenting with similar genetic type among agents isolated from temporally or spatially distinct sources
  - e. Infection due to novel vehicle or mode of transmission
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*Modified from Treadwell, T.A., Koo, D., Kuker, K., Khan, A.S., 2003. Epidemiologic clues to bioterrorism. Public Health Rep. 118, 92–98.*

Similarly, the 2007 death of a wildlife biologist working for the National Park Service from *Yersinia pestis* required a thorough epidemiological investigation. The wildlife biologist was found deceased at his home by colleagues and a subsequent postmortem determined cause of death as primary pneumonic plague (Wong et al., 2009). Epidemiological, ecological, and laboratory investigations concluded the biologist’s source of exposure to *Y. pestis* was most likely during a necropsy that he performed on a mountain lion before his death (Wong et al., 2009). Concerns regarding the potential deliberate use of biological agents such as *Y. pestis* and the presence of emerging infections will continue to complicate efforts to distinguish between naturally occurring disease and disease resulting from deliberate release of a biological agent.

### Molecular strain typing

The microbiology laboratory has made significant contributions to the epidemiology of infectious diseases. Repeated isolation of a specific microorganism from patients with a

given disease or syndrome has helped prove infectious etiologies. In addition, isolation and identification of microorganisms from animals, vectors, and environmental sources have been invaluable in identifying reservoirs and verifying modes of transmission. In dealing with an infection, it is often necessary to identify the infecting microorganism and determine its antimicrobial susceptibilities 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 epidemiological questions. Historically, the typing methods that have been used in epidemiological investigations fall into two broad categories: *phenotypic* and *genotypic*. Phenotypic methods are those methods that characterize the products of expressed genes 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 tool for epidemiological investigations in the 1960s and early 1970s, while phage typing of bacteria and serological typing of bacteria and viruses have been used for decades. Today, the majority of these tests are considered inadequate for epidemiological 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 viruses, such as human cytomegalovirus and measles virus, cannot be divided into different types or subtypes by serology because significant antigenic differences do not exist. Second, the expression of many genes is affected by spontaneous mutations, by 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 tend to vary and, for the most part, have been replaced by genotypic methods. The one major exception is multilocus enzyme electrophoresis (MLEE) (Pasteur et al., 1998; Richardson et al., 1986), which is a robust phenotypic method that performs comparably with many of the most effective DNA-based methods (Pujol et al., 1997; Tibayrenc et al., 1993).

Characteristics of selected phenotypic methods are presented in Table 8.6. These methods have been characterized by *typeability*, the ability of the technique to assign an unambiguous result (i.e., type) to each isolate; *reproducibility*, the ability of a method to yield the same results on repeat testing of a bacterial strain; *discriminatory power*, the ability of the method to differentiate among epidemiologically unrelated isolates; *ease of interpretation*, the effort and experience required to obtain useful, reliable typing information using a particular method; and *ease of performance*, which reflects the cost of specialized reagents and equipment, technical complexity of the method, and the effort required to learn and implement the method.

Extremely sensitive and specific molecular techniques have recently been developed to facilitate epidemiological studies. Our ability to use these molecular techniques (genotypic methods or proteomic methods for prions) to detect and characterize the genetic variability of infectious agents (bacteria, fungi, protozoa, viruses) is the foundation for most molecular epidemiological studies. The application of appropriate molecular techniques has been an aid in the surveillance of infectious agents and in determining sources of infection. The ability to link isolates to sources has direct implications for investigating both

TABLE 8.6 Characteristics of phenotypic typing methods.

Typing system	Proportion of strains typeable	Reproducibility	Discriminatory power	Ease of interpretation	Ease of performance
Biotyping	All	Poor	Poor	Moderate	Easy
Antimicrobial susceptibility patterns	All	Good	Poor	Easy	Easy
Serotyping	Most	Good	Fair	Moderate	Moderate
Bacteriophage or pyocin typing	Some	Good	Fair	Difficult	Difficult
MLEE <sup>a</sup>	All	Excellent	Excellent	Moderate	Moderate

<sup>a</sup> Multilocus enzyme electrophoresis.

