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CHAPTER 21

Use of Host Factors in Microbial Forensics

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INTRODUCTION AND BACKGROUND

Considerable advances have been made in the forensic analysis of microbes and toxins. These advances include sequencing, genomics, and microscopy. An underdeveloped and underutilized area in microbial forensics is how the host interacts with microorganisms in a way that provides unique signatures for forensic use. For investigative and forensic purposes, an immediate goal is to distinguish a potential victim and innocent person from a perpetrator and to distinguish between a naturally acquired or intentional infection. Two principal methods that are sufficiently developed are characterization of the humoral immune response and identification of vaccine-induced immunity or antibiotics that may be present in a possible perpetrator.

This chapter presents central elements of the host response in a simplified fashion and describes a few representative examples that, in the appropriate context, have a high potential of providing evidence that may aid an investigation to distinguish a perpetrator from a victim who has been exposed to a particular microorganism or by-product, such as a toxin. The chapter also presents general information about the immune system so that the interested reader can have a fuller understanding of the immune response in general.

The primary aims of a microbial forensics investigation are to identify the biological agent, its source, and the individuals responsible for the event (1). Analytic approaches will differ when the suspected biothreat agent is encountered in a container or the environment, as opposed to *in vivo* in a human, animal, or plant. Analyses of trace elements, pollens, growth media, latent fingerprints, and microbial and nonmicrobial nucleic acids are all applicable to the container and environmental sample (2). However, once the microorganism or its toxin is in the living host, it is no longer possible to analyze the preceding items except the microbial nucleic acid. However, the host's

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response to the biological agent may be available for analytic clues. This is akin to other forensic studies where physical traces of bite marks, scratches, wound trajectories, and sizes of wounds are often surrogate evidence of the teeth, fingernails, and bullets (3). While the forensic pathologist is familiar with evidence related to determining the manner of death, including the host response, those involved with health care alone are more familiar with the host response. In the context of microbial forensics it is important to integrate all of these with intelligence information so that they may be included in the analytical data and attribution picture.

The host response to a microorganism or other foreign substance is often a well-orchestrated series of events, which may protect the individual from harm or ameliorate its effects (4). At the same time, these host responses may provide clues as to the identity of the offending microorganism or toxin, as well as a rough chronology of when it occurred and for how long it has been persisting. Emerging technologies, such as transcriptional arrays and bioinformatic analysis, will eventually be refined and methods validated to provide even greater help in delineating more of the pathways and components of the host response to an infectious agent (5,6). Other technologies are sufficiently mature to be of use today. The immune system and its components are a mainstay of our protection against infections and malignancies (4,7). Inflammation is often a side effect as the immune system contains and eradicates a microorganism or eliminates foreign tissue. Specific arms of the immune system can be used as markers in support of or against the presence of an infection. The humoral or antibody response to an invading microorganism is one example of a specific immune response that can have forensic value. Some of the antibodies produced may have a protective role together with other parts of the immune system by eradicating the pathogen or neutralizing a toxin. Other antibodies may not be as effective in this role. However, by virtue of their ability to recognize unique and specific microbial antigens, they can serve as indicators that a specific microorganism was recently present or was present in the past. In the case of vaccine-induced immunity, antibodies may recognize highly specific epitopes of one microbe versus those of a related microbe (e.g., influenza virus). This is especially so with different recombinant vaccines and could have forensic importance. Substances such as antibiotics, which can kill a pathogen rapidly, may modify the immune response by removing or reducing the stimulus for a full-scale response. As noted earlier, in clinical and veterinary medicine, measurement of the immune response helps the diagnostician decide what infection was present and how recently. In these situations, the intent is to provide treatment. The forensic scientist may exploit parts of the immune response to discover who is likely a victim of an attack and who might be responsible. This chapter discusses the basics of the host immune response that can have forensic utility.

Examples will provide a sense of what information is obtainable and what is not likely to provide highly significant clues.

Understandably, health care providers are reluctant to compromise a patient's privacy and are normally mandated to guard this privacy by Health Insurance Portability and Accountability Act regulations (8,9). However certain circumstances may compel a health care provider to reveal private information about a patient (8). Nevertheless it is important to understand how valuable information may be in the possession of the treating physician and other members of the health care team. The physician and other health care providers may be among the first to realize that a patient is a victim of a biocrime. In case of a covert attack, it may be the physician or medical examiner who first recognizes the index case. These health care workers are in key positions to preserve critical evidence and thereby contribute to the investigation (10). A number of steps should be followed when the possibility of a biological attack arises, either with the consent of the patient or because individuals are compelled by law to interact with public health and law enforcement.

A joint statement by the Federal Bureau of Investigation (FBI), the Centers for Disease Control and Prevention (CDC), and the Department of Homeland Security (DHS) advises calling the FBI and public health authorities if a suspicious situation arises (11). Some guidelines on the procedure(s) to report of suspicions of biocrimes are provided by the CDC (http://www.cdc.gov), the FBI (http://www.fbi.gov), and the DHS (http://www.dhs.gov) and are detailed in a previous article (10).

