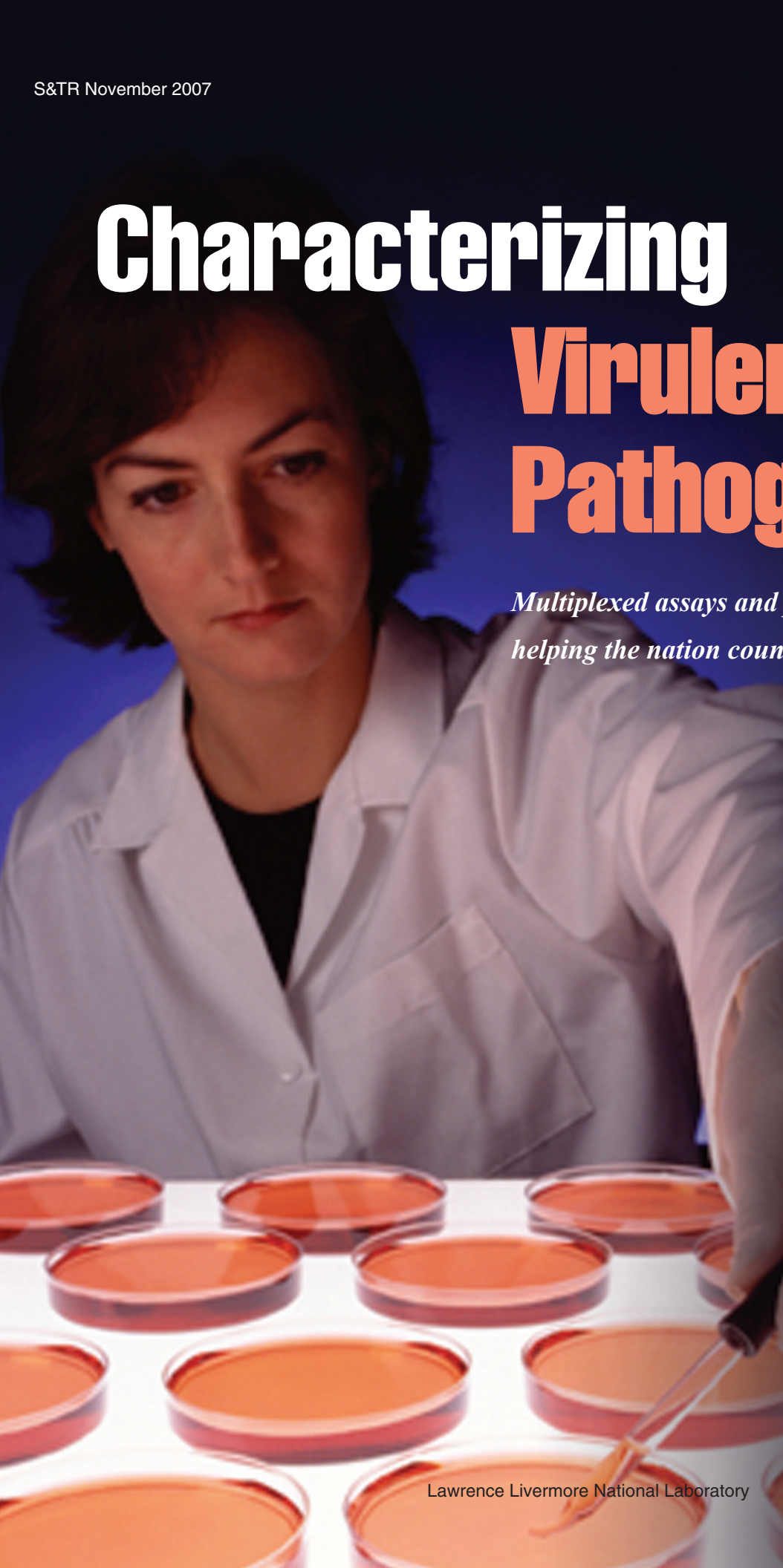
Characterizing Virulent Pathogens



Multiplexed assays and proteomics research are helping the nation counter potential biothreats.

SECURING the nation against potential terrorist attacks is an increasingly complex challenge. Today's global environment requires that homeland security officials be prepared for a range of threats. One concern is that terrorists may use biological organisms to attack the U.S. To address this potential threat, the Department of Homeland Security's (DHS's) Director for Science and Technology allocates more than 40 percent of its budget to chemical and biological countermeasures research.

