NIAID
BIODEFENSE
Preparing Through Research

NIAID Biodefense Research Agenda for Category B and C Priority Pathogens

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U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health
National Institute of Allergy and Infectious Diseases

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PREFACE

On October 22 and 23, 2002, the National Institute of Allergy and Infectious Diseases (NIAID) convened a Blue Ribbon Panel on Biodefense and Its Implications for Biomedical Research. This panel of experts was brought together to provide objective expertise on the Institute's future biodefense research agenda, as it relates to the NIAID Category B and C Priority Pathogens (Appendix 1).

This Blue Ribbon Panel was asked to provide NIAID with the following guidance:

- Assess the current research sponsored by NIAID related to the development of effective measures to counter the health consequences of bioterrorism with a focus on the Category B and C priority pathogens.
- Identify research goals for the highest priority areas.
- Provide recommendations on the role of NIAID in achieving these priorities.
- Provide recommendations on the current NIAID Category B and C Priority Pathogens list.

NOTE: Although the NIAID list of Category A, B and C Priority Pathogens (Appendix 1) closely follows the CDC list of Category A, B and C Biological Diseases/Agents (Appendix 2), the NIAID list highlights specific pathogens identified as priorities for additional research efforts as part of the NIAID biodefense research agenda.

INTRODUCTION

In early 2002, NIAID developed a Strategic Plan for Biodefense Research at the National Institute of Allergy and Infectious Diseases (NIAID). As part of the implementation of the recommendations outlined in the Strategic Plan, NIAID convened two panels of experts to provide advice and guidance on specific areas of research. The first panel prioritized NIAID research plans for the Category A Priority Pathogens (see summary at http://www.niaid.nih.gov/dmid/pdf/biotresearchagenda.pdf). A second group, the NIAID Expert Panel on Immunity and Biodefense, was convened to focus on research related to the innate immune factors important for host protection against potential bioterrorist pathogens (see summary at http://www.niaid.nih.gov/publications/pdf/biodimmunpan.pdf). The recommendations of these panels have provided valuable guidance in the development of new initiatives and in modifying existing solicitations to respond to research needs in the area of biodefense and emerging infectious diseases. Biodefense research is defined as research to understand organisms that are potential bioterrorism threats and to develop new diagnostics, treatments, and vaccines for use in humans who may become infected. Thus, this research is similar to that for other infectious diseases but with emergence the result of a deliberate release rather than a consequence of natural events.

The *NIAID Biodefense Research Agenda for Category B and C Priority Pathogens* builds on the Strategic Plan and provides recommendations relevant to the Category B and C priority pathogens. As with the two previous research agendas, this document focuses on the need for basic research on the biology of the microbe, the host response, and basic and applied research aimed at the development of diagnostics, therapeutics, and vaccines against these agents. In addition, the Agenda addresses the research resources, facilities, and scientific manpower needed to conduct both basic and applied research on these agents.

Because of the number and diversity of the organisms contained in Categories B and C, the document is divided into chapters that include *Inhalational Bacteria, Arthropod-Borne Viruses, Toxins, Food- and Water-borne Pathogens, and Emerging Infections.* These chapters include specific recommendations related to the relevant organisms. The final chapter is a discussion of additional considerations for biodefense that includes recommendations for changes to the NIAID Category A, B, & C list of Priority Pathogens, recommendations on the role of industry in the biodefense research agenda, and research needs related to genetically modified organisms and biodefense.

AREAS OF RESEARCH EMPHASIS

The following areas have been identified as priorities for biodefense research for all Category A, B and C agents:

Biology of the Microbes

Research into the basic biology and disease-causing mechanisms of pathogens underpins efforts to develop interventions against agents of bioterrorism. NIAID supports research to better understand a pathogen's life cycle, as well as the events or processes that are critical to initiating infection or influencing the severity of disease. The application of genomics research, coupled with other biochemical and microbiological information, is expected to facilitate the discovery of new targets for diagnostics, drugs, and vaccines. Comparative genomics (comparing the sequences of different strains of particular organisms) will be a particularly important component of future research, helping to further the understanding of virulence and pathogenicity factors.

Host Response

Research into both innate and adaptive immune responses is critical in the development of interventions against agents of bioterrorism. The identification of innate immune receptors and the functional responses that they trigger will enable targeted activation of the innate immune response and induction of specific adaptive immunity. An enhanced understanding of population variables and their impact on immunity is critical for the design and development of effective vaccines and immunotherapeutics.

Vaccines

Vaccines are the most effective method of protecting the public against infectious diseases. New and improved vaccines against agents of bioterrorism must be suitable for civilian populations of varying ages and health status. In addition, vaccines developed to counter civilian bioterrorist attacks must be safe, easy to administer, and capable of an immediate protective and/or transmission-blocking immune response. Scientists must develop and characterize adjuvants that can enhance these desirable characteristics. A critical component of efforts to achieve these goals is enhanced linkages and partnerships with industry.

Therapeutics

In the event of a bioterrorism incident, effective therapeutics will be needed to address the immediate health needs of the public. Antimicrobial agents for treating many infectious diseases currently exist. However, a broader, more robust arsenal of anti-infective agents is needed to treat the broad civilian population and to intervene against

drug-resistant variants that may emerge. Detailed knowledge of the pathways that are essential for replication and pathogenesis will enhance drug development.

Diagnostics[1]

The initial clinical signs and symptoms of many agents considered biothreats are nonspecific and resemble those of common infections. Therefore, the ability to rapidly identify the introduction of a bioterrorism organism or toxin will require diagnostic tools that are highly sensitive, specific, inexpensive, easy to use, and located in primary care settings. Microchip-based platforms containing thousands of microbial signature profiles have tremendous promise. A centralized database could be constructed to collect this information and allow for the rapid identification of unusual patterns or clustering of illnesses.

Research Resources

Basic research and the development of new vaccines, therapeutics, and diagnostics depend on the availability of research resources. Among the resources needed to conduct counter-bioterrorism research are genetic, genomics, and proteomics information; appropriate *in vitro* and animal models; validated assays to measure immune and other host responses; standardized reagents; and access to biosafety level (BSL) 3/4 facilities. NIAID's research agenda includes training of a new cohort of investigators; establishing the physical infrastructures within which to conduct this research; and having available the technologies, animal models, and reagents necessary to pursue this line of research and clinical testing.

[1] This research does not include environmental detection, which is supported by other institutes and agencies such as the National Institute for Environmental Health Sciences, the Centers for Disease Control and Prevention (CDC), the Environmental Protection Agency, and the Department of Energy.

GENERAL RECOMMENDATIONS

The following recommendations apply to all areas of emerging infectious diseases and biodefense research. Additional general recommendations made by the Blue Ribbon Panel on the Category A Priority Pathogens can be found at http://www.niaid.nih.gov/dmid/pdf/biotresearchagenda.pdf.

Research

- Apply structure-based design, comparative genomics, and structural biology information to the development of new diagnostics and broadly based, cross-reactive therapeutics.
- Evaluate inducers of innate immunity for use as first-line therapies for biodefense.
- Develop approaches to enhance the effectiveness of vaccines in immunologically compromised populations, including the elderly.
- Examine the pathogenesis of microorganisms transmitted through aerosolization in immunized and immunologically naive animal models.
- Develop integrated approaches to understand the factors that lead to the natural emergence of infectious diseases to distinguish them from diseases that emerge through an intentional release.
- Develop methods for rapid detection of antimicrobial susceptibility/resistance.
- Expand research on polymicrobial interactions and the consequences of coinfections.
- Identify host-response profiles for early detection of presymptomatic infections.
- Investigate mechanisms by which organisms evade host immune responses.

Product Development

- Involve the Food and Drug Administration (FDA) and industry in the early planning of the development of vaccines, diagnostics, and therapeutics for biodefense.
- Develop new models that facilitate industry participation in the development of products for biodefense.

- Develop vaccines and immune-based therapies for emerging pathogens, including those that are broadly protective.
- Develop new, broadly applicable therapeutic agents.

Research Resources

- Establish cGMP and GLP facilities capable of producing monoclonal antibodies, vaccines, and other drugs and immunotherapeutics for preclinical development and clinical trials.
- Develop and standardize functional assays for measurement of human immunity.
- Establish genomics and proteomics resources for identification and comparison of new or emerging pathogens, including those that are genetically engineered.
- Ensure adequate numbers of BSL-3 facilities with aerosol-challenge capacity.
- Establish small animal and non-human primate models for emerging infectious diseases.
- Develop a network of centralized repositories for reagents and clinical specimens for emerging and biothreat infections and encourage new strategies to facilitate the shipment of infectious biological samples.
- Identify and develop potential field sites in appropriate endemic areas to study natural history, develop diagnostics, evaluate interventions, and acquire clinical materials.
- Attract new scientific disciplines, such as computational biology and bioinformatics, to biodefense research and expand the research training of a new cohort of investigators.

INHALATIONAL BACTERIA

The Category B and C bacteria with the potential to infect by the aerosol route include *Brucella* species (spp.), *Burkholderia pseudomallei, Burkholderia mallei, Coxiella burnetii,* and select *Rickettsia* spp. Most of these organisms cause zoonotic diseases or infections, i.e., infections or infectious diseases that may be transmitted from vertebrate animals (e.g., rodents, birds, livestock) to humans. The different bacteria infect humans through different routes, including ingestion, inhalation, or arthropod-mediated transmission. However, all of these agents are believed to be capable of causing infections following inhalation of small numbers of organisms. Consequently, these agents are of special concern for biodefense because they may be weaponized to be dispersed as an aerosol.

Brucellosis, caused by *Brucella* spp., is primarily a zoonotic infection of sheep, goats, and cattle, but occurs in certain species of wildlife, such as bison, elk, and deer. Human infections still occur in the Middle East, Mediterranean basin, India, and China, but are uncommon in the United States (U.S.). Natural human infection can occur following occupational exposure or ingestion of contaminated meat or unpasteurized dairy products. The incubation period is variable—from 5 to 60 days. Symptoms are diverse, ranging from acute illness with fever to chronic infections of the brain, bone, genitourinary tract and endocardium. Less than 2% of infections result in death, primarily due to endocarditis caused by *B. melitensis*. Only four of the six *Brucella* spp.—*B. suis, B. melitensis, B. abortus* and *B. canis*—are known to cause brucellosis in humans; *B. melitensis* and *B. suis* are considered more virulent for humans than *B. abortus* or *B. canis*.

Burkholderia pseudomallei, which causes melioidosis in humans and other mammals and birds, is found in soil and surface water in countries near the equator, particularly in Asia. Human infection results from entry of organisms through broken skin, ingestion, or inhalation of contaminated water or dust. Several forms of the disease exist with incubation periods ranging from a few days to many years. Most human exposures result in seroconversion without disease. In acute septicemic melioidosis, disseminated *B. pseudomallei* may cause abscesses in the lungs, liver, spleen, and/or lymph nodes. In chronic or recurrent melioidosis, the lungs and lymph nodes are most commonly affected. Mortality is high—up to 50%—among those with severe or chronic disease, even with antibiotic treatment.

Burkholderia mallei, the organism that causes glanders, is primarily a disease of horses, mules, and donkeys. Although eradicated from the U.S., it is still seen in Asian, African, and South American livestock. Natural transmission to humans is rare and usually follows contamination of open wounds resulting in skin lesions. Infection following aerosol exposure has been reported, leading to necrotizing pneumonia. Systemic spread can result in a pustular rash and rapidly fatal illness.

Livestock serve as the primary reservoir of *Coxiella burnetii*, the cause of Q fever. *C. burnetii* is highly infectious and has a worldwide distribution. Infected animals are often asymptomatic but pregnant animals may suffer abortion or stillbirth. Q fever is considered to be an occupational disease of workers in close contact with infected animals and carcasses, although infections have occurred through aerosolized bacteria in cases where close contact has not occurred. Inhalation of only a few organisms can cause infection. After an incubation period of 2 to 3 weeks, acute illness sets in consisting of fever, headache, and frequently, unilateral pneumonia. The organisms proliferate in the lungs and may then invade the bloodstream, resulting in endocarditis, hepatitis, osteomyelitis, or encephalitis in severe cases. Up to 65% of people with chronic infection may die from the disease. *C. burnetii* can remain viable in an inactive state in air and soil for weeks to months and is resistant to many chemical disinfectants and dehydration.