GENERAL CONCEPTS

In response to a new exposure to a microbe, the innate immune system may be the first line of defense. Then, the immune system starts to generate a humoral immune response. Typically a phagocytic cell (i.e., macrophage) ingests and degrades some of the invading pathogens. It then presents part (antigens) of the microorganism to a helper T cell (a lymphocyte), which then directs other lymphocytes known as B cells to produce antibodies to those antigens of that particular microbe that were presented. It usually takes at least 4 days before any microbe-specific antibody can be detected (12).

Antibodies are a specific form of proteins known as "immunoglobulins" (Igs). IgM, IgG, IgA, and secretory IgA are principal classes of immunoglobulins with prime relevance to this chapter. In response to a new antigen, immunoglobulins usually appear in the order of IgM, IgG, and IgA. B cells first begin to produce IgM, and then some B cells undergo an irreversible switch to ones that produce IgG. Later some of these B cells undergo a switch to become IgA-secreting B cells. Immunoglobulins persist for varying times;

Table 21.1 Immunoglobulin Classes and Properties

Immunoglobulin Class	IgM	IgG	IgA	IgE	IgD
Size (kDa)	900	150	160	190	180
Serum half-life days	5	21–23	5–6	1–5	2–8
Placental transfer	No	Yes	No	No	No
Complement fixation	++	+	−	−	−
Percentage of serum immunoglobulin	13	80	6	0.002	0.2

for example, the half-life of particular IgM antibodies is approximately 5 days, while that of IgG can be as long as 21–23 days (Table 21.1) (7).

At times in ruling in or ruling out a suspect, even specific IgE may be of value in addition to the more universal IgG and IgM responses. Those individuals unfortunate to have allergies have problems due to IgE against allergens (such as ragweed, peanut, or cat dander). In this case the IgE molecules sit on the surface of mast cells and basophils. These cells can release histamine and other allergic mediators when the offending allergen bridges two IgE molecules.

Similar to the immune response to an infection with a live microbe, vaccines can also engender an antibody response. A vaccine can be a live or attenuated microbe, a whole nonproliferating microbe or an antigenic (recombinant) component of the microbe, or a toxoid. Vaccines may contain an adjuvant (e.g., alum) to stimulate the humoral response of the host. Regardless, the intent of immunization is to engender protection, often by the generation of protective neutralizing antibodies. Although the half-life of an individual IgG molecule is less than a month, a population of antibodies of the IgG isotype form may persist for life. Memory B cells can sustain these antibodies and retain the ability to respond quickly by generating the appropriate antibodies when challenged. When the immune system encounters another infection or is subjected to a revaccination (booster), the result is an accelerated production of the particular antibody and an increase in the levels of antibodies that circulate in the blood (Figure 21.1).

Perhaps the pattern of antibody response which has the most forensic value, by providing a time frame, is the appearance of IgM first, followed by a B-cell switch to the longer lasting IgG. During the early phase of exposure, IgM predominates, as time goes on, IgG may wax and wane and IgM is no longer found (Figure 21.2).

The antibody response to a particular agent may be directed to different antigens at different times, that is, early or later after the initial exposure. That

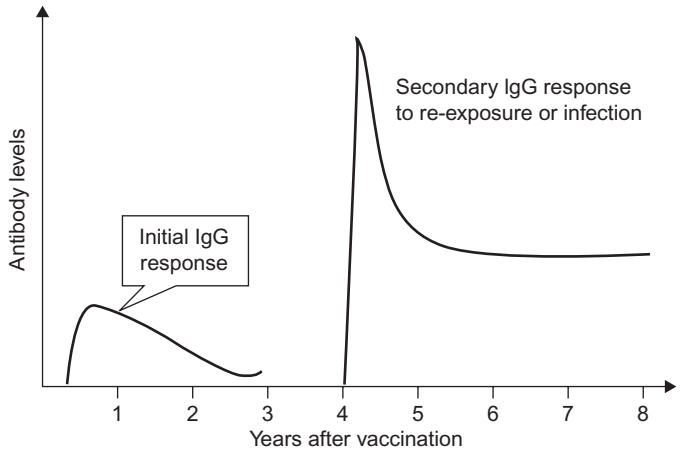

FIGURE 21.1

Illustration of IgG antibody response to a vaccine antigen after first immunization and subsequent exposure by natural exposure to the infectious agent or by another vaccination.

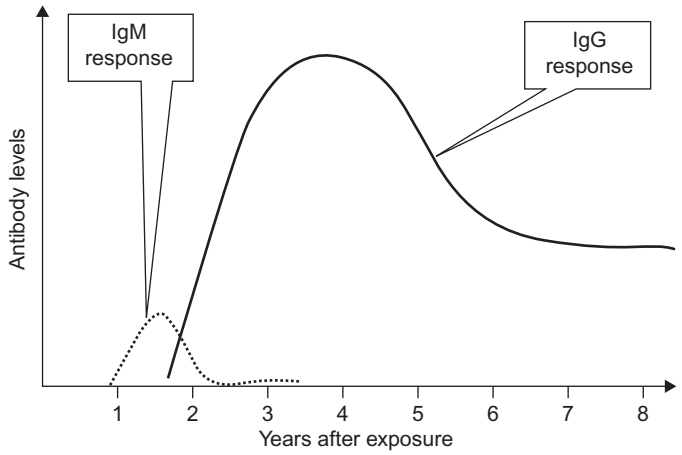

FIGURE 21.2

Illustration of temporal relation of IgM and IgG responses to an infection with IgM as the first and often transient response and IgG as the more sustained response.

