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# Lawrence Livermore National Laboratory Workshop Characterization of Pathogenicity, Virulence and Host-Pathogen Interactions

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Characterization of Pathogenicity, Virulence and Host-Pathogen Interactions
Livermore, CA, United States
September 13, 2006 through September 15, 2006

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LLNL BioSecurity Lecture Series
& Workshops

# Lawrence Livermore National Laboratory

# Workshop

## *Characterization of Pathogenicity, Virulence and Host-Pathogen Interactions*

## September 13-15, 2006

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# Mission Statement

The threats of bio-terrorism and newly emerging infectious diseases pose serious challenges to the national security infrastructure. Rapid detection and diagnosis of infectious disease in human populations, as well as characterizing pathogen biology, are critical for reducing the morbidity and mortality associated with such threats. One of the key challenges in managing an infectious disease outbreak, whether through natural causes or acts of overt terrorism, is detection early enough to initiate effective countermeasures. Much recent attention has been directed towards the utility of biomarkers or molecular signatures that result from the interaction of the pathogen with the host for improving our ability to diagnose and mitigate the impact of a developing infection during the time window when effective countermeasures can be instituted. Host responses may provide early signals in blood even from localized infections. Multiple innate and adaptive immune molecules, in combination with other biochemical markers, may provide disease-specific information and new targets for countermeasures. The presence of pathogen specific markers and an understanding of the molecular capabilities and adaptations of the pathogen when it interacts with its host may likewise assist in early detection and provide opportunities for targeting countermeasures. An important question that needs to be addressed is whether these molecular-based approaches will prove useful for early diagnosis, complement current methods of direct agent detection, and aid development and use of countermeasures.

Lawrence Livermore National Laboratory (LLNL) will host a workshop to explore the utility of host- and pathogen-based molecular diagnostics, prioritize key research issues, and determine the critical steps needed to transition host-pathogen research to tools that can be applied towards a more effective national bio-defense strategy. The workshop will bring together leading researchers/scientists in the area of host-pathogen interactions as well as policy makers from federal agencies. The main objectives of the workshop are:

- to assess the current national needs, capabilities, near-term technologies, and future challenges in applying various diagnostics tools to public health and bio-defense
- to evaluate the utility and feasibility of host-response and pathogen biomarker profiling in the diagnosis and management of infectious diseases
- to create a comprehensive developmental strategy from proof-of-concept, through validation, to deployment of appropriate advanced technology for the clinical/public health and bio-defense environments

The workshop will be a 2-day event (to be held at Livermore, CA) scheduled for September 13 and 14, 2006. An additional day (September 15) will be reserved for laboratory tours and site visits.

LLNL has a long history of successfully developing and deploying technologies and systems for rapid detection and identification of biological pathogens in support of national security. Additionally, LLNL has unique resources such as the High Performance Computing Center (HPCC), Center for Accelerator Mass Spectrometry (CAMS), Livermore Micro-Array Center (LMAC), National Atmospheric Release Advisory Center (NARAC) and a range of other infra-structure capabilities to support development, testing and evaluation of technologies/systems for bio-defense applications. LLNL is committed to researching and continuing to develop the next-generation tools for diagnosis and mitigation of the risk from both emerging infectious diseases and threats from bioterrorism.

# Agenda

**Wednesday, September 13, 2006**

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|---|---|---|
| **7:00 – 7:30** | **Badging at LLNL** | **ALL** |
| **7:30 – 8:15** | **Continental Breakfast** | **ALL** |

**SESSION 1 : ROLE OF HOST-PATHOGEN SCIENCE IN PUBLIC HEALTH & BIO-DEFENSE**

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|---|---|---|
| **8:15 - 8:30** | **Welcome/Introductions** | **Cherry Murray, LLNL** |
| **8:30 - 9:00** | ***Keynote Lecture for the Conference*** | **Michael Kurilla, NIH** |
| **9:00 - 9:30** | ***The Public Health Response to Bioterrorism: Role of Host-Pathogen Science*** | **Stephen Morse, CDC** |
| **9:30 – 10:00** | **Title - TBD** | **Carol Linden, HHS** |
| **10:00 – 10:20** | **Coffee Break** | **ALL** |

**SESSION 2 : MECHANISMS OF HOST RESPONSE**
**Chair : Peter Jahrling, HHS**

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|---|---|---|
| **10:20 – 10:50** | ***Plenary Talk*** | **Peter Jahrling, HHS** |
| **10:50 - 11:20** | ***Transcriptional Control of the Host Response to Pathogens*** | **Richard Jenner, MIT** |
| **11:20 - 11:50** | ***Host Innate Immune Recognition of B. anthracis*** | **Molly Hughes, UVA** |
| **11:50 - 12:20** | ***Innate immune and necrotic pathways mediated by the NBD-LRR family of mammalian proteins*** | **John Lich, UNC** |
| **12:20 – 1:15** | **Lunch** | **ALL** |
| **1:15 – 1:45** | ***Chemokine Receptor CCR5 Mediates Resistance to West Nile Virus Infection in Mouse and Man*** | **Jean Lim, NIH** |
| **1:45 – 2:15** | ***Francisella tularensis: Evading and Provoking Innate Immunity*** | **Martha Furie, Stony Brook Univ.** |
| **2:15 - 4:00** | **Panel Discusison** | **ALL** |
| **4:00 – 6:00** | **Poster Session** | **ALL** |

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| **6:00** | **Dinner** | **ALL** |

**Thursday, September 14, 2006**

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| **7:00 – 8:00** | **Continental Breakfast** | **ALL** |