When Lawrence Livermore began working on the problem of biological threats in the 1990s, few solutions existed for the early detection and characterization of biological agents. Researchers in Livermore's Global Security Principal Directorate attacked the problem by integrating expertise in biology, chemistry, engineering, and computation to develop a succession of increasingly capable, rapid, and rugged biodetection instruments. Anticipating the importance of detection technologies and threat signatures in countering biological attacks, Global Security initiated several forward-thinking projects with Livermore's Laboratory Directed Research and Development

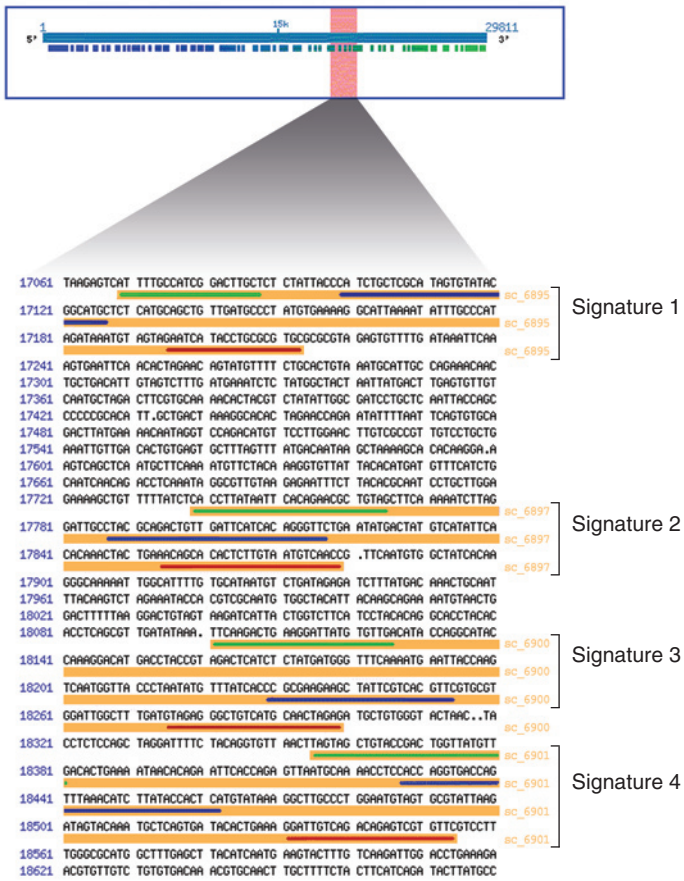
Program. They also formed partnerships with the Centers for Disease Control and Prevention (CDC), other national laboratories, and universities to develop assays for pathogens that might be used in a biological attack and those that could cause a disease epidemic.

Natural or Intentional Outbreak

Determining whether a pathogenic (disease-causing) organism has appeared through natural mechanisms or has been introduced in an act of biological terrorism is challenging. Many microorganisms occur naturally in the environment and cannot be genetically distinguished from those that might be used in an intentional release. Because of the potential impact to the country if a bioterrorist attack were to occur, scientists must be able to quickly characterize the organism in question.

Livemore researchers are addressing some of the knowledge gaps that exist in characterizing pathogens, their disease transport, and host–pathogen interaction mechanisms. "Given the capabilities of modern biotechnology," says computer scientist Tom Slezak, who leads the Laboratory's bioinformatics group, "we don't know if a disease outbreak is due to an intentional release or a natural outbreak until we can check the genome closely."

The 2002–2003 outbreak of severe acute respiratory syndrome (SARS) is a case in point. The outbreak began in China and quickly spread to Singapore, Vietnam, and Canada. Although the casualties numbered fewer than 1,000 people, the rapid spread of the disease and the lack of preparedness for an outbreak caused widespread concern. It took the international public health community about 90 days from the time unusual disease symptoms were first noticed in China until the SARS virus was finally isolated, sequenced, and identified as a new type of coronavirus. Subsequent studies identified numerous wildlife hosts, confirming that SARS was an emerging natural pathogen.



Livermore researchers used the computational design system KPATH to produce four DNA signatures of severe acute respiratory syndrome. Each signature has a forward primer (green underline), a reverse primer (red underline), and an internal probe (blue underline). An entire signature amplicon (a small, replicating DNA fragment) includes all three components.

Computers Speed Signature Analysis

Researchers develop laboratory assays to help detect organisms. The most promising assays are experimentally validated to meet rigid criteria. Researchers start with a map of the microbe's genome and determine a set of candidate signatures—patterns of DNA sequences of nucleotides unique to the organism's genome. Once validated, these signatures enable scientists to rapidly and confidently diagnose the presence of the pathogen.