response often involves IgM at the early stage and IgG later. Late in the course of the disease or during recovery, only IgG to particular antigens may be seen. A classic example of this is the human antibody response to Epstein-Barr virus (EBV) (13), a virus known to cause mononucleosis. During acute early disease, it is common to find high levels of IgM antibodies to the viral early antigen (EA) and viral capsid antigen (VCA). It is rare to find high levels of

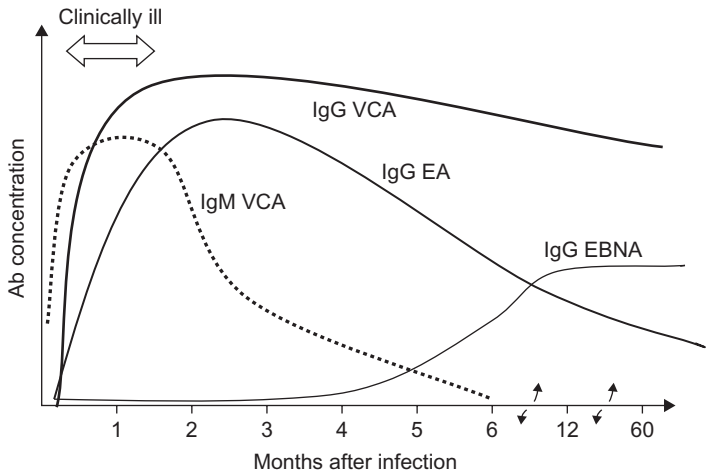

FIGURE 21.3

Schematic response of IgM and IgG to different antigens of EBV over an extended period of time.

IgG to the VCA or to Epstein-Barr nucleic acid (EBNA). As the patient recovers from his/her first infection with EBV, the immune response is characterized by low levels of IgM to EA or VCA, and higher or increasing levels of IgG to VCA. Antibodies to EBNA are often very low during this stage. Several months after clinical recovery, IgM to EA and VCA remain at low levels whereas IgG to VCA and EBNA are present at high levels, often for years. Table 21.3 illustrates this pattern by stage of the immune response to EBV and its particular antigens. Figure 21.3 is a graphic display of these antibody responses. For the clinician or epidemiologist, antibody responses provide a framework to determine where in the course of the infection a patient may be. Tables 21.2 and 21.3 and Figures 21.2 and 21.3 illustrate how responses to a biothreat agent or its toxin may be used to give some chronological indication of exposure. Combining the antibody response with detection of particular antigens can provide further definition as to a time frame of infection or exposure.

ILLUSTRATIVE CONCEPTS

A controlled experiment or normal clinical event illustrates what happens when the immune system sees an infectious agent or a vaccine for the second time. The controlled experiment may be in a laboratory animal or a patient receiving a booster vaccine. The uncontrolled but normal clinical event occurs when the patient is reexposed to the infectious agent. Consider a generic antigen exposure. The first time the immune system encounters antigen X (AgX) it responds as shown in Figures 21.1 and 21.2. Initially, antibodies to AgX are

Table 21.2 Antibody Tests for Epstein-Barr Virus

Stage	Titers
Acute primary infection	
IgM EA and VCA	High
IgG VCA and EBNA	Low
Recovering from primary infection	
IgM EA or VCA	Lower
IgG VCA	Rising
EBNA	Low
After several months	
IgM EA and VCA	Low or normal
IgG VCA and EBNA	Persist at high level for several years

Table 21.3 Antibody Response at Different Time Points to EBV Antigens

Disease Status	Heterophile Ab	VCA-IgM	VCA-IgG	EBNA	EA(D)
Healthy—unexposed	Negative	Negative	Negative	Negative	Negative
Very early infection	Possible	Possible	Possible	Negative	Negative
Active infection	Positive	Positive	Positive	Negative	Possible
Recent infection	Positive	Positive	Positive	Positive	Possible
Past infection	Negative	Negative	Positive	Positive	Possible

barely discernible; then levels rise and later fall to a plateau. If a simultaneous exposure were to occur with AgX and a new AgY from another microorganism, the immune system would quickly mount a brisk response with high levels of Ab to AgX, while the course of Ab to AgY would be slow and delayed, just as it was in the response to the first exposure to AgX. This phenomenon, termed "immunological memory" or an "amnestic response," can be useful when the symptoms and signs of exposure to either X or Y are similar. This is the type of response that can occur with the early flu-like symptoms of pulmonary anthrax (14–16) and with the influenza virus itself (17–19).

Another common example is the repetitive exposure to different strains of influenza virus (17–20). As illustrated in Table 21.4, a person infected for the first time with one strain of the influenza virus has a response to most of its antigens (as a theoretical example, Ag 1, 2, 3, 4, 5, 6). Three years later, the same individual exposed to a partially similar influenza virus responds preferentially

Table 21.4 Response to Theoretical Antigens from Different Flu Viruses at Time of Exposure (Weak vs. Strong)

Infecting Strain and Antigen Composition	Antibody Response: Weak	Antibody Response: Strong
Strain A year 1 (antigens 1, 2, 3, 4, 5, 6)	1, 2, 3, 4, 5, 6	—
Strain B year 5 (antigens 1, 3, 5, 7, 8, 9)	7, 8, 9	1, 3, 5
Strain C year 15 (antigens 1, 3, 8, 10, 12, 13)	10, 12, 13	1, 3, 8

to those antigens that were also present on the original influenza virus (secondary immune response). The person also has a primary antibody response to new antigens, that is, those not shared with the first virus. Ten or 20 years later, during a new flu season and exposure to a third strain of influenza, the most brisk responses would be to antigens previously recognized by the immune system. This is the scientific basis for giving the flu vaccine, which contains a variety of possible antigens common to multiple strains of the flu virus so that a rapid and protective antibody response will occur.