**SESSION 3 : MECHANISMS OF PATHOGEN VIRULENCE AND HOST IMMUNE EVASION**
**Chair : Garry Adams, Texas A&M Univ.**

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| **8:00 – 8:30** | ***Pathomics Discovery Platform: Brucella-Salmonella:Host Transcriptomics, Proteomics & Bioinformatics*** | **Garry Adams, Texas A&M Univ.** |
| **8:30 - 9:00** | ***Alphavirus encephalitis: Determinants of outcome*** | **Diane Griffin, JHU** |
| **9:00 - 9:30** | ***Innate immunity against Francisella tularensis is dependent on the ASC/caspase-1 axis*** | **Denise Monack, Stanford** |
| **9:30 - 10:00** | ***Predictive features of the genome-wide host response to infectious diseases*** | **David Relman, Stanford** |
| **10:00 – 10:20** | **Coffee Break** | **ALL** |
| **10:20 – 10:50** | ***Whole-Genome comparison approaches to the study of virulence*** | **Emilio Garcia, LLNL** |
| **10:50 – 11:10** | ***Beyond Pathogen Identification: Informatics support for characterization of virulence, antibiotic-resistance, host-pathogen interactions, and evidence of genetic engineering*** | **Tom Slezak, LLNL** |
| **11:10 - 12:30** | **Panel Discussion** | **ALL** |
| **12:30 – 1:30** | **Lunch** | **ALL** |

**SESSION 4 : APPLICATIONS TO BIO-DEFENSE (DETECTION, DIAGNOSIS, MITIGATION)**
**Chair : Fred Murphy, UTMB**

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|---|---|---|
| **1:30 - 2:00** | ***Etiologic vs. Non-etiologic (Syndromic) Diagnosis: Fitting into Public Health and Biodefense Systems*** | **Fred Murphy, UTMB** |
| **2:00 – 2:30** | ***Genome-Wide Survey of Host Responses: Use of Computational Analysis to Classify Exposures and Extract Signatures of Unconventional Versus Common Viral Exposures*** | **Marti Jett, WRAIR** |

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| **2:30 – 3:00** | ***Breath as a Diagnostic*** | **Joany Jackman, JHU-APL** |
| **3:00 – 3:30** | ***LLNL Roadmap for Bio-Defense*** | **Bill Colston, LLNL** |
| **3:30 - 5:00** | **Panel Discussion** | **ALL** |
| **5:00 – 5:15** | **Wrap-up/Summary** | **Ray Juzaitis, LLNL** |
| **5:15** | **Adjourn** | **ALL** |

# Abstracts of Oral Presentations

## Keynote Lecture

Michael G. Kurilla, Office of Bio-Defense, DMID, NIAID, NIH, DHHS,
Bethesda, MD 20892

### Abstract

Traditional antimicrobial towards infectious diseases have focused on interference with the ability of the pathogenic agent to replicate within the host. This infection control approach relies on identification of the critical gene functions essential for growth of the organism. These obvious 'targets' naturally emerged from laboratory investigations of these agents. With the advent of genomic sequences as well as resulting transcriptional and proteomic profiling particularly within the context of an animal model, infectious disease is rapidly progressing from an 'infection control' approach to a 'disease modifying' model. With this focus, non-essential genes of the infectious agent may also become potential targets as well as host based targets that modulate pathogenesis while allowing the immune system to function in its normal role of pathogen clearance through a combination of the both the innate and adaptive immune systems. An overview of various research programs exploring potential broad spectrum interventions based on these concepts as well as broad spectrum platforms that should expedite commercial exploitation of these biomedical products will be discussed.

# The Public Health Response to Bioterrorism: Role of Host-Pathogen Science

Stephen A. Morse, Centers for Disease Control and Prevention, Atlanta, GA

A number of events over the last decade have served to focus attention on the threat of terrorism and the use of biological agents against military and civilian populations for the purpose of causing illness or death. Over 1400 species of infectious organisms have been recognized as human pathogens; only a few pose serious problems or are capable of affecting humans on a large scale. Various criteria have been used to identify and prioritize these pathogens for public health preparedness activities. It is possible to introduce biological agents into civilian populations by several means, e.g., aerosol, contaminated food, water, or medical products, or by releasing infected arthropod vectors. A covert release of a biological agent would likely go unnoticed for some time, with those exposed leaving the area long before the act of terrorism becomes evident. Due to an incubation period, the first signs that a biological agent has been released may not become apparent until days or weeks later, when individuals become ill and seek medical care. The "responders" to such an event will likely be the astute clinician, laboratory or public health worker who recognizes the index case or identifies the responsible agent.

Public health emphasizes the prevention of disease and the health needs of the population as a whole. A comprehensive public health response to the deliberate release of a biological agent (whether known or unknown at the time) will involve an epidemiologic investigation, medical treatment and prophylaxis for affected persons. An effective public health response would have to be rapid. The Laboratory Response Network (LRN) was created to facilitate the rapid identification of threat agents. It has a dual function in that it has the ability to detect and respond to agents released by bioterrorists as well as those that occur naturally. The LRN consists of sentinel and reference laboratories. Sentinel laboratories are for the most part, hospital and other community laboratories, which are located at the many widely dispersed hospitals where patients would seek care in the aftermath of a covert release. Reference laboratories are primarily state and local public health laboratories with BSL-3 laboratories where agents and powders can be safely handled. The reference laboratories use standard protocols and reagents for the identification and confirmation of threat agents such that results are public-health-actionable. BioWatch is a DHS environmental monitoring program, which uses many of the LRN laboratories to process air samples collected on filters. Positive results may indicate the aerosol release of an agent and possible exposure. A public health response could potentially be initiated earlier if exposed individuals could be identified before they became symptomatic. Unfortunately, such a test would be difficult to develop, as there are numerous factors that influence both the susceptibility and the response of the host to a given pathogen. Some of these factors are genetic, but others are age, pregnancy, medication, stress, behavioral, and the presence of another disease.

On the population level, a highly sensitive test would be impractical as there would be an unacceptable level of positive results due to other conditions. However, a highly specific test could give public-health-actionable results, but would be more expensive due to the number of host factors being measured. How such a test was used would have to be carefully considered, as population-based testing may not be cost-effective.