Modified from Tenover, F.C., Arbeit, R.D., Goering, R.V., 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. *Infect. Control Hosp. Epidemiol.* 18, 426–439.

TABLE 8.7 Molecular characteristics of genetic diversity at different hierarchical level.

Function	Purpose	Regions of DNA
Discrimination above level of species	Taxonomy/evolution	Highly conserved coding regions (e.g., rDNA)
Discrimination between species	Taxonomy/diagnosis/epidemiology	Moderately conserved regions
Discrimination between intraspecific variants/strains	Population genetics	Variable regions
Discrimination between individual isolates/clonal lineages	“Fingerprinting”—tracking transmission of genotypes/identifying sources of infection and risk factors	Highly variable genetic markers that are not under selection by the host
Genetic markers/linking phenotype and genotype	Identifying phenotypic traits of clinical significance	Genotype linked to phenotype

Modified from Thompson, R.C.A., Constantine, C.C., Morgan, U.M., 1998. Overview and significance of molecular methods: what role for molecular epidemiology? *Parasitology* 117, S161–S175.

natural and deliberate outbreaks (Coleman et al., 2009). These molecular techniques can be used to study health and disease determinants in animal (including human) and in plant populations. Molecular techniques may also be applied to clinical and environmental samples. It requires choosing a molecular method(s) that can discriminate genetic variants at different hierarchical levels, coupled with the selection of a region of nucleic acid, which is appropriate to the questions being asked (Table 8.7).

Genotypic methods are those based on an analysis of the genetic structure of an organism. Over the past decade, several genotypic methods have been used to fingerprint pathogenic microorganisms (Table 8.8). The methods have been described in detail elsewhere (Tenover et al., 1997; Thompson et al., 1998; Soll et al., 2002; Pennington, 2002; Arens, 1999; Foley et al., 2009). In general, molecular typing methods can be divided into three general categories: restriction endonuclease-based methods, amplification-based methods, and sequence-based methods (Foley et al., 2009). Among these methods, restriction fragment-length polymorphism/pulsed-field gel electrophoresis (RFLP-PFGE) and RFLP + probe and ribotyping have been the most commonly used methods for

fingerprinting bacteria (Soll et al., 2002; Swaminathan et al., 2001). Random amplification of polymorphic DNA (RAPD) and karyotyping have been used for fingerprinting fungi (Soll et al., 2002; Soll, 2000). MLEE, RAPD, and polymerase chain reaction (PCR)-RFLP have been used for fingerprinting parasitic protozoa (Soll et al., 2002). Multilocus variable number tandem repeat analysis (MLVA) has been used to subtype *B. anthracis*, *Y. pestis*, and *Francisella tularensis*. MLVA schemes are now available for most bioterrorism agents (Van Belkum, 2007). Single-nucleotide polymorphisms (SNPs) have been used to analyze strains of *B. anthracis* and several gram-negative foodborne pathogens (Foley et al., 2009; Keim et al., 2004). An assay used for scoring SNPs of *B. anthracis* has been shown to have high-throughput capability and can be performed with small amounts of DNA (Keim et al., 2004). Select gene or complete genome characterization, as well as other molecular methods, has been used for viruses (Arens, 1999).

When should fingerprinting be used? Strain typing data are most effective when they are collected, analyzed, and integrated into the results of an epidemiological investigation. The epidemiologist must collaborate with the laboratory

TABLE 8.8 Examples of genotypic methods used in epidemiologic investigations.

## Restriction endonuclease–based methods

- a. Restriction fragment–length polymorphism without hybridization
  - Frequent cutter (4- to 6-bp recognition site) coupled with conventional electrophoresis to separate restriction fragments
  - Infrequent cutter (generally 6- to 8-bp recognition site) coupled with pulsed-field gel electrophoresis to separate restriction fragments
- b. RFLP with hybridization
  - Frequent cutter (4- to 6-bp recognition site) coupled with conventional electrophoresis to separate restriction fragments followed by Southern transfer to nylon membrane. Power and efficacy of typing method depend on the probe.
  - 16S and 23S rRNA (ribotyping)
  - Insertion sequences (e.g., IS6110 of *Mycobacterium tuberculosis*)