Typhus group rickettsiae such as *Rickettsia prowazekii* are transmitted in the feces of lice and fleas, where a form exists that remains stably infective for months. Spotted fever group rickettsiae, including *R. rickettsii* and *R. conorii*, are transmitted by tick bite. Limited studies have suggested that some rickettsial species have low-dose infectivity via the aerosol route. *R. prowazekii* and *R. rickettsii* cause the most severe infections, with case fatality rates averaging 20-25 percent due to disseminated vascular endothelial infection. The case fatality rate for *R. conorrii* and *R. typhi* infections is 1–3 percent, and infected individuals present with similar clinical manifestations including fever, headache, myalgia, cough, nausea, vomiting. A rash often develops three to five days after symptoms begin. The case fatality rate is lower in children.

Biology of the Microbes

Brucella spp. are small, non-spore forming non-motile aerobic gram-negative coccobacilli. Once inside the body, the Brucella spp. are rapidly phagocytized by polymorphonuclear cells (PMNs) and *macrophages*, but may still survive intracellularly and remain viable. The mechanism(s) by which the organisms evade intracellular killing by PMNs is not completely understood; however, it may include suppression of the PMN myeloperoxide-H_2O_2-halide system, and a copper-zinc superoxide dismutase, which eliminates reactive oxygen intermediates. Intracellular survival within macrophages may be due to the inhibition of phagosome-lysosome fusion by soluble *Brucella* products. The smooth lipopolysaccharide (S-LPS) component of the outer cell wall is the major cell wall antigen and virulence factor. Non-smooth strains have reduced virulence and are more susceptible to lysis by normal serum. The genomic sequence of one strain of B. suis strain 1330 has just been completed, and published with the sequence of a second strain associated with sheep brucellosis nearing

completion. The genomic sequence of B. melitensis strain 16M was completed and published earlier in 2002.

Burkholderia mallei and *B. pseudomallei* are both aerobic gram-negative bacilli: *B. mallei* is nonmotile while *B. pseudomallei* is motile. Very little is known about the molecular mechanisms underlying *Burkholderia* virulence. The polysaccharide capsule of B. *pseudomallei* is one important virulence factor, and toxins as well as type II lipopolysaccharides have also been proposed to play a role. The genomic sequencing of *B. mallei* is nearing completion, whereas that of *B. pseudomallei* is in progress.

Coxiella burnetii is a gram-negative, highly pleomorphic coccobacillus. It enters host phagocytes passively through existing cellular receptors, where it survives within the phagolysosome. A low pH is necessary for the metabolism of the organism. In nature, *C. burnetii* is resistant to complement and is a potent immunogen. The cell wall has an immunomodulatory activity that produces toxic reactions in mice. The genomic sequence of the Nine Mile strain of *C. burnetii* has been completed.

Rickettsiae are small, gram-negative, obligately intracellular bacteria that reside mainly in the cytosol of endothelial cells or in cells of their arthropod host. The organism undergoes local proliferation at the site of the louse bite, disseminates through the blood, and then infects endothelial cells of capillaries, small arteries and veins. Spotted fever rickettsiae spread from cell to cell by actin-based mobility, and the infected cells are injured by the production of reactive oxygen species. Typhus group rickettsiae proliferate within the cytosol until the cell bursts. The genomic sequences of *R. prowazekii* (Madrid E strain) and *R. conorii* (Malish 7 strain) have been completed, and those of *R. typhi* and *R. rickettsii* are nearing completion.

Host Response

Host immune responses to many of the inhalational bacterial pathogens in Category B are not well understood. Little is known about the contribution of innate immunity to resistance to infection or early control of bacterial replication and spread.

Infection with *Brucella* spp. leads to acquired immunity, but the duration of the response is not known. The outer membrane S-LPS is the major determinant of virulence and dominates the antibody response. Passive transfer experiments demonstrate that antibodies to S-LPS confer short-term protection. Studies of the human immune response reveal that IgM antibodies appear within the first week of infection, followed by a rise in IgG antibody after the second week. The persistence of IgG levels may be a sign of chronic infection even after treatment.

The nature of the host response to *B. mallei* and *B. pseudomallei* is relatively unknown and requires further study.

Immunity following recovery from infection with *C. burnetii* or *R. prowazekii* is lifelong in most cases. *R. prowazekii* can establish a latent infection, however, that can reactivate after years or decades. Cell-mediated immunity against *C. burnetii* appears to last longer than humoral immunity and may be a more important factor in long-lasting immunity. Cell-mediated immunity is also required for the ultimate clearance of rickettsial infections.

Vaccines

Safe, efficacious human vaccines do not exist for most of the Category B inhalational bacteria. Whole, killed vaccines against *R. prowazekii* and *R. rickettsii* are no longer available. The *R. prowazekii* vaccine was highly effective in reducing typhus deaths among U.S. soldiers during World War II. A spontaneous mutant has been used effectively in the field as a vaccine, but it can undergo reversion to virulence. Although effective attenuated, live bacterial bovine vaccines exist for *B. abortus* and *B. melitensis*, no vaccine against *Brucella* spp. is available for humans. Similarly, no human vaccines exist for glanders or melioidosis.

An Australian *C. burnetii* vaccine has been developed but it is not licensed for use in the United States. The vaccine is well tolerated, but subcutaneous administration sometimes results in severe reactions at the injection site. The vaccine also can cause severe hypersensitivity in people who have had a previous exposure to *C. burnetii,* therefore, requiring pre-vaccination skin test screening. Research is ongoing to find a safer and more effective Q fever vaccine.

Therapeutics

Standard therapeutic regimens exist for all of the Category B inhalational bacteria. Doxycycline, alone or in combination with other antibiotics, is generally considered the drug of choice. Success varies, but is generally good, with one dose of doxycycline sufficient to cure louse-borne typhus. For brucellosis, alternative drugs are available for pregnant women and children, although there is little experience with their use. Standard treatments for *B. mallei* reduce mortality but are not completely effective and have a high failure rate. However, in contrast, melioidosis responds slowly to therapy and treatment must continue for extended periods of time (up to 20 weeks). *B. pseudomallei* is resistant to many antibiotics, including aminoglycosides and beta lactams. Chronic infections of brucellosis or rickettsial diseases may require longer or repeated courses of therapy. This may be due, in part, to sequestration of organisms intracellularly where they cannot be reached by currently formulated antimicrobial drugs.

Tetracycline- and chloramphenicol-resistant *R. prowazekii* are purported to have been developed, and no other antibiotics are known to be effective for treating *R. prowazekii* and *R. rickettsii* infections.

Diagnostics

Since symptoms of acute infection with many of the Category B inhalational bacterial pathogens are non-specific and may resemble other flu-like illnesses, early diagnosis is difficult. Because early drug treatment may be key to recovery and prevention of chronic sequelae, early diagnosis is important to identify those diseases that are amenable to early antibiotic use. Unfortunately, specific, rapid diagnostic tests are not available for most of these bacteria and culture of organisms remains the most definite test. For many of the Category B inhalational bacterial pathogens, only a few reference laboratories have experience isolating these organisms. Cultures also may be time consuming, and for bacteria such as the *Brucella* species, the rate of isolation in culture ranges from 15% to 70% and may require up to 8 weeks.

Serological assays are used for brucellosis and Q fever but may be difficult to interpret as a sign of acute or chronic infection. Enzyme Linked Immunosorbent Assays (ELISA) are available for *Brucella* spp., *C. burnetii*, and *R. prowazekii.* Polymerase chain reaction (PCR) assays are being developed for brucellosis, Q fever, and other rickettsiae. Diagnosis of several of these infections by serology and PCR during acute illness has been hampered by the absence of detectable antibodies and the low quantities of organisms in the blood of many patients. *Burkholderia mallei* and *B. pseudomallei* infection can be identified by serological tests, but the organisms cannot be differentiated. Culturing of the organism is necessary for definitive diagnosis.

Research Resources

Most of the Category B inhalational bacteria cause zoonotic infections and thus infect mammals in addition to humans. Standardized animal models exist for these bacteria. For example, *Burkholderia pseudomallei* and *B. mallei* are infectious for mice and lethal for some strains providing models for virulence and testing of vaccines and therapeutics. The hamster also has been explored as a model for *B. mallei*. The mouse is a suitable model for *B. abortus* and *B. melitensis* pathogenicity and vaccine protection. Most of the Category B inhalational bacteria require BSL 3 biocontainment for propagation and animal work. There are currently three well-characterized mouse models of disseminated endothelial infection by rickettsiae that provide an excellent tool for studies of immunity, pathogenesis, and evaluation of vaccine, therapeutics, and diagnostics. *Rickettsia prowazekii* and *R. rickettsii* do not establish infections in mice, but guinea pig and rhesus models have been developed, including models for very low dose aerosol infectivity for *R. rickettsii.*

Goals for Research on Inhalational Bacteria

Immediate

- Investigate the mechanisms by which the intracellular inhalational bacteria survive.
- Further characterize the mechanisms by which the inhalational bacteria are taken up into cells and cause infection.
- Develop appropriate animal models for all the inhalational bacterial diseases, including models that incorporate aerosol challenge.
- Identify promising drug and vaccine candidates for preclinical development.
- Identify and develop potential field sites in appropriate endemic areas to study natural history, acquire clinical materials, develop diagnostics, and evaluate interventions for inhalational bacteria.
- Evaluate efficacy of antimicrobials in animal models of the inhalational bacterial diseases.
- Initiate and/or develop rapid diagnostic tests for these pathogens including point-of-care diagnostics.
- Develop microarrays for functional genomics studies of inhalational bacteria.
- Initiate and/or complete the genomic sequencing of representative members and strains of the inhalational bacteria and compare them to detect differences that correlate with pathogenesis and virulence.

Intermediate and Long-term

- Expand research on the pathophysiology of the inhalational bacterial pathogens.
- Evaluate the host response to *B. mallei* and *B. pseudomallei* to identify possible correlates of immunity and their potential relevance to vaccine development efforts.
- Identify potential vaccine candidates for *C. burnetii* and *R. prowazekii*.
- Screen licensed antimicrobials for use alone or in combination against *Burkholderia* species.
- Develop new and/or improved rapid diagnostic procedures for *Brucella* and *Burkholderia* species.

- Identify and characterize innate immune responses that occur after exposure to inhalational/aerosolized bacteria.
- Investigate the mechanisms leading to severe reactions with the current *C. burnetii* vaccine.
- Advance the development of rapid, sensitive, and specific diagnostics suitable for clinic and field use.
- Identify and develop a new generation of vaccines for Q fever.
- Investigate biological basis of chronic Q fever and brucellosis.
- Develop new approaches for evaluating drugs and therapies for intracellular inhalational bacteria.
- Develop immunologic reagents for use with non-murine animal models of inhalational bacterial diseases.
- Develop genetic systems for studies of inhalational bacteria.
- Develop cross-protective vaccines within the following groups of inhalational bacteria: *Brucella, Burkholderia,* and the typhus and spotted fever groups of *Rickettsia.*
- Develop humanized animal models for the inhalational bacteria.
- Develop novel approaches to antimicrobial therapy, including pathogenesis-blocking interventions.
- Identify the site(s) of latent *Burkholderia* and *R. prowazekii* infection and the mechanisms of reactivation.

ARTHROPOD-BORNE VIRUSES

Category B and C arthropod-borne viruses (arboviruses) that are important agents of viral encephalitides and hemorrhagic fevers, include the following:

- **Alphaviruses**: Venezuelan equine encephalitis (VEE) virus, eastern equine encephalitis (EEE) virus, and western equine encephalitis (WEE) virus
- **Flaviviruses**: West Nile virus (WNV), Japanese encephalitis (JE) virus, Kyasanur forest disease (KFD) virus, tick-borne encephalitis (TBE) virus complex, and yellow fever (YF) virus
- **Bunyaviruses**: California encephalitis (CE) virus, La Crosse (LAC) virus, Crimean-Congo hemorrhagic fever (CCHF) virus

While arthropod vectors such as mosquitoes, ticks or sandflies are responsible for the natural transmission of most viral encephalitis and hemorrhagic fever viruses to humans, the threat of these viruses as potential bioterrorist weapons stems mainly from their extreme infectivity following aerosolized exposure. In addition, vaccines or effective specific therapeutics are available for only a very few of these viruses.

Many arboviruses are endemic in North America (EEE, WEE, WNV, CE, LAC), South America (VEE, WEE), Asia (JE, CCHF), and Africa (WNV, CCHF), including others which are not listed. The most prominent in the United States at the present time is WNV, which was first identified in North America in New York City in 1999. The virus has spread throughout the continental U.S., causing thousands of cases of disease and over a hundred deaths by the end of the summer of 2002.

Natural infection of humans and other animals by an arbovirus is acquired via the bite of an infected mosquito, tick or sandfly, depending on the virus. In general, the incubation period varies from 3 to 21 days, reflecting a period during which the virus replicates locally and spreads by means of the bloodstream to peripheral sites before invading the brain or other target organ. In the brain, certain of these viruses spread cell to cell, causing encephalitis. Other viruses, such as YF and CCHF, target the liver and other organs, causing hemorrhages and fevers. Relatively little is known about the pathogenesis of these encephalitis and hemorrhagic fever viruses. However, in studies of mice exposed to aerosolized VEE, virus was detected in the brain within 48 hours after infection.

