

The Silent Guardian Demonstration

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From October 2004 through March 2005, the Naval Research Laboratory (NRL) transformed a research protocol for gene-based pathogen identification into a demonstration project called Silent Guardian. Silent Guardian executed a bio-surveillance operation in the Washington, DC, region during the weeks surrounding the 2005 Presidential inauguration. To perform this bio-surveillance, nasal wash specimens were collected from patients presenting with flu-like symptoms at six military clinics in the Military District Washington and taken to NRL for testing. Simultaneous analysis for both common respiratory pathogens and bioterrorism agents was performed 24 hours a day for two months.

The Silent Guardian Demonstration achieved four major objectives:

- Pathogens were unequivocally identified from a general urban population within 24 hours;
- The ability to sequence genes from more than 20 pathogen species was demonstrated in a production mode;
- The capability of moving cutting edge technology from research to production within a six-week period was demonstrated by combined military/civilian teams; and
- A clear path was identified that could lead to an automated, portable, user friendly system.

This is the first documented application of DNA microarray technology to operational, broad-spectrum strain-level pathogen identification in an urban population.

INTRODUCTION

During the winter months, runny noses, fevers, and other flu-like symptoms are common among the general population. After September 11th, 2001, concerns increased over the use of bioterrorist agents, which present clinically with similar symptoms. One approach to surveillance for a bioattack is to monitor for unusual outbreaks of flu-like symptoms. Identification of the causative agent could be subsequently determined by traditional reference methods (usually culture, requiring days to weeks, or a DNA-based identification method known as a polymerase chain reaction or PCR). In the latter, the DNA target or cDNA (DNA copied from RNA targets) is amplified with specific primer pairs. Recently, real-time PCR (using fluorescent signals for real-time monitoring of PCR products) has shown great potential for more rapid pathogen detection. Up to four different targets can be detected simultaneously in one solution by using fluorescent labels of different colors with appropriately configured optical detectors. However, the number of targets that can be measured simultaneously is limited by the number of colors that can be measured simultaneously.

Microarrays provide a means to discriminate among a much higher number of amplified DNA products. In this method, multiple DNA "capture" probes are separated in a geometrically defined manner and capture the different "target" sequences of comple-

mentary DNA in a sample. The most commonly used form of microarrays, known as spotted arrays, however, are subject to false positives resulting from cross talk between spots, and they do not provide specific sequence information that leads to strain identification. NRL has designed a "resequencing array" that uses a high density of micron-sized squares with 25-base DNA capture probes (25-mers). The 25-mers represent consecutive regions in 200-500 base-long gene sequences that are characteristic of particular pathogens. By measuring the binding to the capture probes, the sequence of the genes present can be determined and the pathogen and strain identified.

One requirement for using microarrays is that targets must be amplified prior to hybridizing the target DNA to the immobilized capture probes. The methods for amplification include (1) specific PCR and (2) random amplification. In specific PCR, DNA primers with sequences exactly complementary to the ends of the target DNA are used to start the amplification. The reaction is fast, sensitive, and specific. However, the number of specific primers that can be multiplexed may be limited, and specific PCR may not amplify rapidly evolving species because of changes in the gene sequences. Potential complications caused by cross-reactions in the PCR, possibly affecting sensitivity and specificity for all targets, must be examined for each added primer pair in multiplexed assays. This is not a problem in random amplification since *all* DNA molecules in the complex mixture are amplified.

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However, with random amplification, each individual target is usually amplified fewer times than would be the case if a specific primer were used. When a random amplification and a microarray are combined, a large amount of “noise” can also appear on the array. NRL invented a bioinformatics analysis method to distinguish the sequence and prevent the background noise from producing spurious sequence reads.¹ However, the noise can still interfere with the ability of small amounts of pathogen target to produce a hybridization pattern that is visible above the background, thereby reducing the sensitivity of the assay. Therefore, random amplification is particularly valuable for identifying rapidly evolving species, while specific amplification excels for detecting previously identified species—even at vanishingly low concentrations.

The Respiratory Pathogen Microarray (RPM v.1) used in this project is a custom designed resequencing microarray. It was designed by the Naval Research Laboratory and manufactured by Affymetrix (Santa Clara, California). The RPM v.1 was intended for the detection of common respiratory pathogens (especially Adenovirus, Influenza A/B viruses, and *Streptococcus pyogenes*) encountered in the military training populations (Fig. 1). In addition, the RPM v.1 chips have regions specific for several Centers for Disease Control Category A biothreat pathogens: *B. anthracis* (three regions), Variola major virus (two regions), Ebola virus (one region), Lassa fever virus (one region), *F. tularensis* (two regions), and *Y. pestis* (two regions).

SILENT GUARDIAN DEMONSTRATION

The Silent Guardian Demonstration Project was initiated in October 2004 according to instructions from the Assistant to the Secretary of Defense Dale Klein and Deputy Assistant to the Secretary of Defense Klaus O. Schafer. The demonstration required NRL to transition the bench-top laboratory protocol for individual clinical samples into an “assembly-line process suitable for implementation.” The goal was for the laboratory to have minimal capability within three weeks of receiving initial funds and to be at full operational capability within 10 weeks, including all reagents and equipment needed to process 10,000 samples on site. An entire laboratory infrastructure with supporting Laboratory Information Management System (LIMS), Quality Control/Quality Assurance (QA/QC) measures, biosafety issues addressed for Biosafety Level 2, and a biothreat contingency protocol (in the event of a positive assay) were rapidly designed and implemented to enable this schedule. Implementation involved recruitment of skilled Department of Defense management and technical personnel, facility modification, protocol development, and training of all staff. After these tasks were completed, patient specimens provided by six

military treatment facilities (MTFs) located throughout the Military District of Washington were transferred to NRL for analysis and the results reported to the Air Force Medical Service COHORT electronic surveillance system, a database developed and maintained by the Air Force Surgeon General’s Office. NRL hosted more than 40 active duty Air Force and Navy personnel and provided technical support to implement and operate a central laboratory capable of processing more than 300 clinical specimens per day with laboratory practices consistent with those prescribed by the College of American Pathologists. Meeting these objectives imposed major new requirements for biochemical processing of clinical samples, bioinformatics, and supporting information technology (IT). All five laboratories were minimally operational within three weeks, with one complete processing line for the chips in place.