## Amplification-based methods

- a. Random amplification of polymorphic DNA analysis; arbitrarily primed PCR
- b. Amplified fragment–length polymorphism method
- c. Repetitive element method; variable number tandem repeat fingerprinting

## Sequence-based methods

- a. Multilocus sequence typing
- b. Electrophoretic karyotyping
- c. Single-nucleotide polymorphism analysis
- d. Whole-genome sequencing

scientist when investigating a potential outbreak of an infectious disease. Microbial fingerprinting should supplement, and not replace, a carefully conducted epidemiological investigation. In some cases, typing data can effectively rule out an outbreak and thus avoid the need for an extensive epidemiological investigation. In other cases, these data may reveal the presence of outbreaks caused by more than one strain. 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. Except for whole-genome sequencing (WGS), 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 in the isolates that appeared to give identical patterns but that were unrelated epidemiologically. This is unlikely to occur when a set of epidemiologically linked

isolates are analyzed (Tenover et al., 1997). For this reason, only WGS would provide unequivocal data required for attribution.

The power of molecular techniques in epidemiological investigations can be exemplified by a few examples. PulseNet, the national molecular subtyping network for food-borne disease surveillance, was established by the CDC and several state health departments in 1996 to facilitate subtyping bacterial food-borne pathogens for epidemiological purposes. Twenty-five 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. The PulseNet

network, which began with 10 laboratories typing a single pathogen (*Escherichia coli* O157:H7), achieved full national participation in 2001 and includes 83 food safety laboratories of the US Food and Drug Administration (FDA) and the US Department of Agriculture. Sister networks have also been established internationally (Swaminathan et al., 2001; Gerner-Smidt et al., 2006). Currently, PulseNet USA utilizes standardized PFGE protocols for six organisms with MVLA as a complementary technique: *E. coli* O157:H7, *Salmonella enterica*, *Shigella* spp., thermotolerant *Campylobacter* spp., *Clostridium perfringens*, and *Vibrio cholerae* (Gerner-Smidt et al., 2006). 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. Isolates are subtyped on a routine basis, and data are analyzed promptly at the local level. Clusters can often be detected locally that would not have been identified by traditional epidemiological methods alone. PFGE patterns are shared between participating laboratories electronically, which increases the ability to link apparently unrelated outbreaks and to identify a common vehicle (Centers for Disease Control, 1999). For example, in 2018, PulseNet was critical to facilitating the identification of an *S. Mbandaka* outbreak affecting 100 persons in 33 states in the United States (<https://www.cdc.gov/salmonella/mbandaka-06-18/index.html>). Starting in March 2018, CDC and other public health and regulatory officials linked geographically dispersed *S. Mbandaka* isolates from stool samples of symptomatic patients, which had the same PFGE pattern. The initial epidemiological investigation revealed that many of the patients reported eating cold cereal, and the vehicle was subsequently identified as contaminated Kellogg's Honey Smacks sweetened puff wheat cereal. Additionally, environmental and product samples obtained by the FDA at the contract production facility were positive for

the outbreak strain of *S. Mbandaka*. Without molecular typing, epidemiologists would have found it difficult to identify cases associated with each state cluster and assign attribution to the source. However, the use of PFGE subtyping as part of routine surveillance has benefits beyond outbreak detection. For example, the temporal clustering of unrelated cases is not uncommon, and without molecular typing, valuable public health resources would be wasted investigating pseudo or unrelated outbreaks. Molecular genotyping of food-borne pathogens continues to evolve. PulseNet has transitioned to the use of WGS for *Listeria monocytogenes* and is expanding WGS to other pathogens to improve the level of resolution. In the future, PulseNet will be evaluating metagenomic approaches and other strategies using next-generation sequencing (NGS) technology for direct characterization of patient samples as clinical practice embraces culture-free diagnostic methods. PulseNet remains a powerful tool that can be applied for the early detection of cluster(s) of illness that result from deliberate contamination of food (Gerner-Smidt et al., 2006).

