Autonomous System for Pathogen Detection and Identification


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Autonomous System for Pathogen Detection and Identification

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Abstract

This purpose of this project is to build a prototype instrument that will, running unattended, detect, identify, and quantify BW agents. In order to accomplish this, we have chosen to start with the world's leading, proven, assays for pathogens: surface-molecular recognition assays, such as antibody-based assays, implemented on a high-performance, identification (ID)-capable flow cytometer, and the polymerase chain reaction (PCR) for nucleic-acid based assays. With these assays, we must integrate the capability to:

- collect samples from aerosols, water, or surfaces;
- perform sample preparation prior to the assays;
- incubate the prepared samples, if necessary, for a period of time;
- transport the prepared, incubated samples to the assays;
- perform the assays;
- interpret and report the results of the assays.

Issues such as reliability, sensitivity and accuracy, quantity of consumables, maintenance schedule, etc. must be addressed satisfactorily to the end user. The highest possible sensitivity and specificity of the assay must be combined with no false alarms. Today, we have assays that can, in under 30 minutes, detect and identify simulants for BW agents at concentrations of a few hundred colony-forming units per ml of solution. If the bio-aerosol sampler of this system collects 1000 L/min and concentrates the respirable particles into 1 ml of solution with 70% processing efficiency over a period of 5 minutes, then this translates to a detection/ID capability of under 0.1 agent-containing particle/liter of air.

Introduction

Although we face numerous credible threats, to date the US has no acceptable autonomous system for the detection, identification, and quantification of airborne pathogens. Such a system is needed to provide protection to the occupants of large buildings or other enclosed-airspace facilities, such as subways or other gathering sites.

A detection/identification system could be used in at least three ways: detect-to-warn; detect-to-treat; detect-to-restore. In the detect-to-restore mode, the user may accept a manual or semi-automated-processing instrument that would be used to help delineate the contaminated area and to verify the efficacy of any decontamination procedures; subcomponents of the autonomous system could find use in such operations. In the detect-to-warn and detect-to-treat modes, however, a rapid, autonomous operation is needed. Obviously, planning on the part of the
authorities is needed to be able to respond to an alarm. Relatively inexpensive HEPA-filtered masks could be donned in the detect-to-warn mode, and quarantine/limited travel of exposed persons would be the appropriate in the detect-to-treat mode, followed by the administering of selected antibiotics, etc.

To be acceptable, the autonomous system will have to satisfy the following criteria:

1. It must be able to run, unattended, for 24-hour periods (and much longer periods than this are preferable).
2. It must be capable of the automatic, positive detection, identification, and quantification of the pathogens.
3. It must be capable of positive detection and identification of the items listed under #2 at aerosol concentrations of 1 agent-containing particle/liter of air (ACPLA) or greater in the presence of all normal environmental airborne backgrounds, including diesel exhaust, smoke, pollen, non-pathogenic bacteria and spores, etc.
4. It should detect and identify in less than 20 minutes for 5 ACPLA concentrations or higher, and less than 35 minutes for 1 ACPLA concentration. Quantification of the threat is highly desirable.
5. It must have a false-alarm rate that is less than 1 per year.

We are currently working to build such an autonomous system, which we call the "Bio-Sentry", using flow cytometry for surface-recognition assays, PCR for nucleic-acid-based assays, and sample collection/preparation instrumentation, along with commercial bio-aerosol collectors. We have selected the flow-cytometric and PCR assays because they have proven themselves, worldwide, as the most powerful assays, other than cell culture. We are not including cell culture, due to its long duration for growth of the samples, its labor-intensive identification, and its inability to detect non-culturable organisms.

Performance considerations for \textit{B. anthracis} (\textit{B.a.})

Starting with 1 ACPLA of \textit{B.a.}, where one particle for \textit{B.a.} could be assumed to consist of 15 spores, then a person, at rest (roughly 100 cal/hour being consumed at 33\% overall efficiency), exchanging room air through the alveoli of his lungs at roughly 5 l/min, assuming high efficiency for deposition and germination of spores in the lungs, would receive a life-threatening dose, estimated to be 8000 to 10,000 spores, in roughly 100 minutes. If the person were more active and, therefore, breathing harder, he would obviously receive the life-threatening dose faster. Using these estimates, for a person functioning at light levels of activity in an office building for an entire work day, a 0.1 ACPLA of \textit{B.a.} aerosol would probably be the lower limit for causing a life-threatening dose. Therefore, we will determine what level of performance would be required in the Bio-Sentry to detect all aerosol concentrations of 0.1 ACPLA and higher.

Assuming only 0.1 ACPLA, a collector running with 1000 l/min air throughput at 50\% collection efficiency of the spore particles, collecting over a period of five minutes into a volume of 1 ml of water, would accumulate $2.5 \times 10^2$ particles into its collection fluid. If the subsequent fluidics subsystem that handled and prepared the collected sample (using sonication or other process) were able to disrupt the clumps of spores from each other with 70\% efficiency, then the starting concentration of spores after the five-minute collection would be roughly $2.5 \times 10^3$ spores/ml. Under field-test conditions, the LLNL miniFlo correctly identified and quantified all spore unknowns, over the full test range of $10^3$ spores/ml through $10^6$ spores/ml, using a single-target assay for bacteria.

