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CHAPTER 14

Microbial Forensics Host Factors

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Consider the diagnosis of a disease caused by a biothreat. Multiple questions arise immediately. One question is whether it is a natural event or an intentional attack. Knowledge of the host response can potentially answer some questions depending upon the pathogen involved. Some of these questions include who else was exposed, who else may have been vaccinated for protection, how long ago was the person infected, and were other persons who are close to the individual also infected before or after the index case? Aside from public and personal health concerns, these questions seek to categorize individuals as possible victims versus perpetrators. It is the aim of this chapter to present the elements of the host response in a simplified fashion that in the right context has high potential to bring answers to these questions.

Microbial forensics has a primary aim to identify the biological agent, its source, and the individuals responsible for a biothreat event.[1] Analytic approaches differ when the suspected biothreat agent is encountered in a container or the environment, as opposed to a human or animal. Trace element, pollen, growth media, latent fingerprint, and microbial and nonmicrobial nucleic acid analysis are all applicable to the container and environmental sample.[2] However, once the microbe or its toxin is in the living host, it is no longer possible to analyze all of the preceding items except the microbial nucleic acid. Nevertheless, the host response to the biological agent is available for analysis. 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] The forensic pathologist is already familiar with these. Those involved with epidemiologic and diagnostic issues will be 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 an authenticated piece of a puzzle may be included in the analytical and attribution picture.

Microbial Forensics

The host response to a foreign substance is often a well orchestrated series of events designed to protect the individual from harm. Modern techniques help us elucidate the pathways and components of the host response. The immune system and its components are a mainstay of our protection against infections and malignancies.[4,5] Inflammation is often an unpleasant side effect as the immune system contains and eradicates a microbe or foreign tissue. Specific arms of the immune system can be used as markers in favor or against the presence of an infection. The humoral or antibody response to an invading microbe is an example. Some of the antibodies that are produced have a protective effect with other parts of the immune-inflammatory system and are responsible for eradicating the infection. Other antibodies may not be as effective in this role. However, in their ability to recognize unique and specific structures of a microbe, they serve as beacons that a microbe was recently present or was present in the distant past. Substances such as antibiotics which can rapidly kill a microbe may modify the immune response by removing the infectious driving force for a full-scale response. In clinical medicine and veterinary medicine, measurement of the immune response helps the diagnostician decide what infection was present and how recently. In these situations the intention is to provide treatment. For other pieces of the puzzle, 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 will discuss the basics of the host immune response that can have utility in the microbial forensic sense. Examples will provide a sense of what information is achievable and what is not likely to provide clues with a high degree of certainty.

GENERAL CONCEPTS

In response to an encounter with a new microbe, the immune system first starts to activate the antibody system. Usually a cell known as a macrophage engulfs some of the microbes. It then presents part of the microbe to a helper T cell (a lymphocyte) which then directs other lymphocytes known as B cells to produce antibodies to that particular microbe and even more specifically to that part which was presented. It usually takes at least 4 days before any microbe-specific antibody can be found.[6]

Antibodies are a specific form of the proteins known as immunoglobulins (Igs). IgM, IgG, and IgA are the principal classes of immunoglobulins with relevance to this chapter and will be discussed in more detail. 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 cells that can release histamine when the offending allergen bridges

TABLE 14.1 Immunoglobulin classes and properties

Immunoglobulin Class	IgM	IgG	IgA	IgE	IgD
Size (kD)	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	80	13	6	0.002	0.2

two such molecules. In an infection, 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 those that produce IgG. Later some of this population of cells undergo a switch to become IgA-secreting type B cells. Immunoglobulins persist for varying times; for example, the half-life of particular IgM antibodies is 5 days, while that of IgG can be as long as 21–23 days (Table 14.1).[4,6]

Similar to a live microbe, vaccines can also provoke an antibody response. The vaccine can be composed of a live or attenuated microbe, a whole non-proliferating microbe, or an antigenic part of the microbe. Regardless, the intent of the vaccine is to produce protection, often by protective antibodies. Although the half-life of an individual IgG molecule is less than a month, a population of antibodies in the IgG isotype form may persist for life. Memory B cells can sustain these antibodies and retain the ability to quickly generate 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 increase in the levels that circulate in the blood. Figure 14.1 illustrates this.