## Plenary Talk for Host Mechanisms Session

Peter Jahrling, NIAID/NIH Integrated Research Facility at Fort Detrick, Maryland

Smallpox (variola) virus poses a significant threat as an agent of bioterrorism. To mitigate this risk, antiviral drugs and an improved vaccine are urgently needed. Satisfactory demonstration of protective efficacy against authentic variola will require development of an animal model in which variola produces a disease course with features consistent with human smallpox. Towards this end, cynomolgus macaques were exposed to several variola strains via aerosol and/or intravenous routes. Two strains, Harper and India 7124, produced uniform acute lethality when inoculated intravenously in high doses ($10^9$ PFU). Lower doses resulted in less fulminant, systemic disease and lower mortality. Animals that died had profound leukocytosis, thrombocytopenia, and elevated serum creatinine levels. Following inoculation, variola was disseminated via a monocytic cell-associated viremia. Distribution of viral antigens by immunohistochemistry correlated with the presence of replicating viral particles demonstrated by electron microscopy and with pathology in the lymphoid tissues, skin, oral mucosa, gastrointestinal tract, reproductive system, and liver, resembling that seen in human smallpox. High viral burdens in target tissues were associated with organ dysfunction and multi-system failure. Evidence of coagulation cascade activation (D-dimers) corroborated histologic evidence of hemorrhagic diathesis. Depletion of T-cell dependent areas of lymphoid tissues occurred, probably as a consequence of bystander apoptotic mechanisms initiated by infected macrophages. Elaboration of cytokines including IL-6, and interferon-γ contribute to a cytokine storm formerly known as "toxemia." A more precise understanding of disease pathogenesis should provide targets for therapeutic intervention, to be used alone or in combination with inhibitors of variola virus replication.

# Transcriptional Control of the Host Response to Pathogens

Richard G. Jenner, Whitehead Institute for Biomedical Research, Cambridge, MA
and Division of Infection and Immunity, University College London, London, UK.

We have collated and compared published transcriptional profiling data involving a broad range of host cell types and pathogen species. We found that different host cells respond to pathogens with a broadly similar expression program that we have termed the common host response. This organized set of gene expression changes is observed in macrophages, dendritic cells, peripheral blood mononuclear cells and epithelial cells after treatment with a broad spectrum of bacteria, fungi and viruses. Components of this response may therefore be of use in defining a transcriptional signature of infection.

The common host response likely represents the concerted action of a number of transcription factors acting in a regulatory network. We have begun to map this transcriptional regulatory network in macrophages by using genome-scale location analysis (ChIP-chip) to discover the set of genes bound by NF- B, both before and after treatment with lipopolysaccharide (LPS). These experiments identified 348 genes bound by NF- B, 90% of which were not previously known target genes and many of which form part of the common host response. Our work reveals how the different NF- B family members coordinate their activity and, together with gene expression data, allow us to begin reconstructing the transcriptional regulatory networks that underlie the host response to infection.

# Host Innate Immune Recognition of B. *anthracis*

Molly A. Hughes[1], Lisa Lowchyj-Waggoner[1], Candace S. Green[1], Vanessa K. Grippe[2], Gloria M. Lee[2], Tod J. Merkel[2]

[1]Division of Infectious Diseases, Department of Internal Medicine, University of Virginia Health Sciences System, Charlottesville, Virginia
[2] Laboratory of Respiratory and Special Pathogens, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland

*Bacillus anthracis* is a spore-forming, Gram-positive organism that is the causative agent of anthrax. Innate immune recognition of *B. anthracis* by the host likely plays a key protective role following infection. In our studies, we have examined the host macrophage cytokine response to *B. anthracis* spores and bacilli. Under conditions in which Sterne strain spores do or do not germinate, we have found that spores do not elicit a tumor necrosis factor-alpha (TNF- ) response from murine bone marrow-derived macrophages (BMDMs) whereas bacilli elicit a TNF- response comparable to that of the positive control. We have evaluated the cytokine response of BMDMs exposed to non-toxigenic mutants of *B. anthracis* Sterne strain to better understand the role of lethal toxin and/or edema toxin in the host inflammatory response. Further, we have evaluated the role of Toll-like receptor (TLR) recognition of *B. anthracis* heat-killed bacilli. Specifically, we have examined *in vitro* and *in vivo* the role of TLR2, TLR4, and the TLR adaptor signaling protein MyD88 in the response to *B. anthracis*. Heat-killed *B. anthracis* (HKBa) stimulates TLR2, but not TLR4, signaling in human embryonic kidney 293 cells and stimulates TNF- production in C3H/HeN, C3H/HeJ, and C57BL/6J BMDMs. The ability of HKBa to induce a TNF- response is preserved in TLR2-/- but not in MyD88-/- BMDMs. *In vivo* studies revealed that TLR2-/- mice and TLR4-deficient mice are resistant to challenge with aerosolized Sterne strain spores but MyD88-/- mice are as susceptible as A/J mice. We conclude that, although recognition of *B. anthracis* bacilli occurs via TLR2, additional MyD88-dependent pathways contribute to the host innate immune response to anthrax infection. Ongoing studies are focused on further characterizing the MyD88-dependent pathways involved in the host response to *B. anthracis*

# Innate Immune and Necrotic Pathways Mediated By The NBD-LRR Family of Mammalian Proteins

Lich JD, Williams KL, Willingham S, Bergstrahl DT, O'Connor W, and Ting J Lineberger Comprehensive Cancer Center and the Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599

We first described a large family of mammalian proteins that share structural homology with plant pathogen resistance proteins. These proteins harbor N-terminal coiled-coil domains (CARD/Pyrin/BIR), central nucleotide binding domains (NBD), and C-terminal leucine rich repeats (LRR). Based on this domain architecture, we named these proteins the CATERPILLER or CLR family for CARD, Transcription Enhancer, R (purine)-binding, Pyrin, Lots of Leucine Repeats. Subsequently, this family of proteins has been designated NBD-LRR or NLR. This presentation will describe two NLR proteins that harbor N-terminal pyrin domains and play novel roles in innate immune signaling and cell death.