Livermore's bioinformatics group, which includes biologists, computer scientists, mathematicians, and statisticians, is the largest in the world focusing on pathogen signatures, and it was first to use computers for identifying candidate signatures at a whole-genome scale. (See *S&TR*, April 2004, pp. 4–9.) The group's computational DNA-signature generation and analysis system, called KPATH, uses efficient algorithms to compare the genome of a target pathogen to a library of microbial genomes, searching for areas unique to the target organism or to the family of related pathogens. Designed after the September 11, 2001, terrorist attacks and implemented in 2002, the automated system can deliver microbial signature candidates spanning 200- to 300-plus base pairs of DNA in minutes to hours. SARS was the first natural-outbreak pathogen the group used to test KPATH's capability. When CDC asked the Livermore team to develop candidate signatures for SARS in 2003, Slezak's group did so in just three hours.

During a disease outbreak involving potentially tens of thousands of sick people, time is of the essence. Medical personnel need to know what tests to perform—for example, which bodily fluids to sample to look for the presence of a particular virus. "Clinicians must pick one of five fluid types to test," says Slezak. "Choosing the wrong bodily

fluid to test could have devastating consequences in terms of the number of people who might get sick or die. Any delays can accelerate the spread of disease."

In 2006, a Livermore-developed signature for SARS was used in a landmark study to detect the virus in the bodily fluids of long-tailed macaque monkeys. For this study, researchers from the U.S. Army Medical Research Institute of Infectious Diseases infected the animals with the SARS-CoV *Urbani* strain. A key finding was that researchers did not find the virus where expected. "Conventional wisdom said the virus would appear in the feces," says Slezak. "However, in all but one monkey, the virus was found in the urine." These results will help researchers develop effective SARS vaccines and therapies. "If SARS were to reappear," says Slezak, "clinicians now know where to look for the virus in the body and when."

Nationwide Warning System

Early detection and response to the release of a potentially lethal microorganism are crucial for saving lives. In 2003, DHS launched the BioWatch program, a nationwide early-warning system that detects trace amounts of specific microorganisms in the air. The program is a collaboration of federal and state agencies, including CDC, the Environmental Protection Agency, Los Alamos and Lawrence Livermore national laboratories, the Federal Bureau of Investigation, and state and city environmental monitoring agencies.

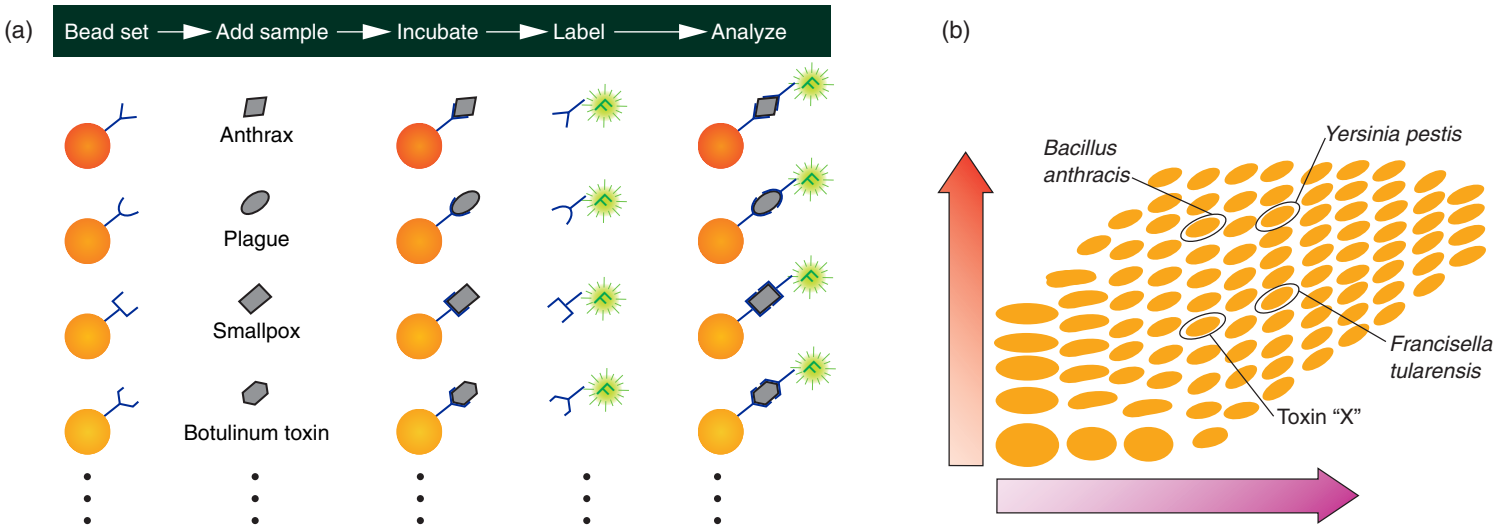
BioWatch detectors, in place in about 30 cities in the U.S., use the architecture originally developed for Livermore's and Los Alamos's Biological Aerosol Sentry and Information System (BASIS) as well as up-to-date versions of the DNA signatures used in BASIS. (See *S&TR*, October 2003, pp. 6–7.) Local agencies monitor BioWatch instruments, and CDC coordinates sample analysis through the nation's Laboratory Response Network. Scientists at participating laboratories analyze the samples using polymerase chain reaction (PCR)—a technique that replicates and amplifies a fragment of DNA to produce copies of a sequence so it can be detected.