In humans, arbovirus infection is usually asymptomatic or causes nonspecific flu-like symptoms such as fever, aches, and fatigue. A small proportion of infected people may develop encephalitis and, although most recover, some may be left with severe residual neurological symptoms such as blindness, paralysis, or seizures. Clinical disease and fatality vary by the specific infecting virus. For example, less than 1% of adults infected with VEE develop encephalitis; on the other hand, the fatality rate is higher among

those infected with JE (25%) or EEE (50%) viruses. With LAC infection, disease is more severe and more common in children. However, with WNV, particularly in the U.S., older and immunosuppressed individuals are at greatest risk of developing serious or life-threatening disease. Several of these viruses, such as VEE, EEE, WNV, and JE, also represent important veterinary diseases, causing highly fatal (up to 90%) encephalitis or other symptoms in horses, birds, and other animals.

Biology of the Microbesı

The transmission cycle of the alphaviruses, flaviviruses, and bunyaviruses generally involves cyclic passage of the virus from an infected vertebrate host (e.g., bird) to an arthropod/insect vector (e.g., mosquito) during feeding of the arthropod on the host. The viruses multiply to high numbers in the arthropod, and are then passed onto and infect a new host when the mosquito feeds/bites again. The transmission cycles of arboviruses are generally not well understood, including the species of vertebrate hosts and arthropod vectors involved in natural maintenance and spread of the virus to new geographic areas and hosts.

The Category B and C arboviruses are all enveloped RNA viruses that replicate in the cytoplasm of infected cells. Viral envelope glycoproteins have been identified that are involved in binding of the virus to host cells, that function in viral tropism, and that serve as targets of host-neutralizing antibodies. The viruses also code for nonstructural proteins, such as enzymes, that are needed in the viral replication process. The number and type of viral structural and non-structural proteins is specific for each virus family; while some have been extensively studied, others have not. Genomic sequencing and other nucleic acid studies have established relationships among certain of these viruses and have led to identification of sites on genes and proteins that are important for virulence, attenuation of virulence, and associated pathogenesis. Crystallography studies of certain alphavirus and flavivirus structural proteins are providing insights into protein function and identification of potential targets for antiviral drug development.

Host Response

Infections with arboviruses elicit long-lasting immune responses. Virus neutralization by antibodies and host T cell responses likely play important roles in recovery from infection. However, host factors involved in innate and acquired immunity, as well as other parameters, such as age of the host, have not been adequately described for arbovirus infections and subsequent disease outcomes. Some specific issues regarding infection with alphaviruses, flaviviruses, and bunyaviruses are addressed below.

Natural infection with an alphavirus results in immunity to the homologous virus. Specific neutralizing antibodies to the equine encephalitis virus envelope glycoproteins (particularly the E2 protein) are identifiable within a few days of infection. Pathogenesis studies in mice suggest that protection might also be mediated by non-neutralizing antibodies that largely are directed at one viral glycoprotein (E1). Passive transfer of neutralizing antisera or monoclonal antibodies has been partially successful in reducing disease in animal studies of VEE; results vary with animal species and route of viral challenge. Data from animal studies suggest that cytotoxic T cells may also play a role in viral clearance.

With flavivirus infections, immune complex formation and production of antibodies against nerve tissue components have been reported to be associated with a poor outcome, suggesting a role for immunopathologic injury in the central nervous system. The phenomenon of immune enhancement has also been described whereby a person with preexisting non-neutralizing antibodies against one flavivirus from an initial infection displays exacerbated severe disease upon a second infection with a related flavivirus.

Host immune responses to the bunyaviruses CE, LAC, and CCHF have not been well described, although there is evidence that CCHF patients with severe hemorrhagic disease have a greater antibody response than those with milder disease. Somewhat more is known about responses to Rift Valley Fever virus and Hantavirus, both Category A bunyaviruses.

Vaccines

A limited quantity of unlicensed vaccines is available to researchers and others at high risk for infection with several alphaviruses: a live-attenuated vaccine for VEE and inactivated vaccines for VEE, EEE, and WEE are currently available from the Department of Defense (DOD) under Investigational New Drug (IND) applications. The TC-83 live attenuated VEE vaccine protects animals from aerosol challenge. However, in ongoing human clinical studies, about 20% of participants fail to mount a minimum neutralizing antibody response and another 20% develop clinical symptoms of disease. The status of the DOD Special Immunizations Program is under review; these unlicensed vaccines are no longer being manufactured and immunizations may cease to be available in the near future. Licensed vaccines for these encephalitis viruses are desirable. Thus, new products would need to be developed, manufactured and evaluated before adequate supplies could be produced in the event of widespread natural infection or man-made threat.

Licensed vaccines are available in the U.S. for two flaviviruses, YF and JE. The live, attenuated YF vaccine (17D), which has been used for many decades, is administered to military personnel, laboratory workers at risk of infection, and travelers in the general

population planning to visit countries where the disease is endemic. The JE vaccine is an inactivated, mouse brain-derived preparation produced in Japan, Korea, Taiwan, Thailand, and Vietnam. The vaccine, which has an efficacy of 91%, is administered to military personnel deployed to endemic areas and travelers considered to be at high risk. Two other JE vaccines, an inactivated cell culture-derived P-3 strain and a live-attenuated SA 14-14-2 vaccine, are only widely distributed in China. Although the supply of YF and JE vaccines is adequate for these intended purposes, manufacturing facilities would need to expand quickly to produce amounts needed for the general population in the event of a bioterrorist threat. Limited quantities of two different licensed vaccines for TBE are available for use in Europe and in Russia and other countries of the former Soviet Union.

More recently, progress has been made on the development of several vaccines against WNV for use in the U.S and elsewhere. Two chimeric live attenuated vaccines are being developed that use other flaviviruses, YF and dengue, as backbones with WNV envelope proteins. A DNA vaccine is also being developed. These candidates have shown promise in animal studies and are expected to advance to clinical trials.

At present there are no licensed vaccines available for the bunyaviruses CE, LAC, or CCHF.

Therapeutics

No specific antiviral therapies are licensed for treatment of the viral encephalitis viruses, although ribavirin has been used under investigational drug protocol to treat certain hemorrhagic fevers. Human immune globulin has been used in Israel to treat patients infected with WNV, but the effectiveness of such treatment is unknown. A clinical trial is underway in the U.S. to evaluate the effectiveness of Interferon Alpha-2b as treatment for disease caused by WNV or St. Louis encephalitis virus. Research is ongoing to evaluate chemical compounds for possible antiviral activity against WNV, VEE, YF, and other viruses.

Diagnostics

Diagnosis of encephalitis viruses is generally made by serologic antibody tests, such as ELISA or virus neutralization tests. Recovery and culture of virus varies: neurotropic viruses occasionally can be isolated from blood obtained early in the course of infection, before the onset of neurological symptoms or the development of antibodies. Reverse transcriptase PCR and/or immunohistochemistry have been used to identify viral genes and proteins of WNV, EEE, YF, and other viruses in blood, cerebral spinal fluid, or biopsied or autopsied brain or liver tissue.

Research Resources

Animal models exist for some of the viral encephalitides. The recent development of the hamster model for WNV will facilitate the testing of new vaccines and therapeutics. Standardized strains of WNV and anti-West Nile virus sera, as well as other arboviruses and corresponding antisera, are available from the CDC and through the NIAID-supported World Reference Center for Arboviruses. Work with CCHF and TBE virus complex require BSL 4 biocontainment; EEE, VEE, WN, and YF viruses require BSL 3 or higher biocontainment.

Goals for Research on Arthropod-Borne Viruses

Immediate

- Expand research on the pathogenesis and biology of arthropod-borne viral infections in animal models.
- Expand research on immune responses to these viral infections, their correlation with protection from disease, and potential for immune enhancement of disease severity.
- Initiate and/or advance the development of vaccines.
- Determine correlates of immunity and evaluate potential for vaccine-induced cross-reactive immunity and immune enhancement of disease severity.
- Expand the *in vitro* and *in vivo* screening capability for effective antiviral drugs.
- Initiate and/or develop rapid diagnostic tests for these pathogens including point-of-care diagnostics.
- Initiate and/or complete the genomic sequencing of representative members and strains of the arthropod-borne viruses and compare them to detect differences that correlate with pathogenesis and virulence.
- Exploit genomic information to design new vaccines and diagnostics.
- Develop human or humanized antibody preparations for passive immunization against arboviruses.
- Expand research on vector biology, ecology and vector control methods.
- Assess the availability of licensed vaccines and vaccine candidates, including production capacity and regulatory status.

- Initiate development of standardized reagents for use with non-murine animal models of disease.
- Attract and train new investigators in laboratory and field-based investigation specific to arboviruses.
- Identify and develop potential field sites in appropriate endemic areas to study natural history, acquire clinical materials, develop diagnostics, and evaluate interventions for arboviruses.

Intermediate and Long-term

- Expand genomic analysis, including proteomics and structural studies.
- Expand research on host factors that contribute to the pathogenesis and transmission of these viruses.
- Continue development and launch clinical trials of new vaccine candidates.
- Develop and optimize human antibodies as passive therapies for arboviruses.
- Enhance research on antiviral drugs using all available technologies (e.g., structure-based design, combinatorial chemistry and libraries, genomics).
- Advance the development of rapid, sensitive, and specific diagnostics suitable for clinic and field use.
- Expand research on the pathogenesis and biology of arbovirus infection in humans.
- Develop animal models of arthropod-borne viral diseases that incorporate aerosol challenge.

TOXINS

The Category B toxins include ricin toxin from *Ricinus communis*, epsilon toxin of *Clostridium perfringens* and Staphylococcal enterotoxin B (SEB). These protein toxins produced by bacteria are the most toxic biologic agents known. *Clostridium botulinum* toxin, the most potent of the biological toxins, is included among the Category A agents. These toxins may be delivered by a variety of routes—contamination of food and water, as well as inhalational exposure to aerosols, are all routes that pose major threats from a bioterrorist perspective.

Ricin toxin is derived from the bean of the castor plant, *Ricinus communis*. The toxin is very easy to produce in massive quantities at minimal cost in a low-technology environment. The lethality of the toxin is approximately 1,000-fold less than *C. botulinum* toxin, but it still represents a significant threat due to its heat stability and its worldwide availability as a by-product of castor oil production. Low dose inhalation among workers exposed to castor dust results in nose and throat congestion and bronchial asthma. While no data exist about higher dose inhalational exposure in humans, nonhuman primates exposed to a ricin aerosol developed severe pneumonia, acute inflammation and diffuse necrosis of the airways, and died within 36 to 48 hours of exposure. Ricin is an example of a multi-chain microbial ribosome-inactivating protein toxin. These toxins inhibit protein synthesis by acting on elongation factors (diphtheria toxin and *Psuedomonas* exotoxin A) or ribosomal RNA (Shiga toxins and ricin). By stopping protein synthesis, these toxins prevent new growth and lead to cell death.

Clostridium perfringens is an anaerobic bacterium found in soil and can infect humans and many domestic animals. Five types of bacteria exist (types A–E) that produce four major lethal toxins and seven minor toxins. Major lethal toxins include alpha toxin (associated with gas gangrene), beta toxin (responsible for necrotizing enteritis) and epsilon toxin (a neurotoxin that leads to hemorrhagic enteritis in goats and sheep). Among the seven so-called minor toxins, theta toxin appears to be the most important, is lethal, and also has been associated with gas gangrene.

Strains of *Staphylococcus aureus* have been shown to produce at least thirteen genetically and serologically distinct enterotoxins, the most widely studied of which is SEB. Although these toxins contribute to the gastrointestinal symptoms of Staphylococcal food poisoning, more severe consequences occur following aerosol exposure. Inhalation results in the rapid onset of extremely high fever, difficulty breathing, chest pain, and headache. Gastrointestinal symptoms, such as nausea and vomiting, are by comparison relatively mild. While inhalation of high doses of these toxins may result in death, much lower inhaled doses can lead to a severe, temporarily incapacitating illness. SEB is an example of a microbial superantigen toxin. Others include Staphlyococcal and Streptococcal exotoxins.