UTILITY OF SEROLOGIC ANALYSIS OF PEOPLE EXPOSED TO ANTHRAX: STRENGTHS AND LIMITATIONS

Our knowledge of the humoral response to infection with biothreat microbes is limited compared with our knowledge of the kinetics of the response to common human infections. Nevertheless, in the appropriate context and with sufficient background information, detection of antibodies to a particular microbe and its antigens can have important value for a microbial forensic investigation. It may have critical probative value or it can guide investigative leads. Absence of a specific antibody response may also have value in a particular investigation. Certainly its importance is increased in the context of information of what organism could be involved, when the exposure was likely to have occurred, the route of exposure, what symptoms and signs are manifesting in the host, and other laboratory data such as presence of antigens and microbial nucleic acids (21). Other information, such as how many hosts (people or animals) have had this infection in the geographic region, what is the incidence, and background prevalence of antibody to the organism in question or a related organism, in the population being studied, is also important.

Vaccination responses can have forensic value. The current anthrax based on protective antigen (PA) vaccine contains small amounts of lethal factor (LF) and edema factor (EF), which are responsible for some of the side effects, so

one might expect to see antibodies against these antigens as well as to PA. Recombinant PA is just PA so anti-LF and anti-EF would be absent in an immunized individual.

The 2001 anthrax letter attacks raised multiple questions for every person infected, potentially exposed, vaccinated, or treated. Some of these questions included how these persons were infected by spores, if at all; that is, through breaks in the skin (cutaneous anthrax), by inhalation of spores [pulmonary anthrax (22)], or by ingestion [gastrointestinal anthrax (23,24)]. Alternatively, were they among the "worried well"?

Consider the situation where a close associate comes down with symptoms compatible with inhalational anthrax after receiving a letter containing powder and that material is no longer available. Until this is shown not to be anthrax, great worry will ensue.

In several cases of documented exposure, there was not enough time for the patient to develop antibody to a specific *Bacillus anthracis* antigen, at least as probed for IgG. Serial serum samples obtained from potentially exposed individuals on November 16, 17, 18, and 19 of 2001 were tested for IgG antibody to the protective antigen (PA) component of the anthrax toxins by enzyme-linked immunosorbent assay (ELISA); all samples were negative. Serial tests for serum IgG antibody to the PA toxin of anthrax were performed on 436 workplace-exposed persons. All but one test was negative. Most of the specimens were collected on October 10 and 17 (25).

It is instructive to look at the positive antibody case in the context of the nature and duration of that individual's symptoms when he developed a positive test. Ernesto Blanco, a 73-year-old mailroom clerk (case 2) experienced fatigue on September 24. He worked in the mailroom of the AMI building and delivered mail to the index case. On September 28, he developed a nonproductive cough, intermittent fever, runny nose, and conjunctivitis. These signs worsened through October 1 when he was hospitalized. In addition, he had shortness of breath with exertion, sweats, mild abdominal pain and vomiting, and episodes of confusion. His temperature was elevated to 38.5°C (101.3°F), heart rate was rapid at 109/min, respiratory rate was slightly fast at 20/min, and blood pressure was 108/61 mm Hg. He had bilateral conjunctival injection and bilateral pulmonary rhonchi. At the time of admission, his neurological examination was normal. No skin lesions were observed. The only laboratory abnormalities were low albumin, elevated liver transaminases, borderline low serum sodium, increased creatinine, and low oxygen content in the blood. Blood cultures were negative on hospital day 2, after antibiotics had been started. The chest X-ray showed a left-sided pneumonia and a small left pleural effusion but no classic mediastinal widening (26). The patient was initially given intravenous azithromycin; cefotaxime and ciprofloxacin

were subsequently added. A nasal swab obtained on October 5 grew *B. anthracis* on culture. Computed tomography of the chest showed bilateral effusions and multilobar pulmonary consolidation but still no significant mediastinal lymphadenopathy. Pleural fluid aspiration was positive for *B. anthracis* DNA by polymerase chain reaction (PCR). Bacterial cultures of bronchial washings and pleural fluid were negative. Immunohistochemical staining of a transbronchial biopsy demonstrated the presence of *B. anthracis* capsule and cell-wall antigens. During hospitalization, his white blood count rose to 26,800/mm^3, and fluid from a second thoracentesis was positive for *B. anthracis* DNA by PCR. Immunohistochemical staining of both pleural fluid cells and pleural biopsy tissue demonstrated the presence of *B. anthracis* capsule and cell-wall antigens. Serial serum samples demonstrated >4-fold rise in serum IgG antibody to the PA component of the anthrax toxins by an ELISA assay. The patient was able to leave the hospital on October 23 and was on oral ciprofloxacin. Table 21.5 illustrates both the clinical and microbial forensic approach and the context in which to analyze such a patient. It is likely to be common to most situations where a biocrime is suspected to have potentially affected individuals. The first set of questions is directed toward whether the person is sick: does the person have any indications of not being well and is laboratory evidence indicative of an infection? The second set of questions asks whether there is any specific and objective laboratory evidence of a particular infection. A third set of questions arise if the cause of infection was an agent on the Select Agent list (27). These questions include (i) was the infection acquired naturally or was it an intentional action that led to the infection and (ii) how did the particular individual acquire it if it was not a natural