Fast Multiplexed Assays

Typical PCR is a singleplex process—that is, it detects one signature on an organism's genome. If the result is positive for a target organism, the technician tests the sample with other signatures related to the organism to confirm the initial finding. To speed the analysis process, a Livermore team funded by DHS and CDC developed a multiplexed nucleic-acid assay that can detect multiple biological threat agents at one time. The team, led by molecular virologist Pejman Naraghi-Arani, includes James Thissen, Alda Celena Carrillo, Jason Olivas, Sally Smith, Linda Danganan, and Lance Tammero.

In developing the assay, Naraghi-Arani asked Slezak's group to screen the available genomic sequence information of selected pathogens and identify regions of interest on the genomes. Candidate signatures were compared with other microorganisms, including related strains and genetic near-neighbors of the target pathogens. The computational screening included DNA from more than 2,300 aerosol samples collected with BioWatch detectors as well as samples from soils, bacteria, insects, animals, and humans to test for cross-reactivity.

Naraghi-Arani's team then developed the multiplexed assay using a bead-based liquid array technology that extracts nucleic acids from the sample and amplifies the DNA. Polystyrene microbeads are tagged with a sequence of



(a) Multiplexed assays simultaneously detect multiple bacterial spores and cells, viruses, and toxins. (b) Using a Luminex instrument, scientists can resolve up to 100 different classes of biological organisms by their fluorescence intensity.

nucleotides that complement the signatures of interest. If a target organism is present, it will combine with a microbead. The beads are embedded with precise ratios of red and infrared fluorescent dyes. When excited by a laser, the two dyes emit light at different wavelengths. The ratio of each dye reflected emits light at a unique frequency that identifies the organism. An additional dye is used to indicate if the bead detected the unique signature DNA of the pathogen of interest.

The multiplexed assay requires only picogram quantities of DNA and contains all verification signatures in one reaction. "The sensitivity is as good as the best singleplex assay," says Naraghi-Arani, "and the cost savings is about 90 percent." The team subjected the multiplexed assay to a series of verification tests. "Even in a mixture containing large amounts of a variety of DNA masking the target DNA, the assay panel still identified the presence of the target organisms," says Naraghi-Arani. "These results demonstrate that with a rigorous bioinformatics process, a multiplexed assay can be simpler to run than a singleplex assay."

Next-Generation BioWatch

DHS and CDC have evaluated the multiplexed assay to determine if it could be incorporated into BioWatch sample screening. In collaboration with the agencies, Thomas Bunt, associate program leader for Livermore's biological monitoring and response group, conducted a six-month pilot study to compare the multiplexed assay performance to that of the existing BioWatch format. Naraghi-Arani's team participated in the pilot. Says Bunt, "The multiplexed assay contained more than 25 pathogen signatures. Labor and reagent costs were dramatically reduced because we were able to combine multiple BioWatch verification assays into a single reaction."

The researchers analyzed more than 12,000 filter extracts. Results demonstrated that the multiplexed assay yielded faster verification results while maintaining sensitivity of pathogen detection. DHS is incorporating the multiplexed assays into the BioWatch system. "Livermore has been involved in every step of the development process, from bioinformatics to assay validation, protocol development, and data management," says Bunt. "We also operate two BioWatch laboratories so we are end users as well."