The first member, Monarch-1, is expressed exclusively in myeloid cells and is an inhibitor of TLR and CD40 signaling. Its mRNA and protein levels are reduced by TLR agonists including bacteria. Functionally, Monarch-1 prevents the hyperphosphorylation of IRAK-1. This leads to the attenuation of TLR-mediated activation of NF-kB and cytokine production, as shown by the analysis of siRNA targeted cells. In addition, Monarch-1 also interferes with NIK-dependent activation of non-canonical NF-kB downstream of CD40. In Monarch-1 expressing monocytes, NF-kB2/p100 is not processed to its active form, p52. This is due to the association of Monarch-1 with NIK, which results in rapid proteasome-mediated degradation of the kinase.

The second member, CIAS1/cryopyrin/NALP3, is also predominantly expressed in myeloid cells. Mutations in this gene are linked to several autoinflammatory syndromes. Patients with defects in this gene exhibit enhanced inflammation characterized by increased IL-1 production. We analyzed three distinct CIAS1 disease-associated variants as well as monocytic cells from patients harboring these mutations. The disease-associated forms of CIAS1 cause enhanced cell death. This requires ASC (Apoptotic speck-containing protein with a CARD) but surprisingly, not IL-1. Thus cell death was not secondary to hyper-IL-1 synthesis. This form of cell death and its relationship to inflammation will be discussed.

# Chemokine Receptor CCR5 Mediates Resistance to West Nile Virus Infection in Mouse and Man

Jean K. Lim[1], William G. Glass[2], David H. McDermott[1], Alexander Pletnev[1], Rushina Cholera[1], Ji-liang Gao[1], Sudkamon Lekhong[3], Shuk Fong Yu[3], William A. Frank[4], John Pape[5], Ronald C. Cheshier[3], and Philip M. Murphy[1].

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West Nile Virus (WNV) is a re-emerging pathogen and a well-known agent of fatal encephalitis in several species, including man, however immunopathogenic mechanisms are poorly understood. Here, in a mouse model of WNV infection we observed upregulation of the chemokine receptor CCR5 and its ligand CCL5 in brain. Infection of CCR5-/- mice was rapidly and uniformly fatal, whereas the majority of CCR5+/+ mice survived. In the brain, CCR5-/- mice had increased viral burden and markedly reduced numbers of $NK1.1^+$ cells, macrophages, and $CD4^+$ and $CD8^+$ T cells as compared to WNV-infected CCR5+/+ control mice, suggesting a protective role of the receptor mediated at the level of leukocyte trafficking to brain. Consistent with this, adoptive transfer of splenocytes from WNV-infected CCR5+/+ mice into infected CCR5-/- mice increased leukocyte accumulation in the CNS compared to transfer of splenocytes from infected CCR5-/- mice into infected CCR5-/- mice and resulted in an overall increase in survival, returning the mortality rate to the level observed in CCR5+/+ control mice. Consistent with the mouse model, homozygosity for *CCR5 32,* a non-functional allele of human *CCR5,* was ~5-fold higher in both of two cohorts of symptomatic WNV-seropositive individuals in the US compared to a control population of healthy Caucasian random blood donors, and was associated with increased mortality. Collectively, these data suggest that CCR5 mediates resistance to fatal WNV infection in mouse and man by coordinating leukocyte recruitment to the infected brain and that *CCR5 32* homozygosity is a genetic risk factor for symptomatic WNV infection in man, the first identified for this disease. The results suggest that pharmacologic blockade of CCR5 may increase susceptibility to disease in WNV infected individuals.

# *Francisella tularensis:* Evading and Provoking Innate Immunity

Martha B. Furie, Center for Infectious Diseases, Stony Brook University,
Stony Brook, NY

Circulating leukocytes respond to infections in tissues by migrating across the endothelial lining of blood vessels and accumulating in the affected area. Endothelial cells play a key role in this inflammatory process through upregulation of adhesion molecules that bind to leukocytes and secretion of chemokines that guide their movement out of the vessels. We found that the live vaccine strain (LVS) of *Francisella tularensis* stimulated such pro-inflammatory changes in cultured human umbilical vein endothelial cells (HUVEC), but it did so in a unique manner. The living bacterium induced only a limited subset of changes, whereas killed *F. tularensis* LVS elicited a wider, more typical array of alterations. We speculate that limited activation of endothelium in response to living *F. tularensis* may facilitate establishment of an initial infection. The fuller activation that is induced by dead organisms may be required subsequently for recruitment of macrophages, which the bacterium invades and co-opts as a protected niche for replication.

Many Gram-negative bacteria activate endothelium via their lipopolysaccharide (LPS), but the structure of *F. tularensis* LPS is atypical. Indeed, we observed that purified LPS from both the LVS and fully virulent strains of *F. tularensis* did not activate endothelial cells, even at very high concentrations. We further noted that intact *F. tularensis* LVS elicited secretion of pro-inflammatory cytokines from human, but not murine, macrophages. Although purified LPS from *F. tularensis* stimulated human macrophages to a limited extent, much greater amounts were required compared to LPS from *E. coli*.

We therefore began to search for alternative pro-inflammatory components of *F. tularensis*. A prime candidate was LpnA, a 17-kDa outer surface lipoprotein that is prominently expressed. The gene encoding LpnA in the LVS was ablated, and the behavior of the resulting LpnA-deficient strain was compared to that of the wild-type organism. When inoculated into mice, the deficient strain was as virulent as the wild-type strain. Moreover, the deficient strain completely protected mice against subsequent challenge with a lethal dose of the LVS. When incubated with HUVEC or human macrophages, the LpnA-deficient and wild-type strains induced secretion of pro-inflammatory cytokines to similar degrees. However, in preliminary experiments, recombinant LpnA stimulated both endothelial cells and macrophages. Perhaps, then, other lipoproteins compensate for the absence of LpnA in the mutant strain.

