Quickly Identifying Viable Pathogens from the Environment

THE intentional release of pathogenic viruses, bacteria, or other biological threat agents in high-traffic areas such as a busy airport or train station could have catastrophic consequences, causing widespread fear and panic in addition to quickly spreading deadly diseases. Biothreat agents can be dispersed in air, water, or food and are extremely difficult to detect and identify. They are relatively easy and inexpensive to obtain or produce, which makes them an appealing weapon for terrorists.

In the event of a biological attack or other contamination incident, laboratory technicians would need to quickly process hundreds to thousands of samples to identify the type of pathogen released and determine the extent of contamination. Clearance sampling conducted before a decontaminated site can be returned to normal operations must be sensitive enough to detect very low levels of live spores in an environment that also contains a high number of biothreat agent spores killed in the cleanup activities. Decision makers need sampling results returned quickly to minimize the time that contaminated areas are closed to the public.

Current techniques for detecting viable pathogens involve several labor- and time-intensive steps, such as pipetting, centrifuging, plating, and colony counting. In addition, laboratories can process only 30 to 40 surface samples per day with these techniques, and confirmed results can take several days to obtain.

Livermore scientists (from left) Gloria Murphy and Teneile Alfaro demonstrate the automated processing of environmental samples using rapid viability polymerase chain reaction (RV-PCR).

To mitigate these efficiency issues, microbiologist Staci Kane and materials scientist Sonia Létant of Livermore's Physical and Life Sciences Directorate are developing a procedure to analyze samples and identify viable pathogens in less than 15 hours—significantly faster than the current process. Their method uses polymerase chain reaction (PCR) to amplify specific DNA sequences before and after culturing, and it can detect quantities as small as a few spores or cells of a deadly biothreat agent. Called rapid viability PCR (RV-PCR), this technique can efficiently distinguish viable spores or cells from dead ones and nonvirulent bacterial strains from virulent strains with the same level of confidence as provided with the traditional approach.

Kane and Létant are also developing robotic techniques to decrease the risk of human exposure to pathogens and increase the number of samples that can be tested at once. Livermore scientists have verified these new techniques using samples spiked with select bioagents and other contaminants, such as dirt, that could be present in specimens collected in the field. "With lab robotics, hundreds of surface samples could be processed per day with confirmed results reported the next day," says Kane, who leads the method development effort for the Laboratory's Interagency Biological Restoration Demonstration.

Technological Challenges for a Quick Assessment

The need for faster identification methods became clear following the 2001 anthrax attacks on several U.S. Postal Service buildings and the Hart Senate Office Building. Contaminated facilities remained closed for months while response teams worked to decontaminate them. To improve the nation's response capabilities, the Department of Homeland Security funded several projects to shorten the time needed to restore a site following a bioattack.

Sonia Létant, a materials scientist in the Laboratory's Physical and Life Sciences Directorate, reviews results produced on samples analyzed with Livermore's RV-PCR technique.

One of these projects is adapting the RV-PCR process to more quickly detect and assess the viability of *Bacillus anthracis*, the Gram-positive, endospore-forming bacteria that cause anthrax. Endospores are dormant, highly resistant structures that can survive extreme environmental stresses such as high temperature, high ultraviolet irradiation, desiccation, and chemical damage, which would normally kill the bacterium. Because of these extraordinary resistance properties, endospores are not readily killed by antimicrobial treatments and thus are of particular concern in decontamination scenarios.

In the Homeland Security project, the Livermore team is developing high-throughput sample processing to detect live *B. anthracis* surrogates in various environmental samples, including wipes, swabs, air filters, vacuum filters, vegetation, and soil. The Environmental Protection Agency (EPA) is supporting a related effort to optimize the technique and verify its ability to detect virulent agents.