Testing for Animal Diseases

DHS is also funding work at Livermore and elsewhere on multiplexed assays to detect agricultural diseases. A number of very serious animal diseases are endemic in other parts of the world but have not appeared in the U.S. for several decades. (See *S&TR*, May 2006, pp. 11–17.) Agriculture is a major sector of the U.S. economy, accounting for more than 13 percent of the gross domestic product and employing more than 15 percent of the U.S. population. Homeland security officials are concerned that terrorists might attempt an attack on the nation's agricultural industry.

Molecular virologist Ray Lenhoff, veterinary epidemiologist Pam Hullinger, and chemist Ben Hindson are collaborating with the Department of Agriculture's National Veterinary Services Laboratory and the Foreign Animal Disease Diagnostic Laboratory at Plum Island, New York, the agency responsible for testing and investigating foreign animal diseases. Plum Island conducts about 300 investigations each year, but during a major outbreak, demand could rise to 100 investigations per week.

The clinical signs of foreign animal disease often closely mimic many diseases that regularly occur in animals. A particular concern is foot-and-mouth disease, an extremely contagious viral disease of cattle, pigs, sheep, goats, deer, and other ruminants. "Traditional tests use a single detection assay to look for one virus at a time," says Lenhoff. "A negative test result could mean either that the assay failed or that the disease wasn't present." The team's first version of the multiplex assay screens for both DNA and RNA viruses and looks for 17 target signatures, including seven major strains of foot-and-mouth disease.

An outbreak of a foreign animal disease, such as foot-and-mouth disease in the U.S., could be devastating to the nation's economy.

The researchers are working on two additional assays: one for diseases affecting cattle and one for those affecting swine. Once they complete the initial development and characterization studies, they will send the assays to Plum Island for additional testing. The team has also developed a high-throughput, semiautomated system that can process more than 1,000 samples in 10 hours using a single line of equipment and two technicians.

In 2006, Hindson led an exercise involving 14 laboratories that belong to the National Animal Health Laboratory Network to evaluate the performance of the multiplexed assays in the hands of end users. Hindson's team "spiked" the test samples with known domestic viruses that mimic foot-and-mouth disease. The laboratories then analyzed the samples

and reported their findings to Livermore. The exercise allowed researchers to test the assays with multiple users and obtain a large data sample to measure the performance of the signatures included in the multiplexed panels.

Identifying Virulent Proteins

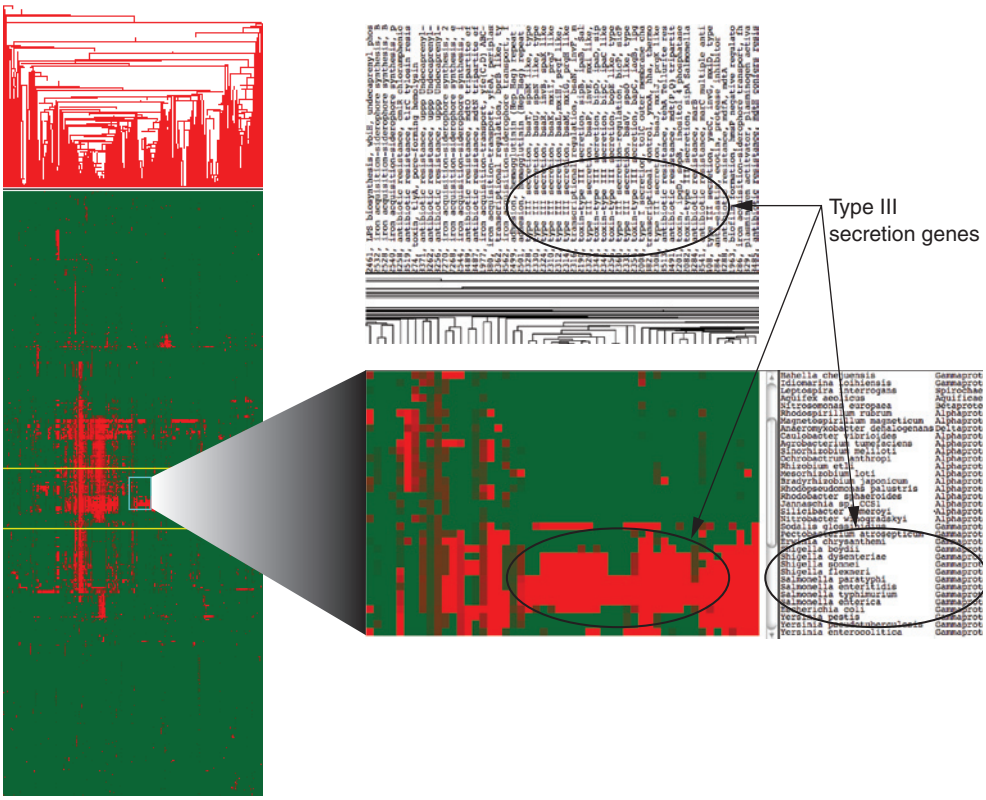
Although an assay can confirm the presence of a microbial species, it is limited in the information it can provide about the species' strain and virulence. For example, of the SARS signatures Slezak's group developed in 2003, it is not clear which, if any, identify genes related to virulence factors or which types of hosts the virus could infect. To develop countermeasures against a particular disease, scientists must understand the pathogen's replication mechanism, including how it confers virulence. Slezak's group is using computational analyses to determine patterns characteristic of an organism's potential virulence mechanisms, including antibiotic resistance. They are applying the data to develop recognition assays using NimbleGen® microarrays, which detect up to 390,000 specific genetic features that serve as signatures for particular functional mechanisms. The microarrays provide a "parts list" of functional elements to more fully characterize a BioWatch positive hit or other sample.