Perhaps the most discernible pattern of antibody response which has forensic value is the appearance of IgM first, followed by a B-cell switch to the longer lasting IgG. During the early phase of exposure, IgM can be seen first. As time goes on, IgG is seen and predominates, and IgM is no longer found. This is illustrated in Figure 14.2.

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 response may involve IgM at the early stage and IgG later. Late in the disease or during recovery, only IgG to particular antigens may be seen. A classic example is the human antibody response to Epstein Barr virus (EBV).[7] EBV is the virus known to cause mononucleosis. During acute early disease, it is common to find high levels of antibody of the IgM isotype to the viral early

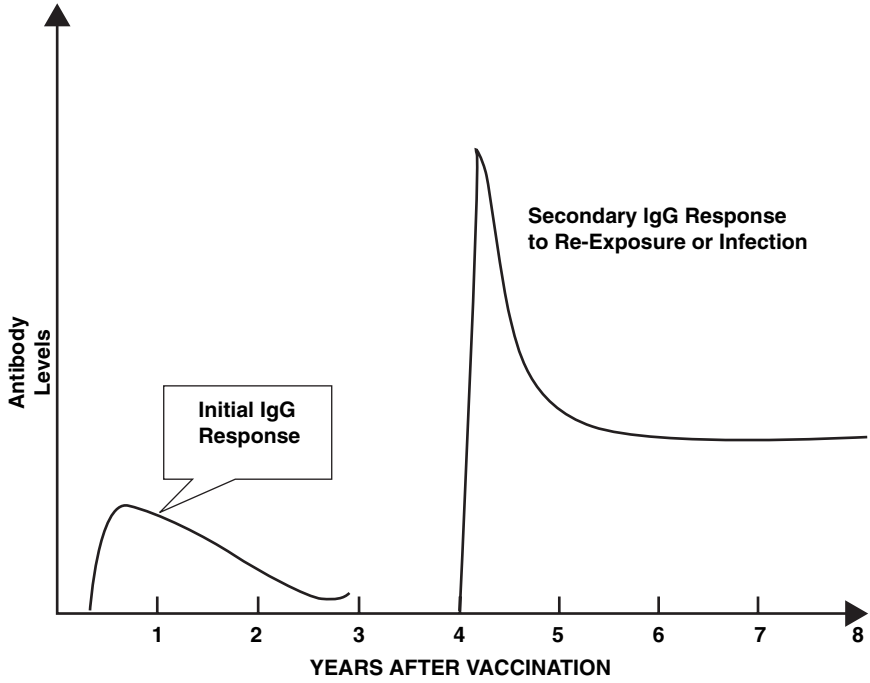

FIGURE 14.1 Illustration of the IgG antibody response to a vaccine antigen after the first immunization and subsequent exposure by natural exposure to the infectious agent or by another vaccination.

antigen (EA) and viral capsid antigen (VCA). It is rare to find IgG antibody to the VCA or Epstein Barr Nuclear Acid (EBNA) in anything but low titers. As the patient recovers from their first infection with EBV, it is rare to find anything but low levels of IgM to EA or VCA, but IgG to VCA in higher or increasing levels is common. Antibodies to EBNA are often very low during this stage. Then after clinical recovery, that is, several months later, IgM to EA and VCA stay at low levels whereas IgG to VCA and EBNA remain at high levels, often for years. Table 14.3 illustrates this pattern by stage of the immune response to EBV and its particular antigens. Figure 14.3 is a graphic display of this. For the clinician or epidemiologist this provides a framework to determine where in the infectious process a patient may be. Tables 14.2 and 14.3 and Figures 14.2 and 14.3 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 the stage of infection or exposure.