To identify additional pro-inflammatory factors of *F. tularensis*, fractionation of material released from killed bacteria was performed by FPLC. Three fractions of approximately 60, 30, and 6 kDa stimulated expression of the adhesion molecule E-selectin by HUVEC. A prominent component in the 60-kDa fraction was a putative heat-shock protein, GroEL. GroEL was removed by immunoprecipitation from the material released by killed *F. tularensis*. The depleted samples activated HUVEC to the same degree as did untreated samples but lost all ability to stimulate human macrophages. GroEL may therefore interact specifically with macrophages to provoke inflammation in hosts infected with *F. tularensis*.

# Pathomics Discovery Platform: *Brucella-Salmonella*:Host Transcriptomics, Proteomics & Bioinformatics

L. Garry Adams, DVM, PhD, DAVCP, Associate Dean for Research, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4461

Understanding the pathogenesis and host-pathogen infection-response pathways is central to the ability to develop improved prevention and intervention strategies as well as medical countermeasures to biological warfare agents. Development of broad based therapeutics will benefit from the identification of common pathways induced in the host by multiple select agents rather than a single agent. The advent of broad scale global technologies that measure either the levels of mRNA expression or protein abundance of all the gene products simultaneously enables a systems level approach to the interpretation of the data. The subsequent application of powerful bioinformatics tools to the integration and interpretation of all the datasets on a global level provides a model of all the pathways that are responding to the infection by the select agents.

The Pathomics Discovery Platform combines host and pathogen gene microarrays and proteomics capabilities to identify and better understand common host pathways of the intracellular bacterial, select agent pathogens infection, *Brucella* spp. and *Salmonella typhimurium*. Initially, we focused on the *in vitro* macrophage cell culture infection model to optimize analytical procedures. These experiments provide data for the creation of a host response model that are subsequently tested in *in vivo* bovine models. Bovine macrophages were infected with *Brucella* spp. or *Salmonella* and at a variety of time points after the infection, infected macrophages were harvested, lysed and mRNAs were extracted for microarray experiments using the custom spotted arrays. Extracted proteins are being processed to peptides and analyzed for proteomic analyses. Bioinformatics approaches are used to integrate these data and extract the host response pathways. Ultimately, we will construct a conceptual *in silico* host response model that describes the common pathways involved in *Brucella-Salmonella* intracellular bacterial infection.

# Alphavirus Encephalitis: Determinants of Outcome

Diane E. Griffin MD PhD
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Alphaviruses are mosquito-borne enveloped, plus-strand RNA viruses that cycle in nature between mosquitoes and birds or mammals and can cause disease in humans or equines. Human disease is manifest as fever, rash, arthritis or encephalomyelitis. We have studied Sindbis virus, the alphavirus type species, in mice as an example of virus-induced encephalomyelitis. The virus infects neurons preferentially and the outcome is dependent on both host and viral factors. Young mice are more susceptible to fatal encephalitis than mature mice and this is independent of the adaptive immune response. Virus replicates more efficiently and causes death in immature neurons, but mature neurons can survive infection. This may be related to the increased expression of interferon pathway genes as neurons mature. In mature neurons that survive infection, the adaptive immune response, particularly antiviral antibody and interferon-γ produced by T cells, can control virus replication in a noncytolytic fashion. Noncytolytic mechanisms of viral clearance are important for recovery because cytotoxic elimination of the infected neuron would result in permanent neurologic damage. Antiviral antibody and interferon-γ are the primary means of noncytolytic clearance, but response is neuron type-specific. Virulent viruses can overcome the defense mechanisms of mature neurons and cause neuronal death. Virulence is primarily determined by amino acid changes in the glycoproteins and 5' NTR of the virus.

# Innate Immunity Against *Francisella tularensis* is Dependent on the ASC/caspase-1 axis

Sanjeev Mariathasan[1*], David S. Weiss[2*], Vishva M. Dixit[1], and Denise M. Monack[2]

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*Francisella tularensis* is a highly infectious gram-negative coccobacillus that causes the zoonosis tularemia. This bacterial pathogen causes a fulminating disease in humans after exposure to as few as 10 cells, and has raised considerable concern as a potential bioterrorist agent. Many of the mechanisms by which the innate immune system fights *Francisella* are unknown, but it is clear that *Francisella* compromises this host response by replicating within macrophages. Here we show that in response to *Francisella* infection, activated macrophages undergo caspase-1-dependent death and concurrently release the pro-inflammatory cytokines interleukin-1 and interleukin-18. Activation of caspase-1 and induction of macrophage cell death required the death-fold containing, caspase-1 adapter, ASC. *F. tularensis*-infected caspase-1- and ASC-deficient mice showed markedly increased bacterial burdens and mortality as compared to wild-type mice, demonstrating a key role for caspase-1 and ASC in innate defense against infection by this microorganism. *Francisella* mutant strains that do not localize to the cytosol did not activate caspase-1 or induce host cell death, suggesting that ASC/caspase-1 specifically responds to intracytosolic infection.

# Predictive Features Of The Genome-Wide Host Response To Infectious Diseases

David A. Relman, Stanford University, Palo Alto, CA 94305

Genomic tools and approaches have enabled a more detailed description of host-microbe encounters, and shed light on fundamentally important processes, including the cellular responses associated with infection. Genome-wide transcript-abundance profiles, like other comprehensive molecular readouts of host physiological state, provide a detailed blueprint of the host-pathogen dialogue during microbial disease, and can reveal functional gene-based modules associated with mechanisms of virulence and host defense. Studies of cancer based on genome-wide transcript-abundance profiles have led to novel signatures that predict disease outcome and serve as useful clinical classifiers. The highly dynamic and compartmentalized aspects of the host response to pathogens complicates efforts to identify predictive signatures for infectious diseases. Yet, studies of systemic infectious diseases so far suggest the possibility of successfully discriminating between different types (classes) of infection and predicting clinical outcome. In addition, host gene expression analysis could lead to the identification of early signatures associated with a protective immune response, both to natural infection and to vaccination. Early explorations in some of these areas indicate the potential feasibility of this approach but also point to important unmet challenges.