Livermore researchers are also conducting proteomic experiments to study virulence mechanisms. Proteomics characterizes all proteins within a cell, including protein expression levels. Some pathogen proteins, known as virulence factors, are responsible for conferring a pathogen's virulence. The key to characterizing a pathogen's virulence potential lies in knowing which proteins and how much of them are expressed.

A Livermore team led by protein chemist Sandra McCutchen-Maloney identified virulence-inducing mechanisms in the *Yersinia pestis* bacterium, the pathogen that causes plague. The *Y. pestis* work draws on earlier Laboratory studies. (See *S&TR*, March 2002, pp. 4–9.) The team, which includes Brett Chromy, Todd Corzett, and Ann Holtz, is collaborating with Walter Reed Army Medical Center, Texas A&M University, University of California at Davis, University of Minnesota, and Pacific Northwest National Laboratory. To augment the gene expression research, McCutchen-Maloney's team provides proteomic data on pathogens and host response to pathogen exposure. The researchers will use these data to improve the assays so that they characterize threat pathogens and can identify exposure in humans and animals before symptoms become apparent.

Naturally occurring plague is transmitted from infected fleas or rodents to humans. Three forms of the disease exist: bubonic, septicemic, and pneumonic. Bubonic and septicemic forms can most often be treated with antibiotics; the disease of pneumonic plague is suppressed at the temperature of a flea (26 °C). However, at 37 °C, the body temperature for humans, virulence factors are expressed. By activating virulence mechanisms in *Y. pestis* through laboratory-induced growth conditions and then measuring cell contents, researchers can link proteomic data with genomic data and better understand pathogen virulence levels.

Dozens of proteins are responsible for virulence in *Y. pestis*. McCutchen-Maloney's team is studying 22 *Y. pestis* strains representing diversity of origin, virulence level, and countermeasure



Computed data compare known virulence and antibiotic-resistance mechanisms (columns) for all sequenced genomes (rows). Red indicates a mechanism is present, while green indicates it is not. Type III secretion is a mechanism that occurs in many pathogens, including *Shigella*, *Salmonella*, *Yersinia*, and *Escherichia coli*.

resistance to determine individual protein expression levels. Adding fluorescent dyes to multiplexed gels allows the researchers to measure differences in the abundance of expressed proteins among the various strains. Then using mass spectrometry, they can identify the proteins of interest—data that will guide the assay development work.