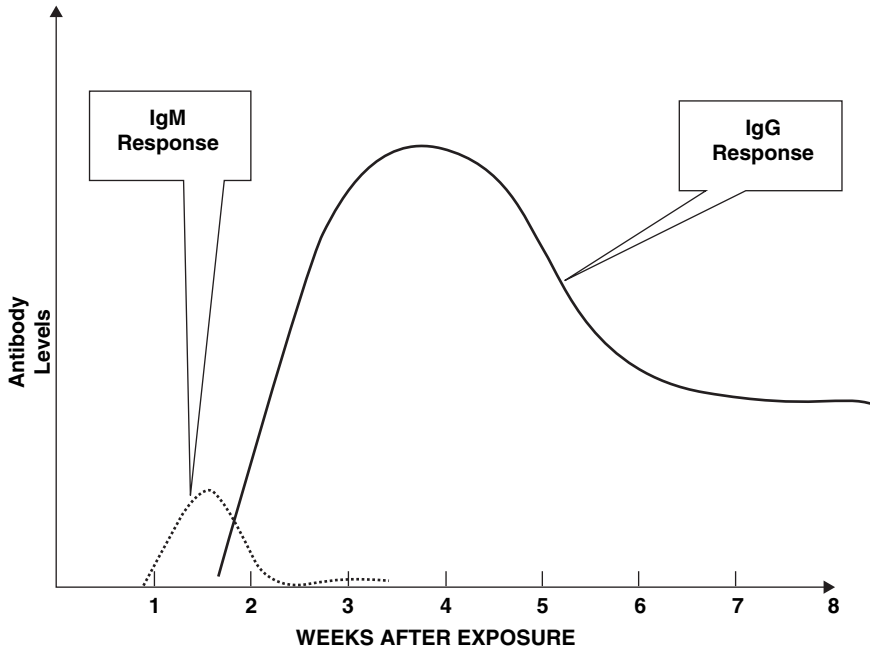

FIGURE 14.2 Illustration of the temporal relation of the IgM and IgG responses to an infection with IgM as the first and often transient response and IgG as the more sustained response.

TABLE 14.2 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 for several years

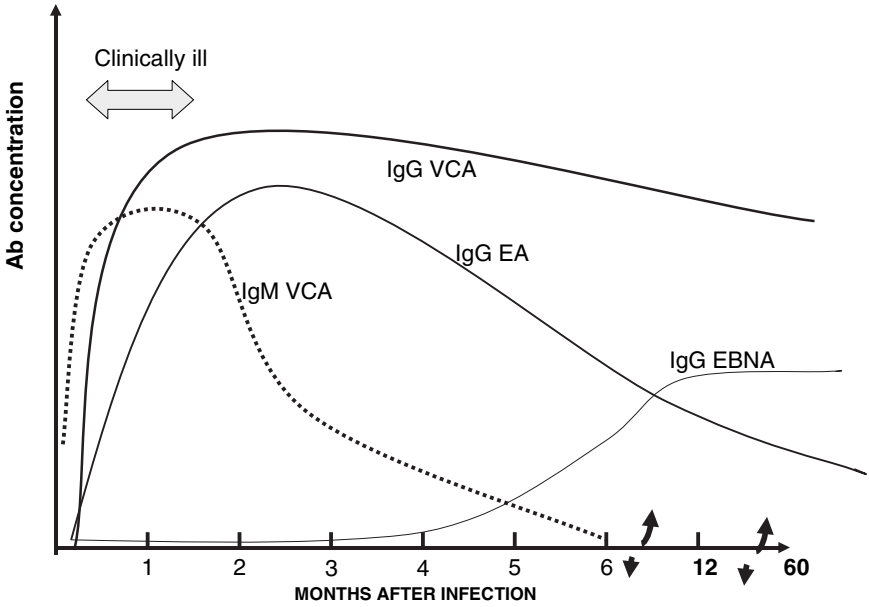

FIGURE 14.3 Schematic response of IgM and IgG to different antigens of EBV over an extended period of time.

TABLE 14.3 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

A controlled experiment or normal clinical event illustrates what happens when the immune system sees the infectious agent or its vaccine representation again. 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 exposed again to the infectious agent for whatever reason. Consider a generic antigen exposure. The first time the immune system sees Antigen X (AgX) it responds as shown in Figures 14.1 and 14.2. At first any antibody to AgX is barely discernible; then the levels rise and later fall to a plateau. If a mixed infectious exposure were to occur with AgX and a new AgY from another microbe, the immune system would quickly mount a brisk and high level of Ab to AgX, while the course of Ab to AgY would be slow and delayed, just as was the first exposure to AgX. For AgX this is a phenomenon termed immunological memory or an amnestic response. This can be useful when the symptoms and signs of exposure to either X or Y are similar. This is the case with the early flu-like symptoms of pulmonary anthrax and the influenza virus itself.