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In order to avoid false positives, however, a threshold for calling a positive might be set higher than $2.5 \times 10^3$ spores/ml. Assuming that the flow assay required 5 minutes after the aerosol collection, which is reasonable, then there would be sufficient time to obtain the results from the PCR assay, using a tiered detection system, for verification of the detection/ID/quantification. The most rapid real-time PCR assays that we have require roughly 15 minutes to detect $10^7$ spores/ml, and require roughly 25 minutes for $10^3$ spores/ml. The inverse of the time (actually the number of thermal cycles) that is required before the automated calling of a positive detection/ID by the instrument’s software is an estimate of the starting concentration of the organism being detected via PCR + Taqman®, and could be used to confirm the results of the flow-based assay. It is our belief that the level of certainty that is required to call an alarm for a public gathering site requires the use of two or more assays with independent, complementary signatures. False alarms are not acceptable, as we have already indicated in criterion #5, above. These considerations demonstrate that a Bio-Sentry should be an achievable system.

**Necessary Subcomponents for the Autonomous System**

**Aerosol sample collector:** There are numerous bio-aerosol sample collectors that are commercially available. Of these, only the XM-2®, from SCP, Inc., has a pre-collection fractionator (PCF) to reject outsized particles. When viewed in a systems perspective, a PCF is very important, since rejecting outsized particles prevents the clogging of fluidic lines that are necessary for sample preparation and assays. Unfortunately, the design of the PCF on the XM-2 is one that requires a large, heavy, power-hungry air pump. Under support from the DoD’s Central MASINT Organization (CMO), we have designed and built a new style of PCF that has performed well. (See Figure 1).

**Fluidics system:** In order to have an autonomous system, we must have the customized, automated fluidics to transport and prepare the collected samples for the assays. We are developing this using small discrete valves, tubing, and chambers (see Figure 2), and we are perfecting the various operational steps that include reagent storage and dispensing, sample preconcentration, incubating with reagents, self-disinfecting, etc., all under computer control that also starts and monitors the assays so that positive detection is automatically called and communicated. There are several companies that have the capability to reduce such fluidic systems to small plastic-based fluidic “circuits”, once we have perfected the detailed, automated operations that are required.

**Detector/Identifiers with quantitative assays:** The world community has already thoroughly investigated the techniques that can be used, under manual operation, to detect and identify pathogens, and these are: culture, nucleic-acid-based assays, and antibody-based assays (Ab-based). The leading nucleic-acid-based assays use the polymerase chain reaction (PCR). Culturing an organism is slow and does not always pick up pathogens that might require a specific host tissue or environment; this is especially true of viral agents. Speaking in a tautology, the culture technique simply does not pick up organisms that are non-viable or non-culturable. Ab-based assays are the fastest to perform, but are confounded by difficulties associated with non-specific binding of the reagents. This problem is exacerbated when using environmentally collected aerosols due to Ab binding to dirt, pollen, molds, spores, etc. which can also generate false positives.

Over the last ten years, PCR has made major inroads into the detection and identification of pathogens, and, while it is typically a slower process than Ab-based assays, it offers highly complementary information that, when used in conjunction with Ab-based assays, provides the most complete signature. In particular, it provides the strongest approach to identifying engineered organisms and allowing attribution of the agent.
False alarms destroy the credibility of detectors, and it is relatively easy to "beat" a single assay. Therefore, we plan on incorporating both Ab-based assays and PCR as the assays to be in the Bio-Sentry. As additional technologies such as mass spectroscopy become more mature and reliable for biodetection, we can incorporate those additional signatures into the system.

Status of the Bio-Sentry

Bio-aerosol collectors

Under the DOE CBNP Program, we have obtained and have performed preliminary evaluations of the performance of the leading aerosol collectors. Figure 1 shows our measurements for three leading aerosol collectors and the all-glass impinger (AGI), a high-efficiency, low-throughput unit. As we mentioned, above, with support from the CMO we designed and built a new type of PCF, that has shown improved performance, with greatly reduced power requirements.

Figure 1. Plot of collection performance for several collectors using dioctyl sebacate (DOS), normalized to power consumption.
Fluidics System

With support from the DOE Chem-Bio Non-Proliferation (CBNP) program, we have designed, built, and tested a small breadboarded fluidics system to perform sample preparation for the flow-cytometric Ab-based assay. This fluidics system, connected to miniFlo, is shown in Figure 3. The focus of our current effort is to build a robust system for mixing the sample solution with the Ab solution for incubation, prior to running the assay.

Figure 2. Photograph of R. Miles, testing the fluidics system connected to the miniFlo.