Table 21.5 Nonspecific and Specific Indications of a Case of Anthrax

Clinical Evidence of an Infection	Nonspecific Laboratory Evidence of an Infection	Specific Clinical Evidence of Infection with *B. anthracis*
Known exposure by proximity to area and infected person	Chest X-ray and computed tomography scan showing pneumonia and pleural fluid	Culture from nasal swab grew live *B. anthracis*
Cough, fever, shortness of breath	Elevated white blood cell count	Positive PCR for *B. anthracis* in pleural fluid on two occasions despite negative cultures
Sweats, abdominal pain, confusion		Positive immunochemical staining for *B. anthracis* capsule and cell wall antigens of transbronchial biopsy, pleural fluid cells, and pleural biopsy despite negative cultures
Abnormal breath sounds		Serum IgG to PA toxin component
Fast heart rate		Serum IgG titer to PA toxin increased within a short time period

infection, that is, was he the target or a bystander? An alternative possibility in the right circumstances is a laboratory-acquired infection.

This case also demonstrates that cultures may be negative at different times from different fluids and tissues because of early administration of antibiotics. However, remnants of the infection, even dead organisms, can be found by probing for antigens and DNA. This patient's response demonstrated a classic principle of infectious disease, a rising antibody titer over time. In this case it was IgG to a particular antigenic component of the anthrax toxins (28,29). The subject's antibody response may have been detected earlier if IgM to this component or to other antigens of anthrax had been sought. The case also points out the utility of integrating the detection of antibody with other indications of an anthrax infection, such as a positive culture, PCR, or antigen detection assay. These take on their greatest significance during clinical illness in someone who was possibly exposed.

Early administration of antibiotics can prevent or interfere with the isolation of a pathogen by culture (30). Of the first 10 pulmonary anthrax cases associated with the 2001 letter attacks, three patients had no isolate of *B. anthracis* from any clinical specimen; however, culture was attempted after initiation of antibiotic therapy. History of exposure in conjunction with compatible symptoms and signs of disease and objective laboratory findings were the basis for the diagnosis. *B. anthracis* was identified in pleural fluid, pleural biopsy, or transbronchial biopsy specimens by reactivity with *B. anthracis*-specific cell wall and capsular antibodies or by the detection of DNA in pleural fluid or blood by PCR (31).

An IgG-based ELISA for anti-PA illustrates the importance of understanding the limitations of an assay used in medicine or forensics (32,33). The ELISA for anti-PA was initially developed at the U.S. Army Medical Research Institute of Infectious Disease and put into operation after optimization and internal validation at the CDC (34) for functional sensitivity and specificity in detecting an antibody response to PA as a surrogate for *B. anthracis* infection. Its major limitations were that only one antigen was used and only IgG was measured. Therefore, a negative result shortly after exposure may, in effect, be a false-negative result. A gap such as this may be filled by development of an assay for antigen-specific IgM or by probing for other *B. anthracis* antigens or epitopes yet to be characterized.

The assay for anti-PA may be very useful in its present form to screen asymptomatic people for possible exposure. The study by Dewan and colleagues (26) provides a contemporary background database on a group of postal workers who may have been exposed to *B. anthracis*. Beginning on October 29, 2001, 1657 postal employees and others who had been to the Washington, DC, postal facility went to the D.C. General Hospital for antibiotics. Added to this number were those people whose treatment began on October 21,

2001. Serum samples were also obtained from the 202 individuals who had been to the Washington, DC, postal facility during the previous 2 weeks. All were negative for anti-PA IgG, including three individuals who reported a remote history of anthrax vaccination. The consistent negative findings may be explained by the fact that antibiotic therapy was initiated before serum was obtained for testing and that there were no baseline serum samples available for testing. Also, the time period from exposure to sampling was very short. Among 28 individuals in the Capitol region with culture-positive nasal swabs who received prophylactic antibiotics immediately, none had a positive culture from a nasal swab repeated 7 days later, and none developed IgG to PA 42 days after exposure. This again emphasizes the limitation and interpretation of a test in someone who had early antibiotic treatment. It does raise forensic utility considerations. Even with these easily disseminated spores, an antibody response may be aborted or modified with antibiotics by early treatment. Furthermore, antibiotics taken prior to exposure would likely be effective in preventing laboratory and clinical signs of an infection. Detection of microbial DNA, antigen, or the organism itself on a person's body, clothing, or possessions should be an indicator for exposure.