Another example common to all of us is repetitive exposure to different strains of flu viruses.[4] As illustrated in Table 14.4, a person infected for the first time with one strain of the influenza virus makes 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 to those antigens that were also present on the original influenza virus. The person also makes a smaller initial antibody response to new antigens, that is, those not shared with the first virus. The initial response is minimal in comparison. 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.

TABLE 14.4 Response to theoretical antigens from different flu viruses at the 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

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 to our knowledge of the kinetics and time 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 microbial forensics. This information may have critical probative value or it can guide investigative leads. The absence of a specific antibody response may also have equal value in a particular investigation. Certainly its importance is increased in the context of knowledge of what organism may 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 hard data points such as detection of antigens themselves, and detection of microbial DNA or RNA.[8] Other information such as how many hosts (people or animals) have had this infection in the geographic region, what is the normal infection rate, and background incidence of antibody titer due to the organism in question or a related organism, is also important.

The 2001 anthrax letter attacks raised multiple questions for every person infected, possibly exposed, vaccinated, or treated. Some of these questions included how these persons were infected, if at all; that is, was it by the skin, which could produce cutaneous anthrax; by inhalation of spores, which would produce pulmonary and systemic anthrax infection; did they ingest any spores, which would produce an initial gastrointestinal infection; or, were they among the "worried well?"

Consider the situation where a close associate comes down with symptoms compatible with anthrax infection after receiving a powder-containing letter. Until this is disproved as anthrax, great worry will ensue. We learned that the limited textbook medicine did not apply. Yet there is useful information to be used in the present while designing future studies.

In several cases of documented exposure, there was not enough time for the patient to develop antibody to a specific anthrax antigen, at least as probed for IgG. Serial serum samples obtained on November 16, 17, 18, and 19 of 2001, well after the potential work place exposure during the first week in October 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 nonreactive. 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 specimens were collected on October 10 and 17.[9]

It is instructive to look at the positive antibody case in the context and duration of that individual's symptoms when he developed a positive test. None of the symptoms detailed below were individually unique to raise suspicion of a particular diagnosis. The 73-year-old man (case 2) developed fatigue on September 24. He was the newspaper mailroom clerk who delivered mail to the first patient (case 1). On September 28, he developed a nonproductive cough, intermittent fever, runny nose, and conjunctivitis. These symptoms 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. 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 that time his neurologic exam 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 "classical" mediastinal widening. The patient was initially given intravenous azithromycin; cefotaxime and ciprofloxacin were subsequently added. A nasal swab obtained on October 5 grew *Bacillus anthracis* on culture. Computed tomography (CT) 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 PCR. Bacterial cultures of bronchial washings and pleural fluid were negative. A transbronchial biopsy showed *B. anthracis* capsule and cell-wall antigens by immunohistochemical staining. During hospitalization, the white blood count rose to 26,800/mm,^{3} and fluid from a second thoracentesis was positive for *B. anthracis* DNA by PCR. Both pleural fluid cells and pleural biopsy tissue showed *B. anthracis* capsule and cell-wall antigens by immunohistochemical staining. Serial serum samples demonstrated a greater than fourfold 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 on oral ciprofloxacin. Table 14.5 illustrates both the clinical and bioforensic approach and context in which to analyze such a patient. These are likely to be common to most situations where a biocrime is suspected to have affected an individual. The first set of questions revolves around whether the person is sick: does the patient have any indications of not being well and any laboratory evidence suggestive of any infection? The second set of questions addresses whether there is any specific and objective laboratory evidence of a particular infection.