Flow Instrumentation

In 1993 we invented a new method to detect scattered light in flow cytometers, the flow-stream-waveguide technique, and built several instruments using this principle.24 When the opportunity arose to build a “luggable” version for use in the DoD’s Joint Field Trials III (JFT III), we did so over a 4-month period. We used this flow cytometer, dubbed “miniFlo”, to run antibody assays for the four BW simulants that were used as unknowns during the detector/identifier portion of the JFT III. The detector/identifier portion of the Joint Field Trials (JFT) III, established by the DoD Joint Program Office for BioDefense and implemented at Dugway Proving Grounds (DPG) in Utah in the Fall of 1996, consisted of the challenge of 36 unknowns per day for 10 days, with results being reported, daily. Each unknown consisted of none, one or two possible simulants from the set of four simulants:

- the spore Bacillus subtilis var. niger, also known as B. globigii, or B.g., in concentrations ranging from 10^3/ml to 10^6/ml;
- the vegetative bacterium, Erwinia herbicola, or E.h., in concentrations ranging from 10^7/ml to 10^9/ml;
- the RNA virus, MS2, in concentrations ranging from 10^5/ml to 10^8/ml;
- and the protein, ovalbumin, in concentrations including 0.1 µg/ml to 100 µg/ml.

The miniFlo and its single-target assays produced both excellent quantification as well as detection/identification against the bacterial simulants B.g. and E.h. The performance of the Ab-
based assay for B.g., the anthrax simulant, at JFT III was particularly good - 100% of all unknowns with B.g. were correctly identified and quantified with no false positives on blanks at $10^3/ml$ sensitivity. Correct identification was also obtained for 94% of the E.h. samples, 77% of the MS2 samples, and 75% of the ovalbumin samples. All samples yielded less than 1% false positives. This illustrates the need for combining Ab-based assays with nucleic acid-based assays to eliminate false positives.

Figure 3. Photograph the miniFlo, the version that we took to the JFT III.

Real-time PCR Instrumentation

Under LLNL's internal support and support from DARPA, LLNL has designed, built, and with the collaboration of Roche Pharmaceuticals, Inc. successfully operated the world's first battery-powered, hand-held PCR and briefcase-sized, real-time PCR instruments, based on thermal cycling chambers made from etched and fusion-bonded silicon. The unit that we sent to the Armed Forces Institute of Pathology is pictured in Figure 4.

Figure 4. Photograph of the battery-powered, real-time PCR analysis instrument.
With CMO and DOE support, the LLNL team has built and field tested a 10-chamber advanced nucleic acid analyzer (ANAA) based on similar PCR instrumentation. This instrument has used the reagents and protocols developed at the Naval Medical Research Institute (NMRI). Figure 5 shows LLNL personnel with the ANAA at DPG during the JFT IV, January 1998.

![Figure 5. Photograph of LLNL personnel with the 10-chamber real-time PCR system at DPG during the JFT IV.](image)

Both the briefcase instrument and the 10-chamber version utilize the Roche-patented Taqman® assay, which adds a second level of confirmation to the PCR assay. PCR normally derives its selectivity from the hybridization required for its primers to anneal to the target sequence before the amplification process can occur. The Taqman® assay adds an independent hybridization step with a separate probe that anneals onto the sequence being amplified. During the PCR amplification, the endonuclease activity of the enzyme cuts the Taqman® probe into separate nucleotides, releasing a fluorescent label from its nearby quencher, causing an increase in fluorescence as the amplification proceeds. Both the briefcase and the 10-chamber instruments monitor the fluorescence from the Taqman® probe during every thermal cycle, providing real-time data from the PCR reaction. This is particularly important when a rapid assay is valuable, since the time it takes to reach a detectable threshold with the Taqman® probe assay is inversely related to the starting concentration of the target organism. That is, you quickly know when there is a high concentration of an agent.

**Integrated System**

We have demonstrated the necessary performance in all of the subcomponents that are required for the Bio-Sentry, and we have built and tested a bench-top integrated test bed, shown in Figure 6, running only with the antibody-based assays. The least mature subsection is the automated fluidics and control system, and this is where the majority of our efforts are focused.
Our initial characterizations of the automated fluidics system has used various sizes of latex beads as simulants for reagents, so that the flow-cytometric assay can measure the efficiency of the mixing and transport processes. An example of the results of such a test are shown in Figure 7. The data are presented in a 2-dimensional histogram or “dot plot”. The vertical axis is the detected fluorescence and the horizontal axis is the small-angle elastic light scattering, or “forward scatter”. The forward scatter is a measure of bead size. The solution being extracted from the aerosol collector contained the non-fluorescent beads. Notice that the three “reagent” beads were mixed into the assay in roughly equal proportions. Our short-term plans are to replace the latex beads with actual fluorescent-labeled antibody reagents and to spray bacterial spores into the aerosol collector. The dot-plot assay would then show two basic groups of particles - bacteria with fluorescence (caused by the binding of the labeled antibodies) and non-fluorescent bacteria. The focus of this effort will be to optimized the system to detect specific aerosolized bacteria at concentrations below 1 ACPLA.
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