The route of infection is important in interpreting results and limitations of the assay used. The example of cutaneous anthrax in Paraguay illustrates this notion, as well as the need to search for other antigens as markers of exposure (35). In an analysis of an outbreak of 21 cases of cutaneous anthrax that followed contact with raw meat from a sick cow, sera from 12 cases and 16 colony and two nonbacterial colony controls were examined by Western blotting for antibodies to PA and LF 6 weeks after the outbreak. An ELISA was used to probe for antibodies to the poly-D-glutamic acid capsule. Of the 12 cases, 11 had antibody to PA, for a sensitivity of 91.7%; none of the 18 controls was positive. Only 6 of 12 cases had antibody to LF; all controls were negative. Anticapsule antibodies were positive in 11 of 12, but were also positive in 2 of 18 controls. Results of this study demonstrate the need to consider other antigens.

CONSIDERATIONS AND CONCERNS RAISED BY ANALYSIS OF OTHER INFECTIONS

Some of the principles discussed earlier are highlighted by a report on severe acute respiratory syndrome (SARS). Appearance of the coronavirus responsible for this disease evoked concern of a possible terrorist origin. A report in the *Morbidity and Mortality Weekly Report* [MMWR (36)] on the "Prevalence of IgG Antibody to SARS-Associated Coronavirus in Animal Traders" discussed the need to validate and interpret tests in appropriate populations. Also discussed was the inability to date the time of infection by the IgG assay and the possibility of assay cross-reactivity to a near neighbor that might be unknown. In a

Promed bulletin, Berger looked at the same data from a different perspective and reported:

> "This week's study in MMWR indicates that animal contact may indeed promote infection; however, the most striking finding seems to have eluded the authors: 1.2–2.9% of individuals in a healthy control group of adults were also found to be seropositive! The population of the Guangdong Province is 86.42 million (2001), of whom 61.14 million are adults over age 14. If we assume that the seropositivity rates among controls is representative of the province as a whole, 734,000 to 1,773,000 adults in Guangdong have at some time been infected by the SARS virus. These figures are 87- to 211-fold the total number (8422) of SARS patients reported worldwide to date!"

This comparison is a good illustration of the advantage of open dissemination and discussion of information, as well as the need to question the methodology of acquisition of data before accepting its application in formulas or for analyses for forensics and epidemiology. It is also of value to remember that many infections with SARS coronavirus may have been asymptomatic or mildly symptomatic.

Plague is a zoonotic infection caused by *Yersinia pestis*, which occurs in the western United States with regularity and has an animal reservoir. The situation with the naturally occurring plague is in contrast to the appearance of a case of smallpox, which would be an immediate indication of a bioterrorist event (see Chapter 15). Cases need to be approached from an epidemiological standpoint first to determine whether it is a naturally acquired case or whether facts point to a deliberate introduction of the organism. Currently, analytical techniques could include genomic analysis of an isolated organism and immunological response of the host. In the new era of rapid and deep sequencing, our capacity to investigate the genomics is growing (37) (see Chapter 27). In consideration of animal reservoirs, ELISA assays were compared with other tests for detection of plague antibody and antigen in multimammate mice (*Mastomys coucha* and *M. natalensis*) (38), which were experimentally infected and then sacrificed at daily intervals. IgG ELISA was equivalent in sensitivity to passive hemagglutination and more sensitive than the IgM ELISA and complement fixation. Antibody was detectable by day 6 after infection using all four tests. IgM ELISA titers fell to undetectable levels after 8 weeks. Plague fraction 1 antigen was detected in 16 of 34 bacteremic sera from *M. coucha* and *M. natalensis*. This antibody pattern comparison shows that the principle of IgM versus IgG to this pathogen works to temporally situate the infection as an early versus late or past event. It also shows that when the information is combined with antigen detection, it engenders more confidence in the results. It should be noted that conclusions from this older reference have been substantiated with more defined antigens and assay technologies.

The context and geographic location where an infection or biothreat occurs may dictate how an infection is viewed and evaluated. An example is provided by melioidosis, which is not endemic to the United States. Melioidosis is caused by *Burkholderia pseudomallei* (39). Key clinical signs and laboratory results may raise the possibility of an infection with this pathogen. Whether it is an acute, persistent, or past infection can be determined by assessing several host responses. Often a simple nonspecific indicator such as erythrocyte sedimentation rate or C-reactive protein (CRP) can raise the clinical suspicion of an infection. In a study of 46 patients with clinical melioidosis, 35 (22 culture positive and 13 culture negative) had relatively uneventful disease courses. Initially, they had elevated serum CRP that decreased with antibiotic therapy and returned to normal as the disease resolved. In another series of patients, IgM and IgG were measured by ELISA in 95 sera from 66 septicemic cases and 47 sera from 20 cases with localized melioidosis (40). Sixty-five sera from culture-negative cases were seronegative for other endemic infections but those suspected of melioidosis were also examined. Other controls included serum from 260 nonmelioidosis cases, 169 high-exposure risk cases, and 48 healthy individuals. The IgG ELISA was 96% sensitive and 94% specific. All sera from cases with septicemic and localized infections and 61 of 63 sera from clinically suspected melioidosis cases were positive for IgG antibody. Sensitivity and specificity of the IgM ELISA were 74 and 99%, respectively. A geometric antibody index for IgM antibody in sera of melioidosis cases was significantly higher in cases compared with that of noncase controls. In another study, a rapid test for IgG and IgM was shown to have clinical utility (41). A study with the intent of evaluating the utility of an IgG assay compared with other assays illustrates how the clinical and temporal context must be integrated for interpretation (42). It also illustrates how there is room for technical improvement in tests but the best setting is often the endemic area itself or at least using samples from that area in which the infection is occurring. These tests were evaluated in the actual clinical setting in an area endemic for melioidosis. Specificity of IgG (82.5%) and IgM (81.8%) assays were significantly better than that of an indirect hemagglutination test (IHA) (74.7%). Sensitivity of the IgG assay (85.7%) was higher than that of the IHA test (71.0%) and the IgM test (63.5%). Specific IgG was found in septicemic cases (87.8%) and localized infections (82.6%). The IgG test was also better than the IgM test and the IHA test in identifying acute melioidosis cases in the first 5 days after admission. IgG antibody to a *B. pseudomallei* antigen remained high for longer than 5 years in recovered, disease-free patients. Because this is a disease that may have an incubation of days to years, an acute case may very well be detected by a rise in specific IgM if it were a matter of days from infection. Although endemic for southeast Asia, if *B. pseudomallei* was used