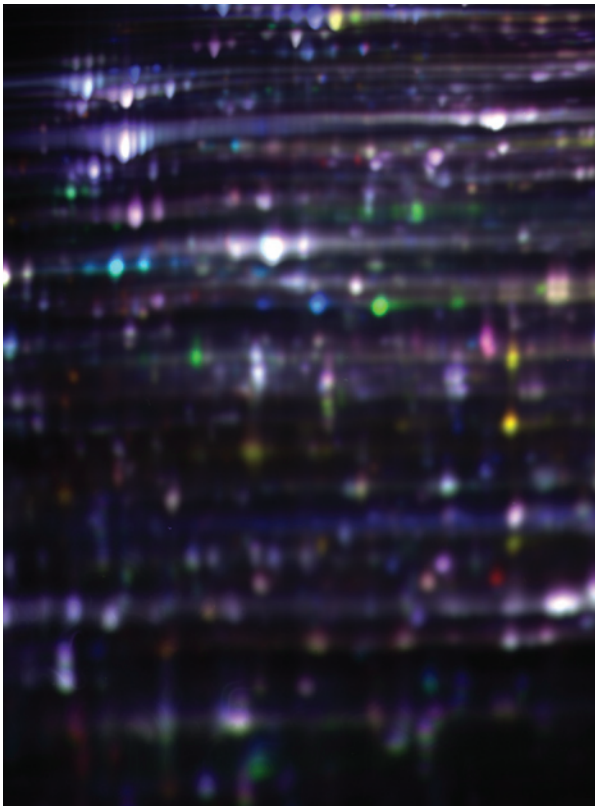
Host–Pathogen Interactions

The team is also attacking the problem from the other end—observing how a host's body interacts with the pathogen. Host–pathogen interactions contain biomarkers such as protein by-products that reveal virulence characteristics. McCutchen-Maloney says, "A lot of people can start showing up sick, as in the SARS cases in 2003. When health officials don't know what they are dealing with, it would be helpful to test patients for biomarkers that could identify the pathogen."

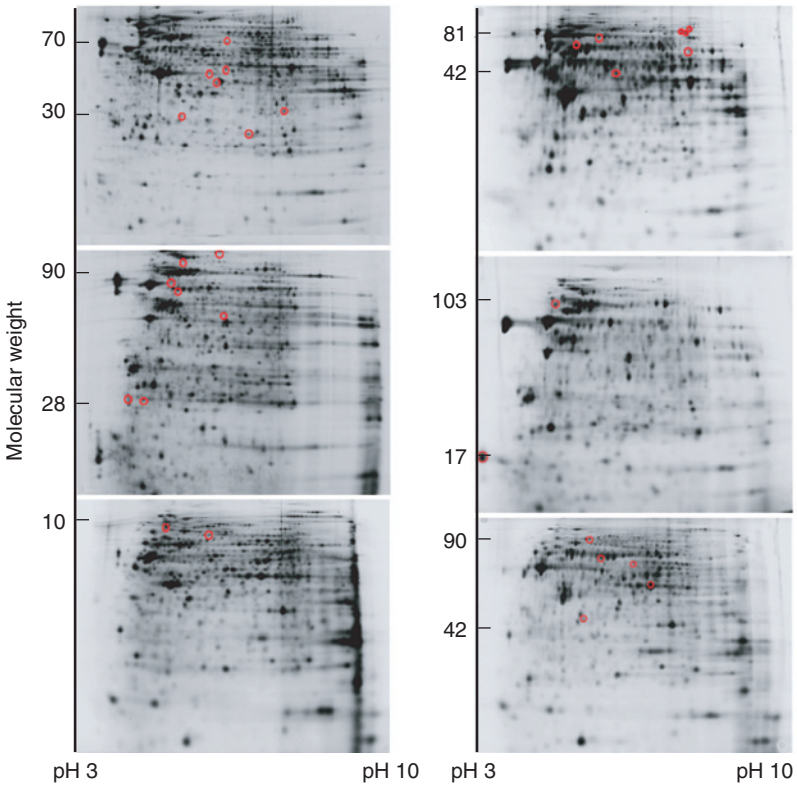
In one experiment, the Livermore team exposed human monocytes to *Y. pestis* and *Y. pseudotuberculosis*. The group identified 16 differentially expressed host proteins in the *Y. pestis* exposure and 13 host proteins in the *Y. pseudotuberculosis* exposure. Only two of the proteins were shared between the two exposures, indicating different immune response mechanisms.

The team also examined virulence mechanisms in two closely related strains of *Y. pestis*, one of which caused pneumonic plague in an animal model. Although the two strains are virtually identical at the gene level, they exhibited more than a 1,000-fold difference in pathogenicity, or virulence level. The research demonstrates that detection alone does not reveal virulence potential. "Near-neighbors can be that important," says McCutchen-Maloney. "It can be the difference between quick death and an upset stomach." The work will help researchers look for better ways to detect and understand virulence within the multiple strains of a species.

The team is working to build a pathogen reference library of information on known, unusual, and emergent pathogens. "The more organisms we can catalog in the



A two-dimensional gel map of 22 strains of *Yersinia pestis* shows more than 2,800 differentially expressed proteins (spots). The colored protein spots represent a *Y. pestis* strain that expressed a large amount of a particular protein.