This case points out that direct cultures may be negative at different times from different fluids and tissues. This may be influenced by the early administration of antibiotics. However the remnants of the infection, even dead

TABLE 14.5 Nonspecific and specific indications of a case of anthrax

Clinical evidence of an infection	Nonspecific laboratory evidence of an infection	Specific clinical evidence of an infection with *B. anthracis*
Known exposure by proximity to area and infected person	Chest X-ray and CT 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

organisms, can be found by probing for antigens and DNA. This patient manifested a classic principle of infectious disease, a rising antibody titer over time. In this case it was IgG to a particular antigen of the anthrax toxin. This antibody response may have been detected earlier if IgM to this toxin or other antigens of anthrax had been sought. The case also points out the utility of integrating the presence of antibody with that of other indications of an anthrax infection. These take their greatest significance during clinical symptoms and signs of infection in a possibly exposed individual.

Early administration of antibiotics can prevent positive cultures from the organism in question. Of the first 10 pulmonary anthrax cases associated with the 2001 anthrax letter attacks, three patients had no culture growth of *B. anthracis* from any clinical samples, but culture was attempted after initiation of antibiotic therapy. The diagnosis was made on the basis of history of exposure in conjunction with compatible symptoms and signs of disease, and objective laboratory findings. *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 DNA by PCR on pleural fluid or blood.[10]

It is very important to understand the limitations of the assay used. An IgG-based ELISA against the anthrax toxin's protective antigen (PA) component illustrates the importance of understanding the limitations of an assay. The ELISA assay was developed at the U.S. Army Medical Research Institute of Infectious Disease (USAMRIID) and put into operation after optimization at the CDC[11] for functional sensitivity and specificity for detecting antibody response to *B. anthracis* infection. Its major limitation is its restriction to one antigen and to IgG. Therefore a negative result at the time of early exposure may in effect yield a false-negative result. This identifies a gap in our knowledge and application that can be filled by development of an IgM assay, and perhaps one that is enhanced by probing for other *B. anthracis* antigens or epitopes yet to be discovered.

The assay may be very useful in its present form to screen asymptomatic people with possible exposure. The study by Dewan et al. gives a sense of this, and provides a contemporary background database on a group of individuals who may have been exposed to *B. anthracis*.[12] They evaluated postal workers. Beginning on October 29, 2001, 1,657 employees and others who had been to the Washington, D.C. postal facility went to the D.C. General Hospital for antibiotics additional to those begun on October 21. Serum samples were obtained from 202 individuals, and all were negative for specific IgG antibody to the PA IgG, including the three participants who reported a remote history of anthrax vaccination. Limitations to this data are the fact that antibiotics were begun before serum testing, and there were no baseline serum samples available for testing. Also, the time period from exposure to sampling was very short. Among 28 individuals with positive nasal swabs in the Capitol exposures who received antibiotics immediately, none had a positive culture from a nasal swab repeated 7 days later, and none had positive serum IgG to PA antigen 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 considerations. Even with this easily disseminated strain, an antibody response may be aborted or modified with antibiotics by early eradication. Furthermore, antibiotics taken prior to exposure would likely be effective in preventing laboratory and clinical signs of an infection or exposure. Detection of DNA, antigen, or the organism itself on a person's body, clothing, or possessions would raise a red flag.

The route of infection is equally important in interpretation of results and the limitations of the assay used. The example of cutaneous anthrax in Paraguay illustrates this, as well as the notion of searching for other antigens as markers of exposure.[13] Analysis of an outbreak of at least 21 cases of cutaneous anthrax developed from touching raw meat of a sick cow was performed. Serum from 12 cases and 16 colony and two noncolony controls 6 weeks after the outbreak were blotted for antibodies to the PA and lethal

factor components of anthrax toxin. An ELISA was used to probe for antibodies to poly-D-glutamic acid capsule. Of 12 cases, 11 had a positive PA screen, for a sensitivity of 91.7%; none of the 18 controls was positive for a specificity of 100. Only six of 12 cases had antibody to the lethal factor; all controls were negative. Probing for antibodies to capsule was positive in 11 of 12, but was positive in two of 18 controls. This study demonstrates the need to consider other antigens.