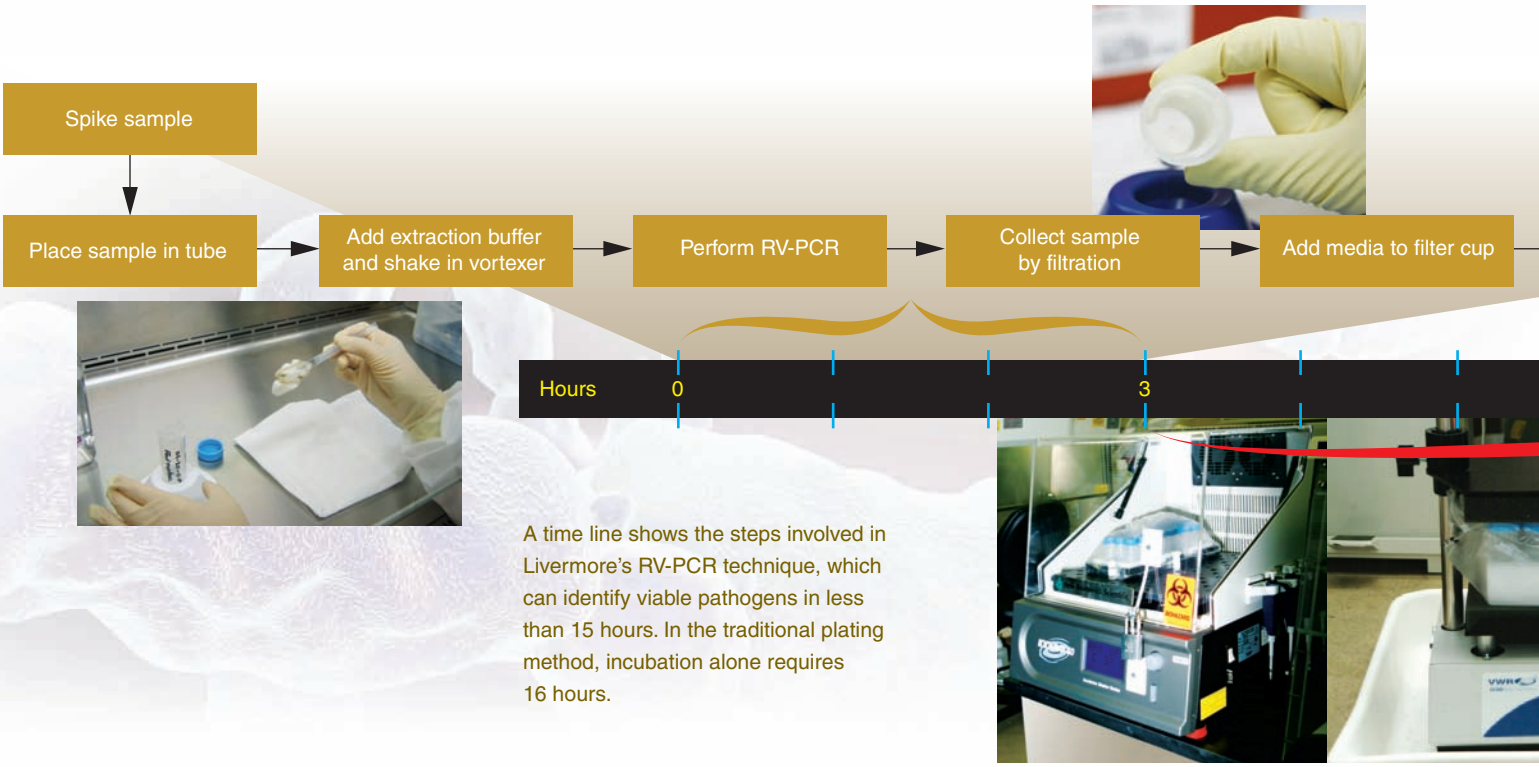
"A key part of the risk assessment after an attack is to determine whether living bioagent spores are present," says Kane. The RV-PCR approach would reduce the time for such assessments, so cleanup activities could be completed more quickly. "Our goal is to validate RV-PCR and get it deployed to the response community," Kane adds. "Our technique could ultimately be adopted by the EPA Environmental Response Laboratory Network for use following a bioagent release."

A Shorter Process with Better Results

Incubating a sample is a critical part of Kane and Létant's technique. By comparing the PCR results before and after incubation, they can determine whether bioagent spores or cells are dead or alive. "If everything is dead, no new DNA will be produced," says Létant, who is leading a team of five researchers for the EPA effort. "The change in PCR response shows us whether viable spores or cells are present."