Biology of the Toxins

The ricin toxin is a 66 kd globular protein, which exists as a heterodimer. Both A and B glycoprotein chains must be associated for toxicity to be manifest. Crystal structure studies reveal a cleft in the A chain that is believed to be the site of enzymatic action of the toxin. The B chain has lectin properties that allow binding to cell surface carbohydrates. Binding of the toxin triggers endocytosis. Once in the cytoplasm, the A chain enzymatically attacks the 28S ribosomal subunit and prevents binding to elongation factor, thereby blocking protein synthesis and resulting in cell death. Ricin toxin is bound very quickly to serum proteins, metabolized before excretion, and quickly cleared.

Epsilon toxin is produced by type B and D strains of *C. perfringens*. The epsilon toxin is encoded on a large plasmid; the toxin is secreted as an inactive single polypeptide prototoxin that is activated by proteolysis in the gastrointestinal tract. The mechanism of action for epsilon toxin is not known, but it appears to increase vascular permeability in the brain, kidneys, and intestine, thus increasing its own uptake. It is unknown if strain types B and C infect humans. Other toxin types have different modes of action. The enterotoxin is a pore-forming toxin that kills enterocytes and probably gains access to tight junctions where it may alter the intestinal barrier function.

Staphylococcal enterotoxins (SE), including SEB, function as microbial superantigens; they bind to T cell antigen receptors and major histocompatibility complex (MHC) class II molecules, which results in overwhelmed T cell stimulation. The massive release of cytokines, such as interferon gamma, IL-6 and tumor necrosis factor (TNF) alpha, is likely responsible for the systemic symptoms that follow exposure. In studies of aerosolized SEB in rhesus macaques, emesis and diarrhea developed within 24 hours of exposure, followed 24 hours later by the abrupt onset of lethargy, difficulty breathing and finally death. Lymphoid tissue studies revealed depletion of B-cell dependent areas and T cell hyperplasia.

Host Response

The ricin toxin is extremely immunogenic; survivors of a ricin attack are likely to have circulating antibodies within 2 weeks of exposure. Immunization of animals with a toxoid of the native toxin or with purified a chain produced measurable antibody responses that correlated with protection from lethal aerosol exposure.

Little is known about the specific host immune response to C. *Perfringens* or toxin. Animal studies demonstrate a protective effect of toxoid and mutated toxin derivatives. Furthermore, veterinary vaccines, consisting of formaldehyde-treated bacterial cultures or filtrates, exist and are effective in preventing disease in sheep, goats and cattle.

Vaccines

No licensed vaccines against ricin toxin, *C. perfringens* epsilon toxin, or SEB are available for humans. A formalin-treated ricin toxoid and the deglycosylated A chain have been studied as candidate vaccines. Prophylactic immunization with two to three doses of ricin toxoid protected against death after inhalational exposure to multiple lethal doses of toxin in animals. Immunization of animals with the toxoid or with a preparation of the purified A chain of ricin produced measurable antibody responses that correlated with protection from lethal aerosol exposure.

Although formalin-treated SEB toxoid and vaccines produced by genetic inactivation of the toxin have demonstrated some degree of efficacy in animal experiments, they have not been approved for human use. The DOD has completed preclinical development of recombinant candidates for Staphylococcal enterotoxin A (SEA) and SEB. The vaccines are derivatives of the toxins that contain minor alterations of the amino acid sequence, retain immunogenicity, and eliminate the superantigen activity of these toxins.

Toxoid vaccines against *C. perfringens* types C and D are recommended for sheep and goats. An equine antitoxin is also available for veterinary use.

Therapeutics

No specific therapy exists for ricin toxin, *C. perfringens* epsilon toxin, or SEB. More than 150 agents have been screened *in vitro* for possible activity against ricin toxin, yet none ultimately proved useful. Efforts are underway to synthesize specific transition-state inhibitors to block the enzymatic effects of the ricin A chain. For SEB, targets to intervene in the cytokine cascade pathways have been proposed as an immunotherapeutic strategy. X-ray crystallography is also being used to identify additional binding sites for targeted drug development.

Diagnostics

Confirmation of inhalational ricin exposure is best obtained through a nasal swab within 24 hours following exposure. Identification of ricin toxin in blood and body fluids is difficult due to its rapid protein binding and metabolism before excretion.

Diagnosis of exposure to *C. perfringens* toxins has centered on culture of the organism or detection of toxins in intestinal tissues. A recently developed multiplex PCR technique allows detection of the four major toxin and enterotoxin genes present in the bacterium in a single assay.

Serum antibody titers against SE toxins are of little diagnostic value due to the widespread presence of detectable levels of antibody that cross-react with several different bacterial pyrogens. SE toxins should be identifiable in nasal swabs for at least 12 to 24 hours after exposure to a respiratory aerosol.

Research Resources

Ricin toxin, epsilon toxin of *C. perfringens,* and SEB are listed as select agents and therefore require compliance with DHHS procedures for possessing, handling and transfer of the toxins by research laboratories. Some animal models exist for these diseases, but additional models that are more reflective of intoxication of humans are needed.

Goals for Research on the Toxins

Immediate

- Collaborate with other agencies to determine research gaps related to the Category B toxins.
- Evaluate potential countermeasures for the Category B toxins.
- Attract and train new investigators in toxinology.

Intermediate and Long-term

- Support the development of improved animal models for evaluating the pathogenic effects of toxins in humans.
- Assess toxin pathology following different routes of exposures (e.g., oral vs. inhalational).
- Expand research on the biophysical and chemical properties that contribute to severity of disease and toxicity for the Category B toxins (e.g., microbial superantigen toxins, multi-chain microbial ribosome-inactivating protein toxins, and *C. perfringens* toxins).
- Identify, characterize, and compare basic biology (cell receptors, internalization, translocation, and trafficking) of the staphylococcal/streptococcal families of microbial superantigen toxins.

- Apply current knowledge of the mechanisms of superantigen activation and related downstream signaling events to the identification of new therapeutic targets for exposure to microbial superantigen toxins.
- Focus studies of C. *perfringens* on the identification of toxins, assessment of potency via different routes, and explore the possible synergistic interactions between C. *perfringens* toxins.
- Develop genomic- and proteomic-based tools to characterize and identify biosignatures and other indicators of toxin exposure.

FOOD- AND WATER-BORNE PATHOGENS

The public health surveillance activities, along with the sewage and water treatment infrastructure and food safety regulations in the U.S., are the first defense against a deliberate contamination of water or food. The centralized production and wide, rapid distribution of food products has increased the risk for outbreaks of disease that can affect large geographical regions of the country. Furthermore, globalization of the food supply increases the potential for exposure to a greater variety of foodborne pathogens. Clearly, water and food are potentially important routes for the dissemination of infectious agents by bioterrorists. A troubling 1984 outbreak of salmonellosis in Oregon illustrates this point: Investigations revealed that members of a religious cult had deliberately contaminated salad bars in area restaurants, resulting in 751 reported cases of illness.

Enteric infections can result from bacterial, viral, or protozoal contamination of food and water. In this chapter, the unique goals and recommendations of each class of organism are addressed in separate sections. The approach used to prioritize the research activities for this large category of pathogens is based on several criteria including availability (e.g., ease of propagation), inoculum size needed, stability in the environment, lethality, degree of incapacitation caused by the disease, possibility of secondary transmission, and availability of countermeasures (e.g., vaccines, therapeutics).

Food- and Water-borne Bacteria

The Category B food- and water-borne bacteria include diarrheagenic *Escherichia coli*, pathogenic *Vibrio* spp., *Shigella* spp., *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, and *Yersinia enterocolitica*. Many of these pathogens are zoonotic, i.e., infections or infectious diseases that may be transmitted from vertebrate animals (e.g., rodents, birds, livestock) to humans.

Nearly all natural infections with these pathogens occur following ingestion of the organism in food derived from infected food-animals or contaminated with feces of an infected animal or person, or through ingestion of contaminated water, or raw or undercooked food and dairy products. Person-to-person spread from close contact with primary cases is also a source of infection. In addition to acute illness, usually characterized by acute diarrheal disease, some of these pathogens are also associated with more serious sequelae or chronic infection. For example, *E. coli* O157:H7 or other strains of Shiga toxin producing *E. coli* (STEC) may lead to hemolytic-uremic syndrome (HUS), and *Shigella dysenteriae* and STEC may lead to dysentery. Certain serotypes of *C. jejuni* have been associated with the development of Guillain-Barrè Syndrome, an acute flaccid paralysis. *Salmonella typhi*, the cause of typhoid fever, can become a chronic infection leading to asymptomatic carriage. Infection of pregnant women with *L. monocytogenes* can result in abortions or stillbirths. Many of these pathogens can induce reactive arthritis.

The fact that most of these diseases occur sporadically in the U.S. at some frequency may make an intentional exposure difficult to recognize quickly. Improved and active public health surveillance characterized by coordinated activities and good communication between local and State health departments and the CDC will be the best monitor for such an event. Availability of improved diagnostics, effective therapeutic strategies, and new preventive measures are the objectives of the NIAID research agenda regarding these organisms.

Two of these bacterial pathogens, *S. dysenteriae* 1 (Shiga bacillus) and STEC, stand out as the most likely potential bioterrorist threats. These two pathogens, which can cause particularly severe disease that is sometimes fatal, infect by ingestion of a few organisms and are transmitted through person-to-person contact. Transmission to humans occurs through ingestion of contaminated food—frequently, inadequately cooked beef and raw milk. Waterborne transmission is also possible. In the case of STEC, cattle can serve as a source of infection. Each of these pathogens has been responsible for large-scale epidemics. *S. dysteneriae* 1 is one of the few bacterial pathogens capable of producing a pandemic outbreak.

Biology of the Microbes

Food- and water-borne pathogens cause disease by colonization of the intestinal track. Some of these pathogens such as enterotoxigenic *E. coli* (ETEC) and *Vibrio cholerae* remain in the intestinal lumen and secrete toxins that lead to diarrhea. Other pathogens such as *C. jejuni*, and *Y. enterocolitica* may cause primarily inflammatory diarrhea, although enterotoxins may also play a role in pathogenesis. *Shigella*, *Salmonella*, *L. monocytogenes* and enteroinvasive *E. coli* (EIEC) invade and damage intestinal mucosa or deeper tissues.

The classic secretory toxin, cholera toxin, is produced by *V. cholerae*. It is a heat-labile enterotoxin that activates adenylate cyclase in the small intestine, resulting in alterations of ion transport across the intestinal epithelium, which leads to secretion of chloride and water by intestinal crypt cells. The result is profuse, watery, rapidly dehydrating, and often life-threatening diarrhea. ETEC organisms secrete one or both of two toxins. One is similar to that of *V. cholerae* and is referred to as LT; the other is a heat-stable toxin (ST) that activates guanylate cyclase. Like cholera, ETEC infection causes a secretory profuse diarrhea.

The classic cytotoxin is produced by *S. dysenteriae* 1 and STEC. Shiga toxin (Stx) may play a role in the destruction of mucosa in the colon and the resulting dysentery caused by this organism. The toxin inhibits protein synthesis and leads to cell death. The elaboration of Stx depends upon the presence of certain phages carried in the bacteria.

Attachment to the gastrointestinal mucosa is a critical step in the pathogenesis of these bacteria. Diarrheagenic *E. coli* must adhere to and colonize the upper intestine before causing disease. The specific adhesion molecules that mediate this attachment are an attractive target for immunization and have been the focus of intensive study. Similarly, invasive bacteria, such as *Shigella* and *Salmonella* spp., must first adhere before beginning the invasion process. While interfering with attachment may be a good vaccine strategy for some vaccines, live attenuated vaccines must undergo some degree of colonization to become sufficiently immunogenic.

Organisms capable of invading the intestinal mucosa have the advantage of escaping immune surveillance and have devised strategies to survive and multiply inside infected cells. *Shigella*, invasive *E. coli*, *Y. enterocolitica, L. monocytogenes,* and *Salmonella* are intracellular pathogens. For certain bacteria like *Shigella*, cytotoxic exotoxins contribute to the destructive properties of the organisms. For others, the goal is to remain in cells without killing them. In these cases, inflammatory cytokine responses often are involved in pathogenesis and apoptosis may lead to the demise of host cells. In other cases, such as *L. monocytogenes*, the pathogen uses host cell systems to its advantage. *Listeria* directs the polymerization of cellular actin at one end of the bacterium as a form of motility and the energy needed for cell-to-cell spread.

The genetic basis for the pathogenicity of these organisms is partially understood, particularly since many have been sequenced. Comparative sequencing of strains exhibiting varying degrees of pathogenicity will lead to identification of new virulence determinants. It is clear that virulence genes exist in chromosomes and as well as on mobile elements such as plasmids and phages. The degree of gene transfer among this group of organisms is striking and leads to the continuing evolution of new strains. This was evident in the emergence of the epidemic O139 serotype of *V. cholerae* that contained a completely new LPS biosynthetic pathway as a result of transfer and incorporation of the entire operon into the chromosome.