CONSIDERATIONS AND CONCERNS RAISED BY ANALYSIS OF OTHER INFECTIONS

Some of the principles discussed above are highlighted by a recent report on SARS. This coronavirus disease also evoked concern of a possible terrorist origin at the onset. A report in the Morbidity and Mortality Weekly Report (MMWR)[14] on the "Prevalence of IgG Antibody to SARS-Associated Coronavirus in Animal Traders" discussed the need to validate and interpret tests in the appropriate populations—the IgG test, discusses its inability to date the time of the infection, and the possibility of reactivity to a near neighbor that might be unknown. In a Promed bulletin, Dr. Berger looked at the data from a different angle 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 percent to 2.9 percent of individuals in a healthy control group of adults were also found to be seropositive! The population of 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 (8,422) of SARS patients reported worldwide to date!" This is a good illustration of the need to question the methodology of acquisition of data before accepting their application in formulas or for analyses.

Yersinia pestis, the cause of plague, is a zoonotic infection which occurs in the U.S. with regularity and has an animal reservoir. This is in contrast to a case of smallpox which would raise an immediate red flag for a bioterrorist event. Cases need to be approached from an epidemiologic standpoint first to determine whether it is an "expected" case or whether the facts point to a deliberate introduction of the organism in a group of people or an individual. Analytic techniques could include genomic analysis of an isolated organism and immunological response of the host. 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*).[15] They 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 shows that the principle of IgM versus IgG to this pathogen works to temporally situate the infection as early versus late or past. It also shows that when the information is combined with antigen detection, it engenders more confidence in the results.

Melioidosis is caused by *Burkholderia pseudomallei*.[16] It is also an example in which key clinical signs and laboratory features raise the possibility of this infection. Related studies and observations are presented here to illustrate some of the temporal issues of the host response and the need to interpret results of an assay in the appropriate clinical and geographic setting. Whether it is an acute infection, persisting one, or past infection can be determined by looking at several host responses. Often a simple indicator of infection such as erythrocyte sedimentation rate or C-reactive protein (CRP) can create clinical suspicion to begin a probe for a specific 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 their 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.[17] Sixty-five sera from culture-negative cases seronegative for other endemic infections but suspected of melioidosis were also examined. Other controls included 260 non-melioidosis 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. The sensitivity and specificity of IgM ELISA were 74% and 99%, respectively. A geometric index for IgM antibody in the sera of the melioidosis cases was significantly higher in melioidosis cases compared to that of the non-melioidosis disease controls. Another study by some of the same authors using a rapid test also showed IgG and IgM to have clinical utility.[18] Another study with the intent of evaluating the utility of an IgG assay compared to other assays illustrates how the clinical and temporal context must be integrated for interpretation.[19] It also illustrates how there is room for improvement in tests and that the best analysis will result from an understanding of the conditions in the endemic area and utilization of samples and controls from that area. These tests were evaluated in the actual clinical setting in an area endemic for melioidosis. Specificity of specific IgG (82.5%) and specific IgM (81.8%) were

significantly better than that of an indirect hemagglutination test (74.7%). The sensitivity of the specific IgG assay (85.7%) was higher than that of the IHA test (71.0%) and the specific IgM test (63.5%). Specific IgG was found in septicemic cases (87.8%) and localized forms (82.6%). The specific IgG test was also better than the specific IgM test and the IHA test in identifying acute melioidosis cases in the first five 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 picked up by IgM versus IgG if it were a matter of days from infection. Although endemic for Southeast Asia, if it were used as a biothreat agent in a different environment, its etiology may not be recognized immediately. The importance of understanding endemic area factors as well as the host to the microbe is further illustrated in another zoonotic example. Rift Valley fever virus (RVFV) can be transmitted via aerosols. One study with the intent at looking for improved tests did show the utility of IgM to determine an early exposure to RVFV.[20] Two ELISA IgM tests 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. ELISA serum IgM on days 6 to 8 were negative or in the lower range of significance; on days 32 to 34 they were strongly positive; on days 42 to 52 they were waning and later 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 above, their data suggest that three doses of RVFV vaccine induced a prolonged primary antibody response. The authors of that study concluded that the ELISA IgM could become an important tool for early diagnosis in 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 for both clinical and forensics use would incorporate endemic 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 the limitations of any analytic process to be applied. This is often considered by understanding the elements of positive and negative predictive values of an assay within a population being tested. On one extreme is the situation that occurred with the onset of human immune deficiency (HIV) in the U.S. First 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 disease at the onset, Kansas, for example. 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. 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). This is because the original tests for what became known as acquired immunodeficiency syndrome (AIDS) involved whole viral lysates in which up to 30% of the HTLV-1 sera cross-reacted. Suspicion to the contrary would 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 clinical 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. The following situations illustrate the concept. Individuals who have syphilis, a bacterial spirochetal infection, can typically have a positive FTA (fluorescent treponemal antibody) test for years. However while infected they would have a positive venereal disease research laboratory (VDRL) test. This reverts to negative with successful antibiotic therapy. There are some notable exceptions related to cross-reactive epitopes or autoimmune diseases. These are readily distinguishable by history and clinical information. Similarly individuals infected with tuberculosis will have a positive skin test (Mantoux), 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 there is a wide difference between the healthy person being tested and a very ill individual being subjected to the same test.