# Whole-Genome Comparison Approaches to the Study of Virulence

Emilio Garcia, Lawrence Livermore National Laboratory, Livermore, CA 94550

Using in-house, recently generated, whole-genome sequences of pathogens of interest and their near-neighbors with reduced or differential virulence, we have explored the ability to identify novel virulence genes or mechanisms of virulence in selected pathogens. Using this approach, we have looked at the role of unique regions acquired or retained during evolution of pathogens such as *Yersinia pestis*, *Francisella tularensis* and *Brucella abortus*. Furthermore, we have constructed whole genome DNA arrays that have enabled us to perform comparative, genome-wide expression studies among the pathogens and their less pathogenic near neighbors. We have identified such unique genomic regions in *Y. pestis* versus *Y. pseudotuberculosis*, *F. tularensis* Schu4 versus *F. tularensis* LVS, and *B. abortus* versus related Brucella species. For *Y. pestis* we have generated individual knockouts of each of the unique regions and tested them for loss of virulence in a murine model.

Differentially inactivated genes (pseudogenes) and non-expressed proteins have served to identify in *B. abortus* target candidates for rapid diagnosis, identification and potential vaccine development. The approaches used with these pathogens are being complemented with parallel studies that measure differential global gene expression patterns in the host during infection by fully virulent and less virulent pathogen pairs. The advantages and limitations of this approach will be discussed.

# Beyond Pathogen Identification: Informatics Support for Characterization of Virulence, Antibiotic-Resistance, Host-Pathogen Interactions, and Evidence of Genetic Engineering

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The LLNL pathogen informatics team has been supporting a wide range of diagnostic assay development efforts over the past 6 years. Initially, the focus was heavily slanted towards pathogen identification diagnostics (e.g., "What organism is this?") The LLNL team has been quite successful at getting large numbers of pathogen assays validated and into daily use around the country in the BASIS and BioWatch programs. This work is now focusing on filling the remaining gaps and will be briefly summarized, along with parallel work on designing protein detection assays.

More recently, we have been focused to begin to answer questions about pathogen characterization (e.g., "What harm can this organism do? What resistance to countermeasures does this organism possess?") and pathogen interactions with hosts (e.g., "What effects does this organism have upon the host's functioning?")

We will describe our recent informatics work supporting several projects that are exploring pathogen functions and host-pathogen interactions. We will also describe a large-scale project that defined nearly 900 Hidden Markov Models (HMMs) to known mechanisms involved with virulence and antibiotic-resistance, as well as vectors indicative of potential bacterial engineering. This work has led to microarray prototypes using over 390K probes that are being used to establish probe design rules and determine effective limits of detection on this platform.

Looking forward, we will summarize some of the current informatics challenges to tackling the problems of detecting and characterizing emerging, unknown, and engineered organisms, both in the environment and within hosts.

# Etiologic *vs*. Non-etiologic (Syndromic) Diagnosis: Fitting into Public Health and Biodefense Systems

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*Gretsky's Law* (as espoused by Charlie Canter): "Skate to where the puck is going to be, not where it is."
*Biodefense Aphorisms*: "Do not assume anything." "Expect the unexpected."

Many facets of the pathogenesis of the infectious diseases caused by agents that may be favored by terrorists, especially those involving innate and acquired host defenses, have been forwarded as candidate diagnostic targets for use in early warning systems and even in the special clinical systems needed to deal with the large numbers of patients that might need emergency care in a bioterrorism episode. As stated in the recent IOM Report, *Globalization, Biosecurity, and the Future of the Life Sciences* (National Academies Press, 2006) we need "a broader perspective on the 'threat spectrum'": (1) "We must recognize the limitations inherent in any agent-specific threat list and consider instead the intrinsic properties of pathogens and toxins that render them a threat and how such properties have been or could be manipulated by evolving technologies"; and (2) "We must adopt a broadened awareness of threats beyond the classical 'select agents' and other pathogenic organisms and toxins, so as to include, for example, approaches for disrupting host homeostatic and defense systems, and approaches for creating synthetic organisms."

However, while delving into the pathogenetic markers of disease processes and host response processes associated with specific infectious agents and the diseases they cause, that is "non-etiologic (syndromic) diagnostic approaches," it seems important to assess just how the products of such research might fit into the diagnostic systems that our national and local institutions and agencies will be using for the foreseeable future. Can completely new approaches be piggy-backed upon traditional systems, which are largely based upon etiologic diagnosis (and associated immunologic/serologic diagnosis), in long-established laboratories? Or, will new laboratories, with separate personnel, be needed to carry out such non-etiologic (syndromic) diagnostic testing? Or, will "curb-side" — "dipstick" etiologic and/or non-etiologic tests be put in the hands of first responders, with all professionally-based diagnostic approaches bypassed? Or, will we have "all-of-the-above," and if so, who will interpret results in the timeframe needed to drive prevention and control actions? Is our leadership up to any such change?

Several perspectives are needed in trying to answer these questions:

(1) Much biodefense technology is rooted in the military / national security culture, not in the very different culture of public health laboratory and epidemiology institutions. After the initial burst of cross-talk following 9-11 and the anthrax episodes of 2001, these cultures seem to have resettled into their separate traditional trenches. Importantly, first responder and emergency services personnel seem to have received most of their training from the military / national security culture (often from retired experts serving as private consultants). Are those experts who are teaching biodefense knowledgeable enough of the complex issues of etiologic vs. non-etiologic (syndromic) diagnosis?