Host Response

While there are unique features of the immune response to each of the enteric pathogens, protection appears to stem from a mixed immune response consisting of secretory IgA and systemic IgG. Cellular immune responses may also be particularly important for intracellular pathogens. There has not been extensive study of the contribution of the innate immune system to protection.

Invasion of epithelial cells by *Salmonella or Shigella* spp., *Y. enterocolitica, L. monocytogenes,* or EIEC triggers a rapid release of cytokines that leads to inflammation. The immune response to some of these pathogens is of particular interest because of its association with reactive arthritis. While certain host response

pathways are induced following infection, bacterial proteins have been identified that have the capacity to inhibit other portions of the host immune response.

Infection with *S. typhi* or *V. cholerae* results in long-lasting immunity against reinfection. *Salmonella typhi* vaccination or illness results in systemic and intestinal antibody production. The specific antigens associated with protection, however, have not been identified, although Vi and O polysaccharides are important. Vaccine studies also point to the importance of intestinal IgA and cellular responses. Further research is needed to more precisely identify the *S. typhi* protective antigens. Historically, most *Salmonella* spp. research has focused on *S. typhi* or *S. typhimurium* in mice as models of typhoid fever. Additional research on vaccines for non-typhoidal *Salmonella* and *S. paratyphi*, as agents responsible for foodborne diarrhea, is also needed.

Infection provides protection only against related or homologous strains or types of other organisms. For example, initial infection by *V. cholerae* 01 of the classical biotype confers protection against either the classical or El Tor biotype, while protection following initial infection with El Tor is limited to that biotype. Immunity to *V. cholerae* serotype O1 does not protect against the other epidemic strain, O139, demonstrating that antibody against bacterial LPS is needed for protection. The best correlate of protection is serum vibriocidal IgG antibody.

Lasting immunity against related strains of *C. jejuni* follows infection; in developing countries, most people acquire immunity in the first 2 years of life. Studies demonstrated that volunteers rechallenged with the homologous *C. jejuni* developed infection but were protected against illness. The specific immune responses necessary for protection against *C. jejuni* remain to be elucidated.

Similarly, persons infected with ETEC acquire serotype-specific immunity. Wide-spectrum ETEC immunity requires multiple infections with organisms of different serotypes, explaining the lack of gastrointestinal symptoms among adult residents of areas associated with traveler's diarrhea. Vaccine studies have shown that protection against ETEC correlates with levels of intestinal IgA specific for colonization factor antigens. For STEC, immune responses against the colonization factor intimin may be important in control of infection. Intestinal IgA is also important in controlling *Shigella* infection; patients recovered from bacillary dysentery due to *Shigella* develop a relative but not absolute immunity to reinfection.

Cell mediated immunity is central to an effective immune response against *L. monocytogenes*, consistent with the organism's role as an intracellular pathogen. Infection with *L. monocytogenes* in immunocompromised individuals, particularly pregnant women, can cause a high degree of morbidity and mortality. The lack of disease among the young and healthy, but exposed, population argues for the

presence of protective immunity. The mouse model of *L. monocytogenes* infection continues to yield important information on host responses to infection.

Vaccines

Few licensed vaccines exist for Category B food- and water-borne bacterial pathogens. Available vaccines have differing efficacy and their use within the U.S. is limited primarily to individuals traveling to endemic areas. However, vaccines might be useful in preventing secondary spread in the event of large community outbreaks and may be of value to the military and first responders.

Vaccines against *S. typhi* generally have been restricted to preventing typhoid fever among travelers and military personnel visiting endemic areas, household members of carriers, and laboratory workers. Two vaccines, a Vi polysaccharide vaccine (ViCPS) developed by the National Institute of Child Health and Human Development (NICHD) and licensed by Pasteur Merieux, and an oral, live-attenuated vaccine (Ty21a) licensed by Berna Biotech, are currently available in the United States. The ViCPS vaccine is given in one IM dose and is protective for 2 years. The oral TY21a vaccine is taken in four doses and offers protection for 5 to10 years. A third licensed vaccine, a parenteral, heat-phenol-inactivated whole cell formulation, is no longer recommended because of reactogenicity. The military also has access to an acetone-inactivated parenteral vaccine. Although each of these vaccines is somewhat effective, none provides total protection; efficacy ranges between 50% and 90% in different studies. Although primarily used as a vaccine to prevent traveler's diarrhea, some studies of Ty21a have not demonstrated efficacy for this purpose. Recently there have been problems with supply of the Ty21a vaccine.

Additional *S. typhi* vaccine candidates have been evaluated in clinical trials. Investigators at the University of Maryland (UMD) have been examining several live attenuated vaccine candidates. Other researchers have developed a series of live attenuated strains. Most of these vaccines have not shown the required combination of safety and immunogenicity. NICHD is testing a conjugate of Vi polysaccharide with protein carriers. Avant is developing a live attenuated vaccine called TY800; phase I trials are expected to begin within the next year. Unfortunately, attempts to use *S. typhi* or other *Salmonellae* as live vectors for multi-valent vaccines have been disappointing, to date.

There are currently no licensed human vaccines available for *E. coli*. Experimental vaccines against ETEC have focused on stimulating immunity against colonization factor antigens (CFAs). A killed whole cell + CTB ETEC vaccine is being produced by Swedish Biological Laboratories (SBL) and tested in trials around the world. A number of additional ETEC vaccines are being investigated, including CFA constructs in

attenuated *Shigella* and *Salmonella*, and *E. coli* LT-B subunit expressed in foods. The NIAID has plans to test a CFA antigen in microspheres in combination with an altered (non-toxic) cholera toxin adjuvant. This vaccine was shown to be poorly immunogenic in Phase I trials in the absence of an adjuvant. There are no vaccines available against other pathogenic *E. coli.* The colonization factor, intimin, is a particularly intriguing antigen, given its critical role in mediating both the attachment and the mucosal effacing lesion induced by STEC. An animal vaccine consisting of intimin expressed in corn is in development.

No licensed vaccines are currently available for *Shigella*. Experimental, live attenuated oral vaccines have undergone limited testing. In general, obtaining sufficient immunogenicity without reactogenicity has been a problem with *Shigella* vaccines. Scientists at the Pasteur Institute have created a promising live attenuated *S. flexneri* 2a vaccine that protected a small number of volunteers against homologous challenge. Investigators at UMD are also pursuing live attenuated mutants of *S. flexneri* in clinical trials supported by NIAID. A vaccine developed at Walter Reed Army Institute for Research against *S. sonnei* (WRSS1) has been in phase I trials and shows promise. The DOD is planning field trials of this vaccine. Investigators at NICHD are also studying parenteral polysaccharide conjugate vaccines, using the detoxified O antigen. This conjugate vaccine is being pursued in field trials in Israel. DOD investigators have also examined subcellular nucleoprotein and LPS-proteosome vaccines. There are many potential vaccine candidates and approaches for vaccines against *Shigella* strains, but a successful strategy has been an elusive goal.

An older cholera vaccine, licensed in the U.S., has been discontinued because it offered only brief and incomplete immunity and was reactogenic. Two new vaccines, a killed oral and live attenuated recombinant strain, have been developed and licensed elsewhere for use by travelers but are not yet available in the U.S. The killed whole cell plus cholera toxin B subunit vaccine is produce by SBL in Sweden. It has been licensed in Scandinavian countries. The live attenuated vaccine is produced by Berna Biotech in Switzerland and has been licensed in some European countries and Canada. Neither of these vaccines is available in the U.S. Whether these vaccines will induce long-lasting protection in endemic populations has not been adequately tested. They will find application as travelers' vaccines. Additional live attenuated vaccine candidates are in clinical trials and have shown promise to date in North American volunteers. Peru 15 is one of those candidates that is about to start trials in the endemic country of Bangladesh.

There currently are no licensed vaccines for *C. jejuni, L. monocytogenes,* or *Y. enterocolitic*a. A killed vaccine against *C. jejuni*, which contains a mutated cholera toxin adjuvant, has been developed and tested by the DOD. Live attenuated strains of *Y. enterocolitica* are being studied as oral vaccines as well as carriers for heterologous

antigens. Vaccine studies have focused on bacterial lipopolysaccharides, such as heat shock proteins.

Therapeutics

Replenishment of fluids and electrolytes is the critical first step in treatment of diarrheal disease. This is particularly true for treatment of *V. cholerae* infection, where enormous, life-threatening fluid and electrolyte losses can develop within hours after the onset of symptoms. While oral rehydration therapy (ORT) continues to save millions of lives each year, it does not help limit transmission of the disease or treat dysentery. Antibiotics may also be helpful in limiting the duration and severity of cholera symptoms. Early administration of an effective antibiotic can affect the severity of *Shigella* infections, so early diagnosis and antibiotic sensitivity testing is important. Antibiotics should be considered for treatment of other enteric infections following identification of the pathogen and determination of its sensitivity profile. Avoidance of antibiotic treatment of STEC infections has been recommended, although study of the effectiveness of antibiotics that can be demonstrated *in vitro* not to induce Shiga-toxin encoding phage seems warranted. Careful monitoring and supportive care is called for to prevent or treat HUS.

Antibiotic resistant strains of food- and water-borne pathogens are emerging as a serious public health issue. Antibiotic resistance in enteric bacteria is being monitored by the National Antimicrobial Resistance Monitoring System (NARMS) and The Foodborne Disease Active Surveillance Network (FoodNet). The following notable findings were observed in 2000: increases in decreased susceptibility and resistance to ciprofloxacin by multiple species of *Salmonella* and increases in the resistance of *Salmonella* species to multiple antibiotics. The prevalence of fluoroquinolone (FQ) resistant *Campylobacter* spp. also has increased. Whereas no resistance to FQ was detected in *Campylobacter* strains in 1990, the incidence rose to about 3% in 1998 and 14% in 2000. Studies suggest that infection with FQ-resistant strains of *Campylobacter* is associated with a prolonged duration of diarrhea. The incidence of antibiotic resistant *E. coli* and *Shigella* strains is also of concern. In addition to the development of new classes of antibiotics, methods to block the acquisition of new antibiotic resistance determinants should be explored.

Two monoclonal antibodies capable of neutralizing either Stx I (produced by *S. dysenteriae* and some strains of STEC) or II (produced by some strains of STEC) have been produced under NIAID contract and are awaiting Phase I safety and pharmacokinetic studies. This product and others in development may be useful in reducing the severity of serious sequelae following infection with *S. dysenteriae* or STEC. Moreover, these antibodies may be beneficial in a Shiga toxin exposure situation.

Diagnostics

Because it is difficult to distinguish the etiological agent from clinical symptoms alone, confirmatory diagnosis is essential for most enteric diseases. The definitive diagnosis for all food- and water-borne Category B bacteria is microbiological culture, which provides the demonstration of viability and isolated organisms for trace-back and other molecular studies. Additionally, the widespread dissemination of multi-drug resistant organisms makes antimicrobial sensitivity testing an essential part of the identification process. While PCR and DNA probes are available for many bacterial pathogens, the assays are limited to research and reference laboratories. Currently, there are no syndrome-based diagnostics (diarrhea and fever) that can identify bacteria, viruses and protozoa in stool and vomitus. Rapid advances in instrumentation technology and the recent availability of genomic sequencing data should stimulate diagnostics development.

Research Resources

None of the Category B food- and water-borne organisms are select agents that require special BSL biocontainment, although Stx itself is a regulated toxin that must be registered with the CDC if quantities on hand exceed a certain amount. A standard reference collection of well-characterized strains of STEC, protocols, and databases are available through the NIAID-funded "Shiga Toxin Producing *E. coli* Strain and Data Repository" (http://www.shigatox.net/stec/index.html).

Goals for Research on Food- and Water-borne Bacteria

Immediate

- Accelerate clinical development of existing *Shigella* vaccine candidates.
- Evaluate licensed antimicrobials for treatment of *Shigella* and STEC infections.
- Expand research on pathogenesis of understudied food- and water-borne bacteria including *Campylobacter, Listeria,* and non-typhoidal *Salmonella* spps.
- Study innate immune responses and their role in combating infection with food- and water-borne bacteria.
- Develop improved diagnostic assays for enteric Category B agents that focus on detection of virulence factors, such as direct detection of toxins.
- Identify potential field sites, including overseas sites, where food- and water-borne diseases are endemic to test new vaccines, diagnostics, and therapeutics.

- Develop syndrome-based diagnostic tests that can identify pathogens (bacteria, viruses, protozoa) in patients presenting with diarrhea or fever.