Another example of the power of molecular techniques is the invaluable information provided during the 2001 anthrax attacks. MLVA was initially used to subtype isolates obtained from patients, environmental samples, and powders. Information from MLVA identified the subtype of *B. anthracis* and was able to link clinical cases to environmental samples and powders, thereby providing information on possible sources of exposure (Hoffmaster et al., 2002). Molecular subtyping also confirmed that clinical cases were caused by the same strain and that suspected cases outside the United States were not linked (Hoffmaster et al., 2002). Both forensic and epidemiological investigations can result in the collection of hundreds of clinical and environmental samples for testing. During this event, MLVA assisted with the identification of potential laboratory contamination of samples because of the large number of samples

requested to be tested (Hoffmaster et al., 2002). MLVA can be used to reliably and rapidly genotype an isolate within 8 h of receipt by the laboratory. Molecular subtyping identified the *B. anthracis* used in the 2001 attack as the Ames strain, a strain rarely found in nature (Keim et al., 2004). This information was a critical epidemiological factor in determining that these cases were most likely the result of a deliberate release (Keim et al., 2004). Additionally, WGS of isolates obtained from spores indicated that the genome and plasmid sequences were identical to those of an Ames strain stored at a US Army research facility (Fricke et al., 2009). The utility of molecular typing methodologies was clearly demonstrated in this forensic investigation involving the deliberate release of a biological agent in the United States.

In 2006, the CDC was notified of two cases of brucellosis in microbiologists who worked in clinical laboratories in Indiana and Minnesota (Centers for Disease Control, 2008). Because *Brucella* spp. are considered category B agents (Khan et al., 2000), infections with *Brucella* spp. should have a thorough epidemiological investigation to determine potential sources of exposure. MLVA was utilized to help identify the source of the *Brucella* infections. The CDC compared blood culture isolates from the two microbiologists with the isolates they handled in the laboratory. The epidemiological investigation revealed that the clinical isolate from the infected microbiologist in Indiana had been forwarded to the clinical laboratory in Minnesota; however, investigation also revealed that the second microbiologist did not handle this clinical isolate (Centers for Disease Control, 2008). Further epidemiological investigation determined that the Minnesota microbiologist had handled unknown isolates later determined to be *Brucella* spp. on an open bench. MLVA confirmed that the source of the Minnesota microbiologist's infection was one of the isolates handled on the open bench (Centers for Disease Control, 2008). The source of the Indiana

microbiologist's infection was an unidentified isolate from a referral laboratory that had requested identification of this specimen (Centers for Disease Control, 2008). Molecular genotyping provided critical confirmation of the source of exposure for these microbiologists and confirmed that these cases resulted from a laboratory exposure.

A radical shift in molecular strain typing occurred with the development of technology that allowed for millions of sequencing reactions to be conducted simultaneously on multiple mixed biological specimens. This advancement in sequencing has been termed NGS (Behjati and Tarpey, 2013). The ability to sequence the whole genome, screen mixed DNA samples at the same time, detect minor alleles very accurately, and identify causes of disease of unknown etiology has improved the value of DNA as evidence in forensic investigations. The throughput diagnostic capacity of NGS technology has the potential to increase the reach and the number of forensic investigations that can be conducted at low cost. NGS is already routinely applied in outbreak investigations to determine the potential source of outbreaks. For example, using DNA sequencing, it was determined that the Haitian cholera epidemic was associated with the introduction of a strain that was closely related to variant *V. cholerae* El Tor O1 strains that had been previously isolated in Bangladesh in 2002 and 2008 (Chin et al., 2011). The 2011 *E. coli* O104:H4 outbreak in Europe was epidemiologically linked to seed shipments from Egypt that were sent to Germany in 2009 (Grad et al., 2012). Sequencing capacity was established in Liberia during the 2014 Ebola outbreak to monitor the evolution of the virus during this outbreak (Kugelman et al., 2015). The technological trend to make portable devices is fueling innovation toward portable NGS devices that are field deployable without the limitations on size, weight, supportive infrastructure, complex sample processing procedures, or need for calibration of sequencing

machines by field engineers. For example, a pocket-sized, USB-powered sequencer (MinION) developed by Oxford Nanopore was successfully used to rapidly sequence Ebola virus at the field diagnostic laboratory in Liberia during the 2014 Ebola virus outbreak in West Africa (Hoenen et al., 2016). Advancements in the field of microfluidics also hold promise for the development of lab-on-a-chip systems with capacity to collect and analyze biological specimens on a miniature device.