(2) Of all the forms of terrorism, bioterrorism stands out as most different, most misunderstood by people who are experts on explosive, chemical and nuclear threats. For example, in a bioterrorism attack there will be no immediate warning, only the later appearance at emergency rooms and clinics of numbers of patients suffering common, perhaps unusual, perhaps not unusual, clinical signs. All this might be localized, or might be part of a multifocal attack. Are those experts who are teaching biodefense knowledgeable enough of the complex issues of etiologic vs. non-etiologic (syndromic) diagnosis as questions and decisions are made in emergency rooms and clinics?

(3) The "astute clinician" (medical or veterinary) and/or the "astute pathologist" will be the first to suspect a malicious attack, and the first to ask for the special laboratory testing called for at this point. The same people will likely be the first to ask for the kind of initial epidemiologic investigation that triggers the full public health response system. Will these clinicians and pathologists be willing to deal with etiologic as well as non-etiologic (syndromic) diagnosis?

(4) Again, after the initial burst of cross-talk following 9-11 and the anthrax episodes of 2001, public health authorities, now rather well funded, have independently developed comprehensive action plans that involve all levels of the public health infrastructure and health care systems, including pharmaceutical stockpiles, but partly because of separate politics, separate funding and separate cultures, additional new connections across agencies and institutions seem to have slowed to a trickle. Can the initial burst of enthusiasm for new approaches be rekindled at the interface between the public health, clinical medicine and counterterrorism communities of our country?

(5) Our leadership suffers from "The View from the DC Beltway," "The View from Atlanta" (i.e., CDC), "The View from The California Office of Emergency Services (OES)," "The View from Local Emergency Services and First Responder Organizations," "The View from the State Public Health Establishment," et al. A major fault uncovered in bioterrorism "war games" (i.e., "table-top exercises") in recent years (Topoff I and II, Dark Winter) has been that our leaders are unfamiliar with the character of bioterrorist attacks, do not understand available policy options and their consequences and likely will turn for advice to trusted associates rather than unfamiliar senior public health and medical leaders. Can decision-makers, via their advisors, fathom the complexities of etiologic vs. non-etiologic (syndromic) diagnosis?

(6) National medical research leadership, especially in regard to funding, seems on the horns of a dilemma—on one side noting a need for pragmatic technology development, and on the other side wishing to continue the tradition of scholarly investigator-initiated research. This has been especially clear in regard to national biocontainment facility needs, and specialized diagnostics and diagnostic technologies needs. Here, the leadership certainly understands the value and potential value of both etiologic and non-etiologic (syndromic) diagnostics, but funding seems quixotic. How does the new U.S. Department of Homeland Security fit into the overall funding base for research in this area?

(7) Yet again, after the initial burst of cross-talk, the special case of the threat of an agricultural bio-terrorism incident seems to have drifted back into the bailiwick of USDA and its state-based subunits. The far more advanced infrastructure and technologies of human public health institutions have not been well co-opted as initially anticipated. Can non-etiologic (syndromic) diagnosis play a role here?

(8) In recent years nearly all emerging infectious disease episodes have involved zoonotic etiologic agents. Further, nearly all infectious agents listed as bioterrorism threats are zoonotic. Public health prevention and control strategies have largely been developed from experiences with vaccine-preventable childhood diseases, sexually transmitted diseases, etc., where traditional surveillance and case-control studies provide the base for intervention activities. Most public health leaders "cut-their-eye-teeth" dealing with such diseases. For the zoonoses, prevention and control strategies have come from a quite diverse and separate base. At the heart of this base have been individual scientists working at the interface between public health and veterinary public health, who have spent whole careers accumulating highly specialized knowledge and experience. These scientists have learned to work with experts in fields far removed from the medical sciences, fields as diverse as molecular virology, bacteriology and parasitology, immunology, pathology, ecology, animal biology, wildlife biology, mammology, ornithology, entomology, meteorology, climatology, geography, epidemiology, sociology, and economics. It would seem that these scientists are barely coping to advance etiologic diagnostics for most of priority zoonotic agents. It seems likely that non-etiologic diagnostic approaches will only come after much more pathogenetic research of zoonotic infections and agents in reservoir hosts and in humans.

With these perspectives in mind, one question before the workshop participants should be, "Will any non-etiologic (syndromic) diagnostic approach, no matter how promising, ever be assimilated into the largely etiologic diagnostic systems used in those institutions whose diagnostic findings actually drive intervention actions?" If proven non-etiologic diagnostic approaches are only used in isolated military or national security settings, then how will key specimens entering the main public health diagnostic stream of our country ever be tested using non-etiologic diagnostic technologies? Even at institutions such as CDC, there is some separation between specimens collected for biodefense *vs.* public health purposes. Unlike the situation with environmental specimens, for example the air sample specimens assayed in connection with the Salt Lake Winter Olympics, where there was no competing turf, there is a long-established turf regarding ownership of diagnostic specimens from humans, and to date this turf seems quite conservatively organized at national, state and local levels. Is this to change? Is this Workshop a place to discuss such change?

# Genome-Wide Survey of Host Responses: Use of Computational Analysis to Classify Exposures and Extract Signatures of Unconventional Versus Common Viral Exposures

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**Background:** Exposures to many unconventional pathogenic agents result in flu-like illness that are initially indistinguishable from common respiratory illnesses and early diagnosis to distinguish among the severe vs common viral infections depends on pathogen proliferation to dangerous, near-untreatable levels. Assessing exposure to a pathogen, in advance of onset of illness or at various stages post-exposure, is invaluable among the diagnostic options. Lymphocytes serve as "whistle blower" indicators as they encounter pathogenic agents even early during the course of infection, registering the encounters in their mRNA. and developing patterns of expression that correspond to each specific pathogen. Time series of gene expression patterns relate to the stage or severity of the infection and are unique for each pathogen.