as a biothreat agent in a different environment, its course and manifestations may not be recognized due to unfamiliarity with the disease.

The example given earlier also points out how the context in which a test is used determines is value. The concept of predictive value is instructive in determining how useful a test may be. In terms of disease detection, a high positive predictive value indicates that the test is useful in determining that the disease is present. A high negativity predictive value would indicate that the test is useful in excluding the presence of the disease.

1. Concept of sensitivity—true positives/true positives + false positives or how many with a positive test actually have the disease.
2. Concept of specificity—true negatives/true negatives + false negatives or how many with a negative test actually do not have the disease.
3. Concept of positive predictive value—how good is the test in predicting disease among a particular population or true positives/true positives + false positives.
4. Concept of negative predictive value—how good is the test in excluding the disease among a particular population under consideration or true negatives/true negatives + false negatives.
5. High + predictive values are seen where disease prevalence is high and is low where disease prevalence is low.
6. Negative predictive values are high when disease prevalence is low and lower when disease prevalence is high.

Another zoonotic agent is Rift Valley fever virus (RVFV), which can be transmitted via aerosols. One study with the intent of looking for improved tests showed the utility of IgM to determine an early exposure to RVFV (43). Two IgM ELISAs detected specific IgM antibodies to RVFV during the first 6 weeks after vaccination. Three inactivated vaccine doses were given on days 0, 6 to 8, and 32 to 34. IgM levels on days 6 to 8 were negative or in the lower range of detection; on days 32 to 34 the IgM levels were strongly positive; on days 42 to 52 they were waning; and in later collected samples were negative. The plaque reduction neutralization test was negative on days 6 to 8 and became positive in later samples. Similar to the examples shown earlier, these data suggest that three doses of RVFV vaccine induced a prolonged primary antibody response. Authors of that study concluded that ELISA IgM may be useful for early diagnosis of acute human infection. Good correlation of a neutralization test and ELISA IgG would indicate a later infection.

Taken together, these examples illustrate that an ideal test or analysis for both clinical and forensics use would incorporate endemic and incident area controls, historical contextual information, knowledge of the route of exposure, background incidence, and kinetics of transmission.

POSSIBLE SCENARIOS OF BIOTERRORISM ATTACKS: DISTINGUISHING VICTIMS FROM PERPETRATORS

Each of these scenarios must take into account multiple factors and limitations of any analytical process to be applied. The start of the acquired immunodeficiency syndrome (AIDS) epidemic provides an example. On one extreme is the situation that occurred with the onset of AIDS from the human immune deficiency virus (HIV) in the United States. Initially, there were no cases, and therefore a precise highly sensitive and specific test with excellent positive and negative predictive values (such as exists now when a combination of tests are used) would not likely yield a positive result in an area where there was little HIV infection and disease at the onset such as, for example, Kansas. A positive test by today's methodologies from a 1970 serum sample from Kansas would be considered a probable false positive and warrant further investigation. Today, several viral and nucleic acid assays are available that would provide a definitive diagnosis in a short period of time (22). However, the same sample tested at the beginning of HIV testing could have been positive if the person had adult T-cell leukemia, which is caused by human T-cell leukemia virus-1 (HTLV-1), because original tests for what became known as AIDS involved whole viral lysates in which up to 30% of the HTLV-1 sera cross-reacted. Questions regarding interpretation of test results could be raised by knowledge of different presentations of the infection. For example, HTLV-1 can actually be used in the laboratory to immortalize cells. In the patient it actually increases the T-cell count, as is the nature with leukemia, instead of decreasing them, as with HIV infections. Other laboratory indicators such as hypercalcemia would now raise leukemia as a consideration.

Interpretation of a positive laboratory test must take into account the health status of the person being tested. This is important for the practice of medicine and can have relevance when extended to forensic analysis (10). The following examples illustrate this concept. Individuals who have syphilis, a treponemal bacterial infection, will typically have a positive fluorescent treponemal antibody test result for years, even after successful treatment. However, while infected they would have a positive venereal disease research laboratory (VDRL) test, which reverts to negative following successful antibiotic therapy. The VDRL test detects nonspecific, anticardiolipin antibodies and can produce false-positive results with other conditions (e.g., pregnancy). There are some notable exceptions related to cross-reactive epitopes or autoimmune diseases. These are readily distinguishable by history and clinical information. Similarly, individuals with active tuberculosis will likely have a positive skin test (Mantoux) or a positive interferon-γ release assay (44), whereas the uninfected healthy person will be negative. In certain instances,

a sick person with a cell-mediated immune deficiency will be anergic, that is, he/she will be negative to multiple skin tests, including common antigens such as *Candida*. The key difference here is that a great difference exists between the *healthy person* being tested and an *ill or immunocompromised individual* being subjected to the same test.