Intermediate and Long-term

- Support development of new candidate vaccines against *S. dysenteriae* 1 and STEC.
- Develop treatments for the prevention of HUS.
- Establish databases of the molecular characterization (e.g., MLST, PFGE, SNP) of potential enteric bioterrorist agents.
- Enhance the understanding of the evolution of bacterial pathogens and the mechanisms of horizontal transfer of accessory elements (e.g. phage, plasmids, transposons) involved in pathogen biology.

Food- and Water-borne Viruses

The Category B food- and water-borne viruses include hepatitis A virus (HAV) and the caliciviruses, such as Norwalk and related viruses. They present bioterrorist threats due to their potential rapid and widespread dissemination, their high level of infectivity, and their morbidity.

Hepatitis A virus causes about 55% of the cases of hepatitis seen in the U.S. annually. Disease is rarely seen in less developed countries, where infection and resulting immunity usually develops by age two or three. Transmission occurs person to person, through contaminated water or food, or by infected food handlers. HAV can survive 3 to 10 months in water. Disease is characterized by abrupt onset of fever followed by bilirubinemia and jaundice. The disease is self-limited, usually resolving in one to two weeks; infection does not result in chronic liver disease and severe cases are rare. HAV superinfection of patients with chronic hepatitis B or C or underlying liver disease increases the mortality rate significantly.

Caliciviruses, including Norwalk, have been identified as a cause of diarrheal disease transmitted by contaminated water or food such as shellfish. The acute vomiting and/or diarrhea usually last only one to three days. Outbreaks may occur in closed settings such as camps, hospitals, ships, and nursing homes. The incidence of person-to-person spread in these settings is high. Caliciviruses have been associated with about one-third of nonbacterial acute gastroenteritis outbreaks in the U.S. The viruses have been identified worldwide: Children in Bangladesh, Ecuador and the Philippines acquire antibodies to Norwalk virus early in life. Antibodies are also found in most adults in the U.S.

Biology of the Microbes

Hepatitis A virus, a member of the Picornaviridea family, is unenveloped and contains single stranded, linear RNA. The capsid is comprised of four polypeptides (VP1 - 4), of which VP1 and VP3 contain the primary neutralization sites. The life cycle of the virus is well understood, as are most molecular aspects of the virus. The exact mechanism by which HAV causes liver damage has not been elucidated and may involve a cell-mediated immune response. The genome of HAV has been completely sequenced.

The caliciviruses are unenveloped particles comprised of a single structural polypeptide with a single strand of positive sense RNA. There are three open reading frames, the first coding for viral RNA polymerase, helicase and protease, and the second coding for the viral capsid protein; the function of the third is unknown. Although the intestinal histopathology of calicivirus infection has been described, the mechanisms underlying the vomiting and diarrhea are not known; no enterotoxin has been identified. There are

many serotypes of viruses: Norwalk, Hawaii, Snow Mountain, and Lordsdale viruses are frequently identified in outbreaks. Their shedding from infected individuals for prolonged periods after symptoms have resolved, the low infectious dose, and their persistence in the environment make them particularly good potential agents of bioterrorism.

The genomes of Norwalk and many other caliciviruses have been completely sequenced and their capsid proteins expressed and purified. These capsids self assemble into virus-like particles that have been used to generate type-specific antibodies that can be used in detection and typing assays. PCR primer sets have also been developed for detection and typing assays. These reagents are used for research purposes and have not been applied for public health or widespread surveillance activities. The availability of these reagents has led to a recent appreciation of the diversity, ubiquity, and significance of the caliciviruses to the health care burden attributed to this class of viruses.

Host Response

Infection with HAV confers lifelong immunity. Titers of serum neutralizing antibodies are high in convalescing patients, beginning to rise about one month after infection; the role of mucosal or cellular immunity is not clear. Passive immunity is provided by immune serum globulin for up to 3 months.

Immune responses to caliciviruses are not well documented. Natural infection results in virus-specific serum IgG, IgA, and IgM, resulting in resistance to disease by homologous virus for several months. Only the IgG antibodies persist, but the duration of protection is not clearly defined. Mucosal and cellular immune responses to Norwalk and related viruses are not well studied. Additional work is needed and NIAID is supporting the preparation and testing of a new Norwalk virus challenge pool that can be used in clinical studies of pathogenesis and determination of vaccine efficacy. Similar challenge pools for other caliciviruses would be helpful in future vaccine studies and for measuring the cross protection afforded by vaccine candidates.

Vaccines

Two vaccines for HAV are licensed in the United States, with several others available throughout the world. These vaccines contain inactivated virus particles and provide protection for 10 or more years. The cost of the vaccines currently prevents universal immunization.

There is no licensed vaccine for Norwalk or other caliciviruses. Current approaches to vaccine development include purified virus-like particles and viral capsid protein

expressed in transgenic plants as edible vaccines. The lack of identified long term immunity after natural infection and the presence of multiple serotypes will present challenges to vaccine development. An association between blood group antigens and susceptibility to infection with particular strains of calicivirus also makes the development of a vaccine approach more difficult.

Therapeutics

No specific antiviral therapies are available for HAV or calicivirus infection. Both diseases are self-limited and rarely severe. Replacement of fluids and electrolytes is recommended for Norwalk virus and other calicivirus infections.

Diagnostics

Hepatitis A virus infection is diagnosed primarily by detecting IgM in serum; IgG without the presence of IgM indicates prior infection and protective immunity. Active HAV infection can also be detected by electron microscopy of stool samples. PCR is not routinely used to confirm a diagnosis, but its sensitivity makes it useful for identifying environmental contamination and for tracing outbreaks. HAV is difficult and expensive to culture, and is too slow to be useful for diagnoses.

The identification of caliciviruses in clinical specimens is not routine. Immunoelectron microscopy can detect calicivirus in stool samples in a research setting. PCR assay, ELISA, and radioimmunoassay (RIA) are available that can be useful for detection and typing, but these assays are also more typically used in a research setting. Development of rapid assays that can by used widely is a goal.

Research Resources

Neither hepatitis A virus nor the caliciviruses are select agents requiring special biocontainment. Hepatitis A virus is difficult to culture; and Norwalk and other caliciviruses cannot be cultured.

Goals for Research on Food- and Water-borne Viruses

Immediate

- Pursue Phase I testing of candidate calicivirus vaccines.
- Characterize the available challenge pool for Norwalk virus that can be used in future vaccine efficacy studies.

- Develop and evaluate rapid, broadly-reactive diagnostics for identifying caliciviruses, including those capable of distinguishing animal and human caliciviruses.
- Investigate immune responses to Norwalk virus and other caliciviruses.

Intermediate and Long-term

- Explore the relationship between blood group antigens and susceptibility to caliciviruses.
- Investigate methodologies for culturing caliciviruses (both *in vitro* and animal models).
- Use genetic information to identify antigens for development of cross protective calicivirus vaccines.
- Explore the reasons for increased pathogenesis of HAV in persons over 50 years of age.
- Continue to support development of alternative, less expensive HAV vaccines.
- Develop challenge pools for additional calicivirus serotypes.

Food- and Water-borne Protozoa

Enteric protozoa and protists are included among the category B agents due to their potential for dissemination through compromised food and water supplies in the United States. Many of these organisms infect domestic and wild animals. These organisms include the protozoa *Cryptosporidium parvum*, *Cyclospora cayetanensis, Giardia lamblia, Entamoeba histolytica,* and *Toxoplasma gondii*, and the protists Microsporidia species such as *Encephalitozoon* and *Enterocytozoon*. Although infections by most of these organisms are usually asymptomatic or self-limiting in otherwise healthy persons, clinical symptoms occur in immunosuppressed persons.

The most important organisms in terms of bioterrorist potential include *C.parvum*, *E. histolytica* and *T. gondii*. These organisms can infect large numbers of people through contaminated water and/or food. In addition, all these infections (with the exception of toxoplasmosis), can be easily transmitted person-to-person and are difficult to diagnosis. Also most can be genetically manipulated to increase virulence or resistance to anti-infectives.

Biology of the Microbes

The life cycles of most Category B food- and water-borne protozoa and protists are well understood. However, experimental studies of some of these organisms are limited by difficulties with *in vitro* cultivation and by the lack of animal models.

Ingestion of *C. parvum* oocysts leads to infection of intestinal epithelial cells, where the organism replicates within protective vacuoles. Because autoinfection can occur when released oocysts are released from the cells, ingestion of only a few oocysts can lead to severe and persistent infections in immunocompromised patients. The mechanism of pathogenesis is not well understood, but *C. parvum* may disrupt intestinal ion transport. Two distinct genotypes of *C. parvum* infect humans, with the sequencing of genotype I almost complete and work on genotype II in progress.

Cyclospora cayetanensis was identified in association with diarrheal disease in 1979 although its taxonomical classification was not resolved until 1993. Oocysts are the infectious form and are resistant to both freezing and chlorination. The oocyst contains two sporocysts that each hold two sporozoites. Infection of the small intestine can result in atrophy of the villi and inflammatory infiltration of the lamina propria. It is not known whether *C. cayetanensis* pathogenesis is due to a direct effect on enterocytes or involves a secreted toxin.

The trophozoite form of *G. lamblia* colonizes the small intestine after ingestion of as few as 10 to 25 cysts. The trophozoite consists of four flagellae and a sucking or adhesive disc, including microtubular structures that serve as important antigens for host recognition. The mechanism of adherence to epithelium is uncertain, but may involve specific receptors. Trophozoites undergo antigenic variation by changing a cystein-rich surface protein to variant specific surface protein (VSSP); these surface proteins also bind metals, such as zinc, that are important for brush border enzymes. Cell-mediated immune responses may play a role in histological damage of the intestine; no enterotoxin has been identified. There is a genome project for *G. lamblia* and gene expression data are also available.

Like *Giardia,* the life cycle of *E. histolytica* consists of trophozoites and cysts. Information about the pathogenesis of *E. histolytica* has been expanding rapidly due to development of new culture media. Adherence to intestinal epithelium is critical in pathogenesis as trophozoites kill target cells only on direct contact; adherence is mediated by the parasite's surface lectin. Other parasitic factors have been identified that degrade secretory IgA, mucins, and other host cell surface glycoproteins, and contribute to cell killing. Sequencing of the *E. histolytica* genome is in progress.

Toxoplasma gondii exists in three forms: oocysts, tissue cysts containing bradyzoites, and tachyzoites. Oocysts form only in the intestines of infected cats. Following ingestion, sporozoites, released from oocysts, penetrate and multiply in intestinal epithelial cells. Invasion of epithelial cells appears to be mediated via the conoid, a cone-shaped structure on the tachyzoite. Tachyzoites are contained within vacuoles within the epithelium, protected from lysosomal fusion, and destroy the host cell before spreading to lymph nodes and other tissues. Cyst formation occurs in infected tissues, including brain, retina, and muscles. Delayed-type hypersensitivity reactions result in rupture of the tissue cysts and necrosis of surrounding tissue, which can be clinically important in the retina. In immunocompromised hosts, reactivation can lead to significant tissue damage and result in death. Transplacental infection can also occur, and fetal infection occurs in 30% to 40% of women first infected with *T. gondii* during pregnancy. Genomic sequencing of *T. gondii* is in progress, with an extensive database of genomic and EST sequences now available.

Microsporidia are a unique group of intracellular, spore-forming protists. Microsporidia species that infect humans include *Encephalitozoon intestinalis, Enc. hellem, Enc. cuniculi*, and *Enterocytozoon bieneusi*, which is resistant to therapy. The spore consists of a resistant wall, one or two nuclei, sporoplasm, an anchoring disk, and a spiral coiled polar tube. During infection, the polar tube everts, piercing the host cell and injecting the sporoplasm. Replication results in an increasing number of mature spores, which eventually rupture the cell. As with *C. parvum*, the potential for autoinfection increases production of the spores. Infection is usually limited to the intestine except in immunocompromised individuals where many tissues may be involved. The complete genomic sequence of *Enc. cuniculi* has been completed and sequencing of *Ent. bieneusi* is planned.

Host Response

Immune responses to food- and water-borne protozoa result in varying degrees of immunity. For example, infection with *E. histolytica* or *T. gondii* results in long-lasting protection in immunocompetent individuals, while infection with *C. parvum* or *G. lamblia* leads to partial immunity. Little is known about host immune responses to Microsporidia or *Cyclospora*. Although systemic antibody responses have been detected, their role in controlling disease is not known.

Specific host immunity against *C. parvum* is poorly understood. Interferon gamma, interleukin (IL) 5 and IL-12, as well as CD4+ and CD8+ cells appear to be important in the immune response. Although specific antigens have been identified, antibodies to these have not been correlated with protection.