Additional advances in molecular laboratory techniques have been used for the rapid detection of antimicrobial resistance. In one prospective study on methicillin-resistant *Staphylococcus aureus*, automated clonal alerts based on real-time subtyping were faster than traditional methods (Sintchenko and Gallego, 2009). At present, however, the direct identification of resistance genes by PCR or similar methods is of limited use because only a few resistance genes are strongly associated with phenotypic resistance (Jorgensen and Ferraro, 2009). PCR followed by electrospray ionization mass spectrometry has been used to detect quinolone resistance in *Acinetobacter* spp. (Hujer et al., 2009). However, this technique must be further evaluated, and limitations must be acknowledged, such as whether detection of a resistance gene indicates that a resistant phenotype is always present (Hujer et al., 2009). The ability to establish antimicrobial susceptibility patterns rapidly is particularly critical for providing the appropriate antimicrobial agents for treatment or postexposure prophylaxis in a situation where the deliberate dissemination of a potentially engineered drug-resistant organism is being considered. Because there are numerous mechanisms for antimicrobial resistance in bacteria, current phenotypic methods will likely continue to be the basis for laboratory determination of antimicrobial susceptibility patterns for the foreseeable future (Jorgensen and Ferraro, 2009).

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## Challenges

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Unfortunately, molecular genotyping information exists in multiple databases and in a variety of formats. Although PulseNet and other systems have web-based access, integration and sharing of data among multiple databases remains a challenge. As information and databases expand, data will also become more challenging to analyze. Therefore, there is a need to refine analytic methods including the use of artificial intelligence to improve pattern recognition and integration of multiple streams of epidemiologic and laboratory data so that outbreaks and bioterrorism events can be detected quickly. Informatics capacity at local, state, and federal level requires continued investment to maximize the integration of epidemiology and laboratory information.

Finally, the threat of bioterrorism has initiated the development of mechanisms to quickly identify the presence of biological agents in the environment to rapidly initiate public health and medical response efforts. Molecular technologies allow for the rapid identification of genetic material of biological agents from collection devices such as those used for outdoor and indoor air monitoring. Public health, forensic, and laboratory assessments must be made based on material collected in a distinct area covered by the monitor or sensor. Because these detectors or devices do not preserve the viability of the agent, the assessment cannot indicate that a live organism was released, that individuals were exposed, or that a deliberate release occurred. As a result, it is critical that information from public health and epidemiological investigations be considered when interpreting information from environmental monitors. Public health must consider the limits of these new technologies, previous history of environmental detection of a biological agent in each area, and environmental sampling



methods. As the recent Institute of Medicine report on “Effectiveness of National Bio-Surveillance Systems: Biowatch and the Public Health System” indicated, the challenge is “understanding the clinical context in which disease detection and reporting occurs and the factors that shape the decision-making process for the state and local public health officials who must interpret the data” from these systems as well as that from traditional public health surveillance systems ([Institute of Medicine and National Research Council of the National Academies, 2009](#)).

### Summary

With few exceptions, a careful epidemiological investigation will be required to determine whether an outbreak of infectious disease is due to the intentional (or unintentional) release of an agent or is naturally occurring. A number of molecular methods have been developed for subtyping microbes that complement the epidemiological investigation as well as identify related cases. For example, since the establishment of PulseNet, the routine use of molecular subtyping by PFGE has improved both the sensitivity and the specificity of epidemiological investigation of food-borne outbreaks at the state and local level ([Hedberg and Besser, 2006](#)) and MVLA was critical in identifying the origin of the anthrax attack. As current subtyping methodologies evolve, applications and uses in the public health response to deliberate releases of biologic agents must be considered and applied.

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