Tests may also discriminate between the length of the infection (i.e., acute or chronic); limitations of these tests may lead to different interpretations unless one is familiar with those limitations. An example of this occurred with the bacterial infection by *Borrelia burgdorferi*, which causes Lyme disease. Antibiotics can abrogate the antibody response because ELISA results are negative in 30% of patients with known disease who were treated early (45). In early cases, reactivity to a unique antigen, OspA, was also negative in serological assays, despite a demonstrable T-cell response (46). Analysis of these same sera found that there was antibody to *B. burgdorferi* but it was below the threshold of detection by conventional assays. It was detectable in its bound form in immune complexes (47,48).

Anthrax can be used as an example where investigatory leads can be generated by considering a scenario *in toto*. The elderly woman who died in Connecticut from inhalation anthrax clearly had no occupational exposure nor was she known to have had contact with anyone who had anthrax. It was possible that she had contact with cross-contaminated mail. However, if this case had occurred as the index case or out of context of the mail attacks, it would have been reasonable to question her travel history; what her work, if any, was; or if she received or used spore-contaminated products from an anthrax-endemic area. Similarly, the Vietnamese woman who died of inhalation anthrax in New York City would also have had these questions investigated. It would have been useful to search for direct or indirect evidence of anthrax by physical examinations of her contacts or close neighbors. Inspection and cultures from her workplace, apartment, and apartment complex (especially contiguous neighbors) are important for detecting the presence of *B. anthracis.* Co-workers, friends, neighbors, and other contacts could have had their serum analyzed for antibody to antigens of *B. anthracis*. These samples could have been frozen so that if one were positive it would be available for a subsequent comparison study. At a minimum, these types of studies could serve as future control data for the geographic region. With molecular methods, even trace amounts might be detectable (49), although parallel investigation using background controls would be necessary. Although hypothetical, several results could have occurred, and each will be considered separately. First example: a close contact is positive for IgM to one of the *B. anthracis* antigens, such as PA. This finding would suggest that this person had recent exposure and, if nothing else, should be treated. This individual could conceivably be the one who knowingly or unknowingly passed the spores to the patient. Given the October 26 onset of illness, which

is late in the mailing sequence, it would be less likely that this individual was a perpetrator but rather a recent victim. However, if this person were IgG positive, then there are several other possibilities. Perhaps this person had past exposure in an endemic region and was treated (e.g., Haiti, where anthrax is known as "charcoal disease"); this person could have been vaccinated for bona fide reasons, such as a researcher who received it to protect against occupational exposure; or this person could have obtained the vaccine originally for legitimate or illegal purposes but was nevertheless vaccinated. The vaccine usage may have been for a clinical trial or for animal experimentation. Animal vaccines may be more obtainable without strict record keeping. This person could have loaded the mail with relative impunity if there was protective antibody generated from the vaccination. Situations similar to this one will require intelligence information regarding access, ability, and motive. In an area where recombinant vaccines are being developed or used, the antibody response would be different between someone using one type of recombinant vaccine as compared to someone using another type of vaccine. Nevertheless, finding IgG to one or more antigens of *B. anthracis* could point investigators toward such a seropositive individual, whereas an IgM finding could justify critical therapy. Where information points to a particular individual, investigation could be extended to search for ingestion or injection of antibiotics as illustrated later in the ciprofloxacin example. Questions would be raised regarding access to antibiotics, recent ingestion/injection of them, half-life of the antibiotic, half-life of the metabolites of the antibiotics, and in which body fluids or tissues can the residual be found. As illustrated from data in the earlier sections, someone with antibiotics in his/her system may be protected following exposure to a potential pathogen. This person would be antibody negative and likely antigen and microbial DNA/RNA negative, as the infection would have been eradicated before the organism could proliferate to any significant level. The widespread prophylactic use of ciprofloxacin during the period following the anthrax mailing attacks is illustrative of an understudied area. Ciprofloxacin has been increasingly associated with tendonitis and ruptured Achilles tendons (50,51). In the future, better methodology to follow pharmacokinetics of an anti-infective compound may have forensic implications. In the last example, someone who takes an antibiotic prophylactically while manipulating a lethal microbe may exhibit side effects that, in proper context of an investigation, may add to the picture of possible culpability. This area is far from established at this point in time.

Strategies can be employed to examine suspicious but possible accidental transmission of infections. This approach is illustrated by a recent study of avian influenza using a multitude of assays. Tools to determine person-to-person spread as the mode of transmission included viral culture, serologic analysis, immunohistochemical assay, reverse-transcriptase/polymerase chain reaction analysis, and genetic sequencing (52).

It is likely that future understanding of the immune system and evolving technologies such as microarrays will bring new analytic power to the field, but in the interim we can make good use of proven principles for forensic purposes.

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