Secretory IgA appears to play an important role in the control of *Giardia*, possibly through binding to trophozoites and preventing adherence. Failure to develop an intestinal IgA response has been correlated with chronic giardiasis in humans. Cell mediated immunity helps clear the parasite by coordinating the IgA response and possibly through anti-IgA cytotoxicity. Individuals who have been cured of colitis or liver abscess due to *E. histolytica* appear to be immune to recurrent or invasive amebiasis. Cell mediated immunity, particularly macrophage cytotoxicity and secretory IgA, appears to be important in limiting invasive disease. The role of antibodies is less clear. Six highly conserved antigens have been identified and serve as potential targets for vaccine development.

Immunocompetent individuals previously infected with *T. gondii* appear to be immune to development of disease after subsequent reinfection. Infection stimulates both cellular and humoral responses. Cellular immunity involves Th1 helper T cells, CD4+ and CD8+ T cells, natural and lymphokine-activated killer cells, and gamma-delta T cells. Within the central nervous system, astrocytes and microglia also are important. Although antibodies are produced in response to infection, animal studies suggest that they offer limited protection.

Vaccines

The lack of understanding of the basic mechanism of pathogenesis and the lack of appropriate *in vitro* culture techniques and animal models have hindered vaccine development for most of the Category B food- and water-borne protozoa. However, some progress has been made with *E. histolytica* and *T. gondii*. Experimental recombinant vaccines capable of eliciting cell mediated immunity, secretory IgA or humoral responses against *E. histolytica,* have been studied. The galactose-inhibitable amoebic lectin involved in adherence is a prime target for vaccine design and has shown some promise in animals. Similarly, animal studies using a temperature-sensitive *T. gondii* mutant are encouraging. A major surface antigen, p30, has been cloned and shown to stimulate cytotoxic lymphocytes *in vitro* and to protect mice against *T. gondii* challenge.

Therapeutics

Since disease caused by the Category B food- and water-borne protozoa and protists is usually self-limited, immunocompetent individuals are generally not treated. However, treatments are needed for immunocompromised individuals. A number of drugs have been tested or used to treat protozoa infections or reactivation in HIV-positive populations, although new, less toxic regimens are needed. In addition, new therapies for *Ent. bieneusi* and drug resistant *G. lamblia* are also required. Treatment

of pregnant women infected with *T. gondii* remains problematic because of the potential teratogenic effects of otherwise effective drugs.

Diagnostics

Identification of the organism in fecal smears is the primary diagnostic method for *Cryptosporidium, Cyclospora, Giardia, E. histolytica*, and Microsporidia. While *Ent. bieneusi* can be distinguished from other Microsporidia in stained smears, electron microscopy or PCR are important to confirm exact identity. Commercial immunofluorescence (IF) and ELISA assays for Cryptosporidium and *Giardia* and a serum ELISA test for *E. histolytica* are also available. PCR assays are being developed for *Cryptosporidium, Cyclospora*, and *E. histolytica*.

The diagnosis of *T. gondii* is complicated and may differ with clinical situations. Acute infection is diagnosed by isolation or PCR from blood, or characteristic serologic results. PCR detection has been successfully used to diagnose ocular, cerebral, and disseminated toxoplasmosis and has revolutionized detection of intrauterine infection.

Research Resources

Cryptosporidium parvum oocysts are available from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. Other NIAID-funded resources are available to evaluate potential therapeutic agents for *C. parvum* and certain Microsporidia in a Severe Combined Immune Deficiency (SCID) mouse model or piglet model. *Toxoplasma gondii* isolates and molecular clones for gene expression studies and genetic studies are also available from the Reagent Program. For all of the enteric protozoa there is an immediate need for post-genomic activities to exploit the genome sequence information of enteric protozoa. These include genome annotation, curation, microarray DNA chip production, distribution, and analysis. In addition, standardized reagents are needed for Entamoeba and *Giardia*.

Goals for Research on Food- and Waterborne Protozoa

Immediate

- Expand the understanding of the relationship of parasitic genotypes to virulence and disease severity.
- Evaluate currently available therapies for use against a broad number of enteric protozoa.

- Evaluate validated candidate vaccine antigens, (e.g., *T. gondii* p30*, E. histolytica* Gal/GalNAc lectin and SREHP proteins) in clinical studies
- Complete sequencing of protozoa currently underway.

Intermediate and Long-term

- Characterize molecular mechanisms of pathogenicity and immune evasion.
- Identify host genetic variations that underlie disease susceptibility.
- Develop new vaccine delivery systems and adjuvants, particularly those that elicit mucosal immunity.
- Develop new therapies for *Ent. Bieneusi,* drug resistant *G. lamblia,* and the latent form of *T. gondii.*
- Develop new, rapid, sensitive diagnostics for the Category B protozoa.

EMERGING INFECTIOUS DISEASES

NIAID is the primary Institute at the National Institutes of Health (NIH) that conducts and supports biomedical research on emerging and/or re-emerging infectious human pathogens, including the agents of bioterrorism. It was once believed that infectious diseases could be conquered. However, new infectious diseases continue to emerge and this goal remains elusive. In addition to the continual discovery of new human pathogens, old infectious disease enemies are reemerging. Natural genetic variations, recombinations, and adaptations allow new strains of pathogen to appear to which the immune system has not been previously exposed and is therefore not primed to recognize (e.g., influenza). Furthermore, human intervention plays a big role in re-emergence. Increased and sometimes imprudent use of antimicrobial drugs and pesticides has led to the development of resistance, allowing many diseases to make a comeback (e.g., tuberculosis (TB) and food- and water-borne infections). Moreover, many important diseases have never been adequately controlled on either the national or international levels. Infectious diseases that have posed ongoing health problems in developing countries are reemerging in the U.S. (e.g., food- and water-borne infections, dengue hemorrhagic fever and West Nile virus). New human diseases can also emerge from animal pathogens (e.g., hantavirus). Organisms that are highly infective, transmissible and virulent and that can circumvent our current armamentarium of antimicrobial drugs and/or vaccines represent potential biothreats. Multi-drug resistant tuberculosis (MDR-TB) and influenza offer two examples of such organisms on the current NIAID list of Category C Priority Pathogens (see Appendix 1).

Influenza

Influenza A is a major pathogen of both humans and animals, and recent advances in genetic engineering have raised concerns about the use of influenza as a biological threat agent. While epidemics of influenza result in approximately 20,000 deaths each year in the United States, the sudden emergence of a novel influenza virus could result in global outbreaks (pandemic) of disease in which morbidity and mortality rates would significantly increase. The devastating impact of the 1918 influenza A pandemic, which killed an estimated 21 million people worldwide and more than 500,000 in the U.S., provides a stark illustration of the potential consequences of the emergence of natural mutations in the influenza genome or the deliberate manipulation and release of a highly pathogenic influenza virus.

Biology of the Microbe

Influenza viruses, a member of the family Orthomyxoviridea, are classified into three types; A, B, and C, with influenza A causing the most severe disease in humans. Nine structural proteins have been identified in influenza A viruses, with two surface proteins, the hemagglutinin (HA) and neuraminidase (NA), playing key roles in the pathogenesis

of the virus and the host's immune response. Although only two influenza A subtypes currently cocirculate globally in humans (H1N1 and H3N2), at least 15 distinct antigenic subtypes of HAs (H1 to H15) and nine NAs (N1 to N9) have been identified in wild aquatic birds. Numerous influenza viral genomes have been completely sequenced, including the sequences for four gene segments from the deadly 1918 strain, which have recently been reported. In spite of the severity of influenza disease, little is known about the role these individual proteins play in the pathogenicity of the virus.

The characteristic epidemic and pandemic patterns of influenza A infection are a result of antigenic drift and antigenic shift, respectively. Antigenic drift refers to the relatively minor antigenic changes that occur continuously within the HA and NA. In contrast, antigenic shift results in the emergence of an influenza A virus subtype against which the population has no inherent immunity. In 1997, an outbreak of avian H5N1 virus in Hong Kong resulted in the death of 6 out of 18 people who were infected. This outbreak, which represented the first evidence of direct transmission of an avian influenza virus to humans, was controlled by the central slaughter of all poultry in Hong Kong. The possibility that purposeful manipulation of influenza A genes can be used to create an influenza virus with a novel HA subtype makes influenza A relevant to biowarfare concerns.

Host Response

While infection with influenza virus in healthy adults results in immunity against the homologous virus, it induces little to no protection across subtypes. Anti-HA antibodies neutralize virus infectivity providing some degree of protection against strains showing antigenic drift within a subtype. In contrast, antibodies to NA do not neutralize virus but may impede the release of virus from infected cells thereby decreasing viral shedding and disease severity. Infection with the influenza virus or receipt of live-attenuated influenza virus vaccines results in the generation of mucosal antibody responses, including IgA and IgG. The local IgA response, in particular, plays an important role in protecting the upper respiratory tract from infection.

Vaccines

Inactivated influenza vaccines were developed more than 50 years ago, and three manufacturers are currently licensed for distribution of the vaccine in the U.S. Current influenza vaccines are trivalent (H1N1, H3N2, and B) and are grown in embryonated eggs and then chemically inactivated. The composition of the inactivated influenza vaccine is reviewed and updated yearly and vaccine strain changes are made based on antigenic drift in circulating viruses.

Live, attenuated influenza vaccines have been developed and evaluated extensively in humans. The first application for licensure of an intranasal, live-attenuated influenza vaccine in the United States is currently under review by the Food and Drug Administration. Potential benefits of the live vaccine include ease of administration and induction of a broader and longer lasting immune response.

Therapeutics

Four licensed antiviral drugs are available for the prevention and/or treatment of influenza in the U.S. Amantadine and rimantadine are active against influenza A, while zanamivir and oseltamivir are active against influenza A and B. Approximately one third of all influenza patients treated with amantadine or rimantadine develop drug-resistant viruses. Resistance to zanamivir and oseltamivir has been documented in laboratory studies; several clinical isolates with reduced susceptibility to one of these two drugs have been identified.

Diagnostics

Diagnostic tests for influenza include viral culture, serology, antigen detection, IF and PCR. Viruses can most readily be isolated from nasopharyngeal specimens. Several commercially available rapid diagnostic tests are available; however, their specificity and sensitivity can vary widely and are lower than those measures obtained by viral culture.

Research Resources

The Department of Agriculture (USDA) requires BSL 3 Agriculture (BSL-3AG) biocontainment for work with highly pathogenic avian influenza viruses of U.S. or non-U.S. origin.

Goals for Research on Influenza

Immediate

- Expand animal influenza surveillance, including natural history studies, on emergence of pandemic strains.
- Develop high-growth vaccine viruses for selected avian influenza subtypes.
- Produce and evaluate pilot lots of vaccine against avian influenza viruses with pandemic potential.
- Expand research on the preclinical development of influenza vaccine candidates including strategies to enhance the immune response.

- Continue to support the development of alternatives to egg-based vaccines, including cell culture-based platforms.
- Expand research to identify host genetic factors that influence susceptibility to influenza disease.

Intermediate and Long-term

- Develop a plasmid library of high-growth vaccine viruses for HA and NA subtypes with pandemic potential.
- Develop influenza vaccines that provide improved protection for high-risk populations.
- Utilize genomic information to identify new targets for the development of antivirals and diagnostics.
- Continue to support the characterization of the 1918 influenza virus to determine the genetic basis of its virulence.
- Support the development of diagnostics to distinguish influenza from other diseases that present with "flu-like" symptoms.

Multi-Drug Resistant Tuberculosis

Multi-Drug Resistant Tuberculosis (MDR-TB) is an emerging public health threat. *Mycobacterium tuberculosis* (Mtb) bacteria, the causative agents of TB, are spread from person to person by airborne droplets expelled from the lungs when a person with TB coughs, sneezes, or speaks. Outbreaks may therefore occur in closed settings and under crowded living conditions such as homeless shelters and prisons.

It is estimated that one-third of the world's population ($1.86 billion people) is infected with Mtb, and 16.2 million people have TB disease. In 1995, the year with the highest TB casualty rate to date, nearly 3 million people died worldwide from the disease. While MDR-TB currently represents a small percentage of all TB cases in the U.S., large regional clusters of MDR-TB cases exist globally with the potential to spread widely.

Identification of both drug-sensitive and drug-resistant Mtb is time consuming and not easily implemented in resource-poor settings. Treatment of MDR-TB requires taking more expensive, second-line antibiotics for up to 2 years—an outbreak of MDR-TB would put an immense strain on the public health infrastructure. At the peak of the TB epidemic in New York City from 1988–1992, there were over 3700 cases, of which at least 19% were MDR-TB. The containment of this outbreak cost approximately $1 billion. In 1997, it was estimated that the average cost of medical care for a patient

with MDR-TB can be as high as $180,000. Epidemics of MDR-TB would likely result in casualty rates similar to those seen when TB is not treated.