Part of the EPA project was dedicated to choosing the optimal PCR assays for *B. anthracis*. The requirements for effective assays include selectivity, sensitivity, and robustness. The Livermore bioinformatics group, led by computer scientist Tom Slezak, used computational techniques to analyze assays from multiple sources. Létant and her team evaluated the top 10 assays and ultimately selected three—one for the *B. anthracis* chromosome and one for each of its plasmids. These assays had sensitivities below 10 genome copies, they were selective, and they were not affected by the presence of growth medium and cell debris in the PCR reaction.

With the RV-PCR method, samples such as surface wipes, air filters, water, and soil are placed in tubes and sent to a laboratory



A time line shows the steps involved in Livermore's RV-PCR technique, which can identify viable pathogens in less than 15 hours. In the traditional plating method, incubation alone requires 16 hours.

for processing, either manually by trained laboratory personnel or mechanically by a robotic platform. The technician (or robot) adds an extraction buffer to the sample inside the tube, and a machine called a vortexer shakes the tube, which releases spores from the sample material into the buffer solution. The sample is then transferred to a cup with a filter that collects the released spores. The filter is washed to remove contaminants, and growth medium is added to the sample. A portion of the mixture is withdrawn to serve as a baseline. The remainder is transferred to an incubator for 9 hours. After incubation, a second PCR sample is withdrawn.

All of these samples, or aliquots, undergo a chemical process called lysing, which ruptures a cell membrane to release the cell's DNA. Samples are then magnetically "cleaned" to remove the remaining debris and concentrate the spores' DNA. Only germinated spores and resulting cells respond to the lysing process, so DNA from dead or intact spores is not detected. "The concentration of DNA increases with the number of live *B. anthracis* cells in the sample," says Létant.

Improving on the Standard Approach

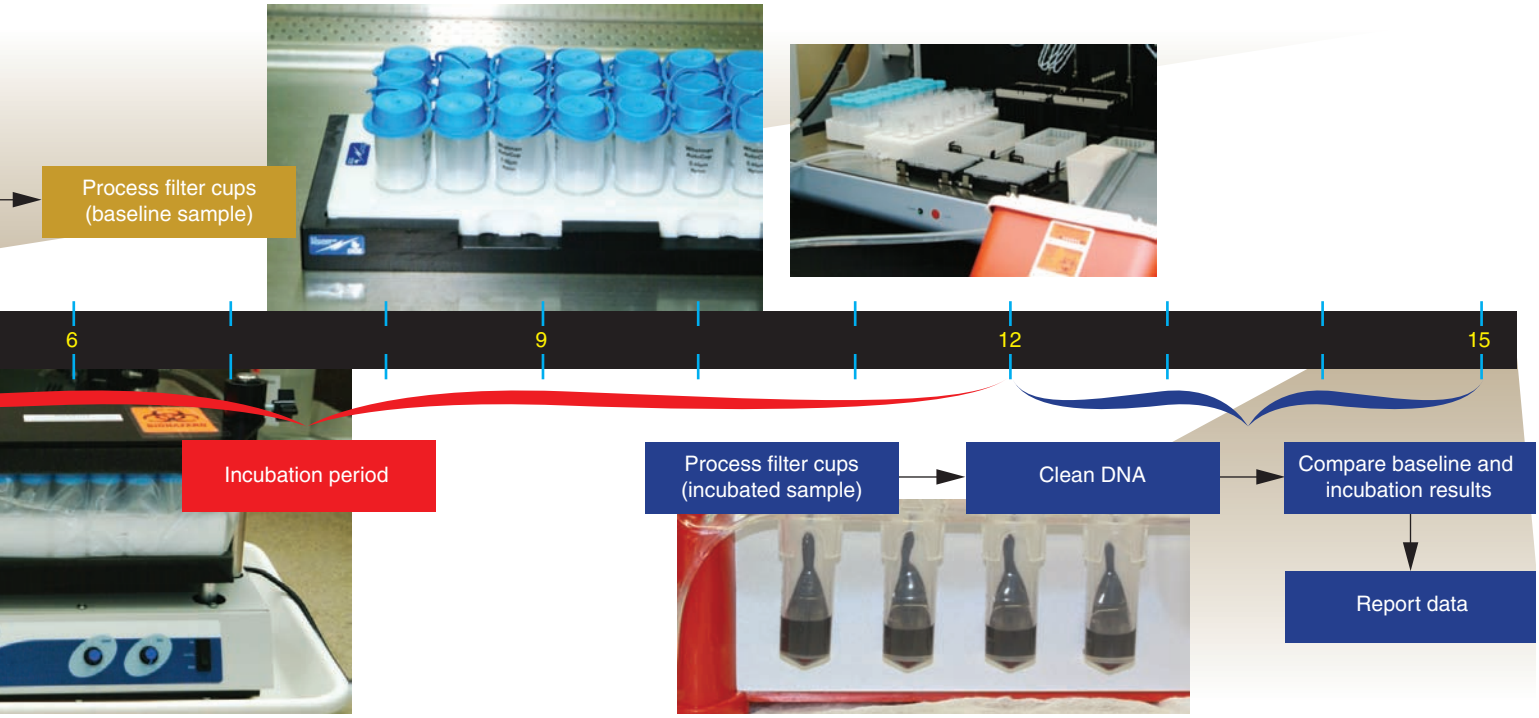
The current standard for identifying viable biothreat agents is the plating method. With this technique, cells are grown in a Petri dish on solid media containing nutrients. The plating method requires additional steps to prepare the samples and to confirm the results. Because this method is not as sensitive as PCR analysis, samples must be incubated for 16 hours or more to grow enough cells for detection. By comparison, RV-PCR can determine pathogen viability after only 9 hours of incubation time. The plating method also takes several days to confirm those initial results, whereas the RV-PCR approach generates confirmed results in 15 hours.