Tests may also discriminate between the time of the infection as acute or chronic, and its limitations may lead to different interpretations unless one is familiar with those limitations. An example of this occurred with the bacterial infection of *Borrelia burgdorferi*, which causes Lyme disease. Dattwyler's group showed that antibiotics could abrogate the antibody response because ELISA results were negative in 30% of patients with known disease who were treated early.[21] Another group showed that in early cases reactivity to a unique antigen, OspA, was also negative in serological assays despite a demonstrable T-cell response.[22] Our own group had an opportunity

to analyze the same sera and found that there was antibody to *B. burgdorferi* but it was below the threshold of the conventional assays. It was detectable in its bound form, in immune complexes.[23,24]

Anthrax can be used as an example where investigatory leads can be generated by considering a scenario *in toto*. The elderly lady who died in Connecticut from anthrax clearly had no occupational exposure nor was she known to have had contact with anyone who had anthrax. It was possible that she received 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 products from an endemic area for anthrax. Similarly the Vietnamese woman who died 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 presence of anthrax. Coworkers, friends, neighbors, and other contacts could have had blood samples analyzed for antibody to anthrax antigens. These samples could have been frozen so that if one were positive it would be available for a comparison study in the future. At a minimum these types of studies could serve as future control data for the geographic region. Although hypothetical, several results could have occurred, and each will be analyzed separately: First example: a close contact is positive for IgM to one of the *B. anthracis* antigens, e.g. PA. This 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 too. However if this person were IgG-positive on the assay, then there are several other possibilities. Perhaps this person had past exposure in an endemic region with a subclinical or treated illness (e.g., Haiti, where anthrax is known as "charcoal disease"). Or this person could have been vaccinated for bona fide reasons such as a researcher who received it for occupational protection. Or this person could have obtained the vaccine originally for legitimate or illegal purposes but was nevertheless vaccinated. Animal vaccines may be more obtainable without strict record keeping. This person could have loaded the mail with relative impunity if there were protective antibody generated from the vaccine. These situations require intelligence information regarding access, ability, and motive. However the IgG finding could point investigators towards such an individual, whereas an IgM finding justifies critical therapy. Coming from the other direction, where information points to a particular individual, investigation could be extended to ingestion of antibiotics. Questions would be raised

regarding access to antibiotics, recent ingestion of them, half-life of the antibiotic, half-life of the metabolites of the antibiotics, and in which body fluids or tissues the residual can be found. As illustrated from the data in the earlier sections, someone with antibiotics in their system could be protected from exposure to a sensitive microbe. This person would be antibody-negative and likely antigen- and microbial DNA/RNA-negative, since the infection would have been eradicated before the organism could proliferate in any significant quantity.

Similar strategies can be employed to examine suspicious but possible accidental transmission of infections. This is illustrated by a recent series of aviant flu. Tools to determine a person to person spread as the transmission mode included viral cultures, serologic analysis, immunohistochemical assay, reverse-transcriptase-polymerase-chain-reaction (RT-PCR) analysis, and genetic sequencing.[25]

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

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