Gel images of human macrophage cells exposed to *Yersina pestis* (left) and *Y. pseudotuberculosis* (right) display distinct profiles. Proteins from three different protein components are measured according to their molecular weight and pH level. Scientists then perform mass spectrometry on selected areas (red circles) to measure the amount of expressed proteins.

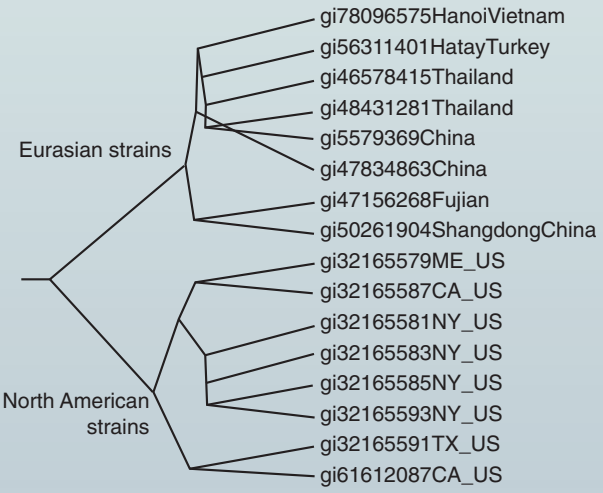
Nature's Ever-Changing Organism

Influenza, or the flu, sickens millions of people and is responsible for up to 500,000 deaths worldwide each year. New influenza viruses are produced by mutation or by reassortment—a process in which two similar viruses infect the same cell and their genetic material mixes. New flu vaccines are formulated every year based on the strains observed in the previous year and on expert opinion about which strains are expected to dominate worldwide in the next flu season.

In 2004, the Centers for Disease Control and Prevention called on Livermore scientists to develop candidate signatures for human and avian influenza viruses. Health officials are concerned about the possibility of a flu epidemic from any host since the influenza virus mutates so rapidly. Beth Vitalis, lead biologist for Livermore's pathogen bioinformatics group, explains, "Numerous strains of influenza circulate through the population every year, and a concern is that one of these strains could acquire an extreme virulence potential similar to the 1918–1919 influenza strain. A potentially more serious threat is that a lethal avian strain will mutate such that it can become readily human transmissible." The 1918–1919 pandemic killed approximately 50 million people worldwide. With today's travel patterns, an unchecked similar pandemic could be even more devastating.

As in the 1918–1919 flu pandemic, the body's immune system often accelerates the host's death. For example, when the body is attacked by a pathogen, one of its defense mechanisms may be to produce a higher level of inflammatory cytokines—proteins that are activated by immune cells and cause inflammation. The resulting inflammation can cause overwhelming damage to body tissues and organs.

Avian influenza infections of humans are relatively rare and have yet to occur in North America. Nevertheless, health officials want to prepare for the possibility that a strain of avian flu could become transmissible from human to human. "The influenza virus evolves rapidly," says Vitalis, "so diagnostics and vaccines must be continually evaluated and updated to be effective against circulating strains."



A computational clustering technique is used to group H5 avian influenza strains based on geographic lineages. All avian strains of influenza infecting humans have been of Eurasian lineage. To date, domestic avian influenza is not transmissible to humans.

library, the better we will be able to say with confidence, 'this looks like *Bacillus anthracis*, but it's not *anthracis*,'" says McCutchen-Maloney.

One of McCutchen-Maloney's next goals is to study viruses, which are more diverse than bacteria and can mutate or change much more rapidly. (See the box above.) As with bacteria, the mechanisms that drive viruses must be understood if researchers are to develop detection methods and countermeasures against them. However, viral mechanisms are more complicated than bacterial processes. Bacteria essentially carry all the parts they need to infect their hosts. In contrast, viruses must recruit or hijack some parts from a host's cells to do their damage.

By developing assays screened by a rigorous bioinformatics process and identifying pathogen replication mechanisms and host-response biomarkers, Livermore researchers are helping DHS and other health and security agencies strengthen the nation's biodefense preparedness programs. Events such as the 2003 SARS outbreak and the potential for an avian influenza outbreak or epidemic provide on-the-job training that prepares Laboratory scientists for rapid response. "We don't want to be unprepared, whether a disease outbreak is intentional or unintentional," says Slezak. "Our research is aimed at helping public health and homeland security officials make informed decisions about responding to and limiting the effects from any type of outbreak."

—Gabriele Rennie

Key Words: biological agents, BioWatch, foot-and-mouth disease, foreign animal disease, influenza, KPATH, multiplexed assays, pathogen, severe acute respiratory syndrome (SARS), virulence.

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