**Results**: We are using the host blood for determination of whole genome regulation in response to various viral agents to extract features and signatures that can be used for point-of-care diagnosis of various viral infections (common respiratory, arena, flavi-, alpha- and other viruses). These data also have the potential to provide stage-appropriate therapeutic targets. These studies utilized exposure time sequences of host gene expression. Series #1 contained common respiratory viruses (influenza A, parainfluenza, rhinovirus, respiratory synctical virus). Series #2 focused on 5 arena viruses (the highly virulent hemorrhagic virus, Lassa, and 2 additional pairs of virulent/avirulent arena viruses). Series #3. includes flaviviruses (West Nile virus and 4 serotypes of Dengue), and Series #4, 2 alpha viruses, among which was Venezuelan equine encephalitis (VEE). The "training sets" were constructed from in vitro exposures to purified peripheral blood leukocytes from ~6 human leukapheresis donors for each virus described above. Numerous modeling / mathematical techniques were applied to these datasets in order to identify signature patterns indicative of each. The "shrink/grow" modeling approaches were used as well as other algorithms that have shown success for signature extraction. For the "grow" algorithm, genes are individually selected aiming at those with the best discriminating power; the first of those frequently show properties unique for specific viral infections.

Our first question related to the fact that, by necessity (due to the lack of human samples from exposures to biothreat pathogens), we were training on in vitro exposures to these many pathogenic agents. Our thesis was that such an exposure would provide a biochemical signature, related to the mechanistic course of action of each pathogenic agent and it should be reflective of at least early events from in vivo exposures (to non-human primates-NHP).

Our first "test" data set was from host gene expression responses in peripheral blood (leukocytes) from NHP exposed to VEE. This is a mosquito-borne viral disease characterized by fever and one or more of the following: severe headache, back pain, myalgias, prostration, chills, nausea, vomiting, weakness and other flu-like symptoms. The aerosol form of VEE is highly infectious, making VEE a potential biowarfare agent. This could be especially worrisome if strains are altered genetically to increase pathogenicity. If this virus was deployed efficiently, it could incapacitate thousands of people for a week or more and cause untold psychological stress as well as being physically debilitating. Diagnosis of VEE relies on virus isolation from acute phase serum or from spinal fluid, or on detection of VEEV-specific IgM in the cerebrospinal fluid in cases of encephalitis. We carried out 2 different approaches using blood from 12-25 control (unexposed) NHP along with 8 samples from VEE-exposed NHP. Although no signs of illness were apparent by day 3, gene expression patterns identified these NHP as having mild exposure to the virus. To increase the level of confidence in the methodology, the NHP samples from 12 control and 5 VEE challenged NHP were blinded as "test" datasets. All of the 17 samples were correctly categorized as to the nature of the exposure. Using both types of training/test methods, one control NHP was categorized correctly as a "control" but had at least some indicators of common viral exposure.

Additional studies are underway to apply the most instructive of these algorithms to arena viruses (NHP exposed to Lassa virus), people exposed to dengue virus or West Nile virus or influenza A.

**Conclusions:** Oligonucleotide (70 mer) arrays or cDNA microarray studies were effectively used for "training" datasets to address the following questions: 1) Are there common genes modulated under both NHP in vivo and human in vitro conditions in response to VEE exposure? 2) Does this common set of genes permit sufficient discrimination of a test data set based on an in vivo pathogen exposure against a database derived from in vitro exposures to several pathogens especially respiratory and arena viruses? Our successes in correct identification of exposures in NHP to VEE provide a framework for further work in validating and differentiating viral exposures from other pathogenic agents using blood from in NHP or human exposures.

Today's fast growing sphere of bioinformatics involves retrieving precise information from massive datasets to achieve in-depth understanding of systems biology. Using our program, GeneCite, scientists can interconnect two input files via any of the three available Boolean operators at NCBI web domain. The other tool, PathwayScreen, takes a list of Gene ID numbers and outputs a file listing the pathways that those genes are in and a link to any appropriate resources, namely BioCarta.com. These and other tools (BioSPICE.org) provide a systems biology approach to understanding pathogenesis through inferences of the host responses.

This study is part of our larger quest to create a library of host gene expression responses that broadly distinguish among common and unconventional pathogenic agents, showing severity of responses via a molecular pathogenesis blueprint that will enable us to design novel methods for intervention. New technologies for multiplexing genomic responses have decreased the time required for such analyses to nearly "point of care" applications. Finding a small number of predictor genes that can accurately classify exposures can offer new options for early detection upon exposure to pathogenic agents

# Breath as a Diagnostic

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Generally, detection of infection occurs when signs are observed and symptoms are reported by exposed people. To effectively protect troops and civilians against attacks with biological weapons, a rapid method to detect infection prior to the onset of illness would be beneficial. We have proposed to develop such a method for rapid evaluation of infection using the markers in breath to aid in the determination of infection. Collection of breath has several advantages including that it can be obtained readily from all people and sampling is non invasive and therefore, low risk. There were three stages to this approach. First we wanted to demonstrate that biological molecules produced by the host in response to infection could be rapidly detected. Second, we wanted to demonstrate that these molecules could be used to distinguish infection i*n vitro* and *in vivo*. Third we wished to show that these host response molecules as a group do not appear in uninfected populations. To this end we have demonstrated that the early responses of cells to infection vary with infectious agent. We showed the appearance of cytokines and other proteins in exhaled breath (EB) derived from pathogen exposed pigs using ELISA and mass spectrometry (MS). In these studies, their appearance preceded the onset of symptoms and correlated with exposure to agent. For this technology to be an effective diagnostic, immune markers which appear as a result of exposure to agents should be absent in uninfected populations. Finally, we demonstrated that early immune markers which correlate with infection are not randomly detectable in the baseline breath samples of apparently health swine populations by MS or ELISA assays This data further supports the operational concept of using EB condensates to rapidly evaluate health status.

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