Detecting *B. anthracis* colonies in a sample containing an abundance of other bacterial colonies can be a challenge with the plating method. "Plates can be overwhelmed by growth from organisms that are naturally present in the environment," says Létant. "The RV-PCR method is selective and can detect the organism of interest in a very high background of other live organisms." RV-PCR also allows technicians to analyze a larger portion of the sample and to detect smaller concentrations of spores than they can with plating.

In the automated version of the RV-PCR method, a robot performs the liquid-handling steps, including mixing and transferring buffer-sample extracts to filtration cups for spore collection, washing filters, adding growth medium to the filter cups for culturing, and sampling cultures for PCR analysis. Using robots is more accurate and less time-consuming than the manual operation. In addition, the automated process is safer because it reduces a technician's risk of exposure to deadly pathogens.

Testing the Technique

To test the accuracy and speed of the RV-PCR method, the team conducted a laboratory verification study designed to evaluate various scenarios, including decontamination. In this experiment,



the team processed 200 samples, including wipes, air filters, and water—all spiked with live, virulent *B. anthracis* spores. The samples, which were divided into eight batches, also contained contaminants ranging from dirt and debris to live, nontarget spores and microorganisms and dead *B. anthracis* spores. "Including positive and negative controls ensures that no cross-contamination occurs during analysis," says Kane.

The RV-PCR method processes the first batch in under 15 hours, consistently detecting at a level of 10 or more spores per sample—one order of magnitude below the detection limit demonstrated by the traditional plating method. However, says Létant, "When hundreds of samples are processed, each batch after the first one adds 3 hours to the turnaround time for results."

The Livermore team also tested a variation of the new technique, called most-probable-number RV-PCR, using *B. anthracis* surrogates and compared the results with those from the traditional culture method. This test was designed to quantitatively estimate the *B. anthracis* spore levels in various sample types generated by the Centers for Disease Control and Prevention in a national validation study of the plating method. In the comparison tests, most-probable-number RV-PCR accurately identified all the samples in less than 24 hours, and the number of spores it detected was within the same order of magnitude as the traditional culture method.

An exercise conducted at the San Francisco International Airport in January 2006 demonstrated how the RV-PCR technique could be used in a bioattack scenario. In July 2010, a Seattle demonstration deployed RV-PCR inside a semitruck set up as a mobile laboratory. Called the Biothreat Response Vehicle, the truck contains robotics, PCR equipment, and biosafety cabinets for processing samples.

"Time is of the essence in responding to a bioattack," says Thomas Bunt, a program leader in Livermore's Global Security Principal Directorate. "The nation has a critical need for fast analysis methods and mobile laboratories, not only to characterize the extent of an attack but also to verify that decontamination efforts have cleared facilities for normal operations. Tools such as RV-PCR and the Biothreat Response Vehicle are valuable assets, protecting the public from exposure to deadly biothreat agents."

—Kristen Light

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