Biology of the Microbe

Mycobacterium tuberculosis is a transmissible, slow growing, acid-fast bacterial pathogen with a waxy outer layer. This pathogen infects and multiplies inside host white blood cells. Humans are a natural reservoir of Mtb but bacteria can be propagated in a variety of experimental animals. The transmission and course of tuberculosis infection and disease appears to be identical with drug-sensitive and drug-resistant Mtb. Other members of the genus *Mycobacterium* include pathogens that cause Leprosy, skin ulcers and, in AIDS patients, complicating infections. The genomes of two strains of Mtb, as well as other mycobacterial species, have been sequenced and the sequence data are publicly accessible for comparative analyses and to identify potential targets for intervention and diagnostics.

Host Response

Infection with Mtb does not always lead to development of TB. Initially, Mtb infection takes root in the air sacs of the lung. Approximately 90% of persons who get infected with Mtb do not develop disease. In these individuals, the microbes are contained by the immune system which may lead to a lifelong, asymptomatic infection. If the immune system becomes weakened from HIV infection, malnutrition, aging, or other factors, these “latent” bacteria may reactivate and spread within the lungs and/or to other tissues resulting in TB disease. Infection with Mtb does not make the host resistant to re-infection or disease.

Vaccines

Currently, there is only one licensed vaccine against TB in the United States but it is not recommended for use. This vaccine, Bacillus Calmette-Guérin (BCG), is reportedly highly variable in its efficacy to prevent adult pulmonary TB. However, it is considered effective in preventing death from TB in young children. Vaccines against TB are expected to be effective against MDR-TB and would offer the best long-term solution to prevent disease after natural or intentional exposure. Several animal models are available to test TB vaccine candidates but researchers do not know which animal model is most predictive of the human response or what immunological markers predict suitable protection.

Therapeutics

Drug resistant variants of Mtb can develop when patients do not complete the prescribed course of antibiotics. The current TB treatment regimen includes up to four antibiotics daily for 6 to 9 months. MDR-TB is much more difficult to treat and involves second-line, less well-tolerated antibiotics given under supervision, over the course of up to 2 years. Since MDR-TB is harder to treat and drug resistant Mtb more difficult to eradicate from the host, patients with MDR-TB remain infectious longer. Clinical trials are being conducted to expand the repertoire of effective antibiotics, and to simplify and shorten chemotherapy against TB.

Diagnostics

Standard diagnosis of TB involves a chest X-ray, and identification of the causative pathogen from patient specimens either microscopically or by culture. Molecular biological techniques to identify Mtb are available, but rapid diagnostics to identify drug resistance are not; serological techniques are being developed. Currently, identification of MDR-TB requires culture in the presence of antibiotics. This method is time- and resource-intensive, and the results are only available after several weeks.

Research Resources

Both drug-sensitive and drug-resistant strains of Mtb must be cultured and handled under BSL3 biocontainment conditions. Currently accepted animal models of TB use mice, guinea pigs, rabbits and non-human primates and may involve infection via aerosol under BSL3 conditions.

Goals for Research on Multi-Drug Resistant Tuberculosis

Immediate

- Exploit genomic and proteomic information to identify new targets for vaccine, drug, and diagnostics development.
- Develop and standardize animal models that better predict vaccine and drug efficacy in humans.
- Develop faster, more robust microbiological and serological diagnostics for drug sensitive Mtb and MDR-TB.
- Increase capacity for testing vaccine candidates in standardized animal models.
- Expand the infrastructure for conducting clinical trials for therapeutics and vaccines, including education and training of personnel in high-burden countries.

Intermediate and Long-term

- Identify surrogate markers of infection and disease to facilitate clinical trials of vaccines and therapeutics.
- Expand research on the pharmacology of current and new TB therapeutics.
- Conduct preclinical safety and efficacy studies for TB vaccine and drug candidates.
- Conduct clinical specificity and sensitivity studies for novel diagnostics.

ADDITIONAL BIODEFENSE CONSIDERATIONS

A number of issues raised by Panel members will need additional discussion with other agencies and/or the scientific community. In some cases, the Panel was divided on a final recommendation. The issues discussed by the Panel include recommendations for additions or deletions and changes to the NIAID list of Category A, B and C Priority Pathogens, the role of industry in the biodefense research agenda, and the consequences of genetically modified organisms.

Recommendations on Niaid Priority Pathogens List

Although the NIAID list of Category A, B, and C Priority Pathogens (Appendix 1) closely follows the CDC's list of Biological Diseases/Agents (Appendix 2), the NIAID list highlights specific pathogens identified as priorities for additional research efforts as part of the NIAID biodefense research agenda. During the Panel's deliberations, the following recommendations related to the NIAID Priority Pathogens list were made:

- Transfer of specific Other Rickettsias (i.e., *R. rickettsii*, *R. typhi*, and *R. conorii)* from Category C to Category B.
- Addition of *Coccidiodes* spp. and hepatitis E virus[2] to Category B.
- Replacement of Staphylococcal enterotoxin B and ricin with the following two groups of Category B toxins, respectively:
  - Microbial superantigen toxins (Staphylococcal enterotoxins and exotoxins, and Streptococcal exotoxins)
  - Multi-chain microbial ribosome-inactivating protein toxins (Shiga toxins, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A)
- Addition of *Clostridium perfringens* toxins in addition to the epsilon toxin to Category B.
- Transfer of Crimean Congo Hemorrhagic Fever virus from Category C to Category B.

[2] In humans, infection with hepatitis E virus (HEV) usually results in an acute, self-limiting condition; death from HEV is relatively uncommon. However, women in the third trimester of pregnancy are especially susceptible to acute fulminant hepatitis, with a case fatality rate approaching 20 percent. Viruses closely related to HEV have been identified in animals including rodents, swine, lambs, and chickens; the swine virus is transmissible to non-human primates making zoonotic transmission a possibility and raising concerns about xenografts. HEV occurs in both epidemic and sporadic-endemic forms and is usually the result of drinking contaminated water; person-to-person transmission is rare. Specific antiviral therapies and licensed diagnostic tests are not currently available for HEV.

- Addition of Herpes B, Ehrlichia, and Hendra virus to Category C.
- Removal of multi-drug resistant TB from the list and addition of multi-drug resistant bacteria with high human pathogenicity to Category C.
- Transfer of dengue hemorrhagic fever virus from Category A to Category C.
- Maintenance of rabies and Nipah viruses on Category C.
- Emphasis of research on monkeypox, camelpox, and Omsk Hemorrhagic fever in Category A.
- Emphasis of research on enterohemorrhagic *Escherichia coli* (EHEC) in Category B.

Role of Industry in the Biodefense Research Agenda

Over the last 10 years, the number of companies actively involved in the development of antimicrobials or vaccines has decreased significantly. The Panel expressed concern that while many small companies are conducting important and innovative research, some may have difficulty carrying a candidate through product development to licensure. Thus, the Panel recommended that NIAID work with industrial representatives to develop a new paradigm for collaborations between the government and industry. This includes the need for a clear statement of the highest priority products and identification of ways to assist industry in developing these products for use. In addition, the Panel recognized the need for increased interactions between industry and the FDA early in the development process. The Panel also recommended that industry make available existing chemical libraries for screening against biodefense pathogens. Finally, the Panel recommended a coordinated effort to test existing therapies for new indications related to biodefense.

Genetically Modified Organisms

Since the early 1970s, when scientists discovered how to transfer genetic elements from one organism into another, there has been concern that this technology could be used to create new bioweapons. Many virulent factors have their origins in bacteriophages, and they are easily amenable to genetic manipulation. Research, particularly in genomics and immunology, has created a wealth of new knowledge that could be used to produce organisms that have enhanced pathogenicity, infectivity, and transmissibility. The diseases caused by these modified organisms might initially be hard to diagnose and resist treatment with current antimicrobials. Additionally, currently available vaccines could be rendered ineffective. Recently, Australian scientists inadvertently created a new virus that had significantly increased virulence for mice by

splicing a gene for interleukin-4 (IL-4) into mousepox virus. Addition of the IL-4 gene apparently suppressed the normal immunological response against the mousepox virus infection. Additionally, the bio-engineered poxvirus may be able to evade vaccine-induced protection.

Although these scientists used a mouse virus, it may be possible to similarly engineer human viruses or other microorganisms, creating new or modified organisms with enhanced pathogenicity, infectivity and transmissibility. The Panel recommended several areas of research that might help counteract these organisms.

Research Needs

- Develop diagnostics for rapid detection of antimicrobial susceptibility/resistance.
- Develop robust genomic tools to detect genetically modified organisms and the presence of virulence factors associated with bacteriophage.
- Initiate and/or complete genomic sequencing of virulent bacteriophages to identify virulence factors and new drug targets.
- Develop multiple and combination approaches to counter effects of genetic modifications that enhance pathogenicity, infectivity and transmissibility.

Appendix 1

NIAID CATEGORY A, B, AND C PRIORITY PATHOGENS

NIAID Category A, B, and C Priority Pathogens

Category A

Bacillus anthracis (anthrax)
Clostridium botulinum (botulism)
Yersinia pestis (plague)
*Variola majo*r (smallpox) and other pox viruses
Francisella tularensis (tularemia)
Viral hemorrhagic fevers

- Arenaviruses
  - LCM, Junin virus, Machupo virus, Guanarito virus
  - Lassa Fever
- Bunyaviruses
  - Hantaviruses
  - Rift Valley Fever
- Flaviviruses
  - Dengue
- Filoviruses
  - Ebola
  - Marburg

Category B

Burkholderia pseudomallei (melioidosis)
Coxiella burnetii (Q fever)
Brucella species (brucellosis)
Burkholderia mallei (glanders)
Ricin toxin (from *Ricinus communis*)
Epsilon toxin (of *Clostridium perfringens*)
Staphylococcal enterotoxin B
Typhus fever (*Rickettsia prowazekii*)
Food- and Water-borne Pathogens

- Bacteria
  - Diarrheagenic Escherichia coli
  - Pathogenic Vibrios
  - Shigella species
  - Salmonella species
  - Listeria monocytogenes

- Campylobacter jejuni
- Yersinia enterocolitica

Viruses

- Caliciviruses
- Hepatitis A

Protozoa

- *Cryptosporidium parvum*
- *Cyclospora cayatenensis*
- *Giardia lamblia*
- *Entamoeba histolytica*
- *Toxoplasma*
- *Microsporidia*

Additional viral encephalitides

- West Nile virus
- LaCrosse
- California encephalitis
- Venezuelan equine encephalitis
- Eastern equine encephalitis
- Western equine encephalitis
- Japanese encephalitis virus
- Kyasanur forest virus

Category C

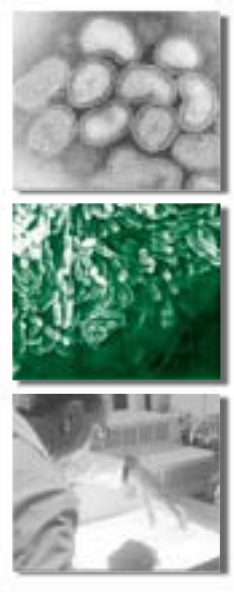
Emerging infectious disease threats such as Nipah virus and additional hantaviruses.

Tickborne hemorrhagic fever viruses

- Crimean Congo Hemorrhagic fever virus

Tickborne encephalitis viruses
Yellow fever
Multi-drug resistant TB
Influenza
Other Rickettsias
Rabies

Appendix 2



CDC BIOLOGICAL DISEASES/AGENTS LIST

CDC Biological Diseases/Agents List

Category A

Anthrax (*Bacillus anthracis*)
Botulism (*Clostridium botulinum* toxin)
Plague (*Yersinia pestis*)
Smallpox (*Variola major*)
Tularemia (*Francisella tularensis*)
Viral hemorrhagic fevers (filoviruses [e.g., Ebola, Marburg] and arenaviruses [e.g., Lassa, Machupo])

Category B

Brucellosis (*Brucella* species)
Epsilon toxin (of *Clostridium perfringens*)
Food safety threats (e.g., *Salmonella* species, *Escherichia coli* O157:H7, *Shigella*)
Glanders (*Burkholderia mallei*)
Melioidosis (*Burkholderia pseudomallei*)
Psittacosis (*Chlamydia psittaci*)
Q fever (*Coxiella burnetii*)
Ricin toxin from *Ricinus communis* (castor beans)
Staphylococcal enterotoxin B
Typhus fever (*Rickettsia prowazekii*)
Viral encephalitis (alphaviruses [e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis])
Water safety threats (e.g., *Vibrio cholerae, Cryptosporidium parvum*)

Category C

Emerging infectious disease threats such as Nipah virus and hantavirus.

Appendix 3

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