Prior to the start of Silent Guardian, specimen analysis consisted of a single technician running 1-20 samples over 2 days from start to finish. To process the projected 300 specimens/day (with a surge capacity to 450 samples/day), an assembly-line type of operation was established at NRL, operating 24 hours a day, 7 days a week. Laboratory staffing comprised NRL staff (Code 6900 and Code 6100), active duty Air Force staff, NRL contractors, and Navy reservists. The protocol was divided into four operations: receiving/nucleic acid extraction, RNA/DNA processing and amplification, chip hybridization, and data acquisition/analysis, performed in five rooms designated as Labs A-D. Figure 1 shows a flow chart of the protocol and photographs from each room.

The IT systems posed one of the biggest challenges. The Silent Guardian Demonstration required the development of a system to track all samples, from patient through analysis and storage, while maintaining confidentiality and chain of custody. At the time of sample collection, volunteer donors completed a questionnaire that was then entered by MTF personnel into the COHORT system. COHORT notified NRL of arriving samples. The NRL Laboratory Information Management System (LIMS) system then tracked each sample through Labs A, B, and C based on scans of the bar code on each sample tube. In addition, the high-resolution DNA microarray chips produced photolithographically by Affymetrix, Inc., were separately barcoded so that the microarray images could be associated in LIMS with information from the procedure. When the procedure was complete, the image was automatically evaluated to read the gene sequences off the chip and identify the pathogen based on best fit to sequences in the public gene sequence database, GenBank. The final pathogen identification was checked by a senior staff member (notified of a positive result by a beeper) prior to release to COHORT.



Room A: Extraction



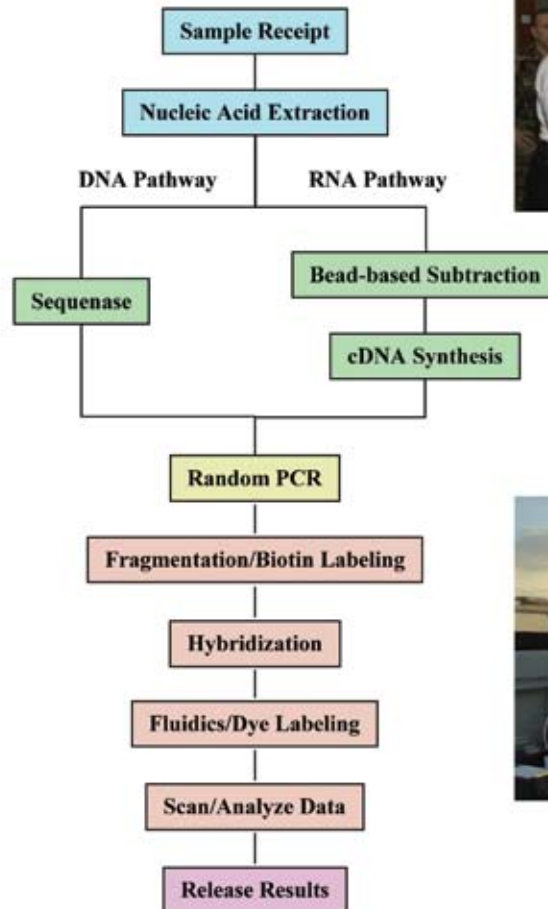
Room A: Receipt of Samples



Room B: RNA Pathway



Room C1: Random PCR



Room C: Scanning



Room D: Release of Results

FIGURE 1

The Silent Guardian Standard Protocol. The major steps involved in pathogen identification are diagrammed in the center. Photographs from the five laboratories used in the protocol are shown.

RPM V.1 SAMPLE PROCESSING AND ANALYSIS

Each nasal wash aliquot was subjected to total nucleic acid extraction (Lab A), isolation of pathogen RNA (and conversion to cDNA) and DNA (Lab B), amplification (Lab C1), and microarray hybridization and image analysis (Lab C). Figure 2 shows false color and real images of the microarray chip after hybridization. The false color is used to identify regions on the chip for each pathogen.

Prior to amplification (Lab C1), each sample was split; half of each sample was carried through the rest of the analysis using the random amplification protocol as the standard protocol; the remaining split was frozen for later analysis using a different primer mix. Random amplification without removal of human DNA, prevalent in nasal wash samples, resulted in an increase in background noise. To improve sensitivity, specific primers were added to the unused split for more efficient amplification. Two groups of specific

primers were used: UberMix (for all regions on the chip) and BTA (for bioterrorism agents, Adenovirus and Influenza). Use of these “splits” enabled direct comparisons between results obtained with the different amplification protocols.

A rigid set of criteria was imposed for a result to be released to COHORT and thereto back to the MTFs. For a “negative” result to be released, two positive controls, added prior to processing, were required to produce hybridization signals on their respective tiles. Thus, any sample producing a positive pathogen response and/or two positive controls was released to COHORT. If the sample was negative for both pathogen and at least one control, a duplicate aliquot was thawed and reanalyzed.

PATIENT RESULTS

Patient samples were collected at six Military District Washington MTFs from both Emergency Department and clinic settings. Adult patients (active duty

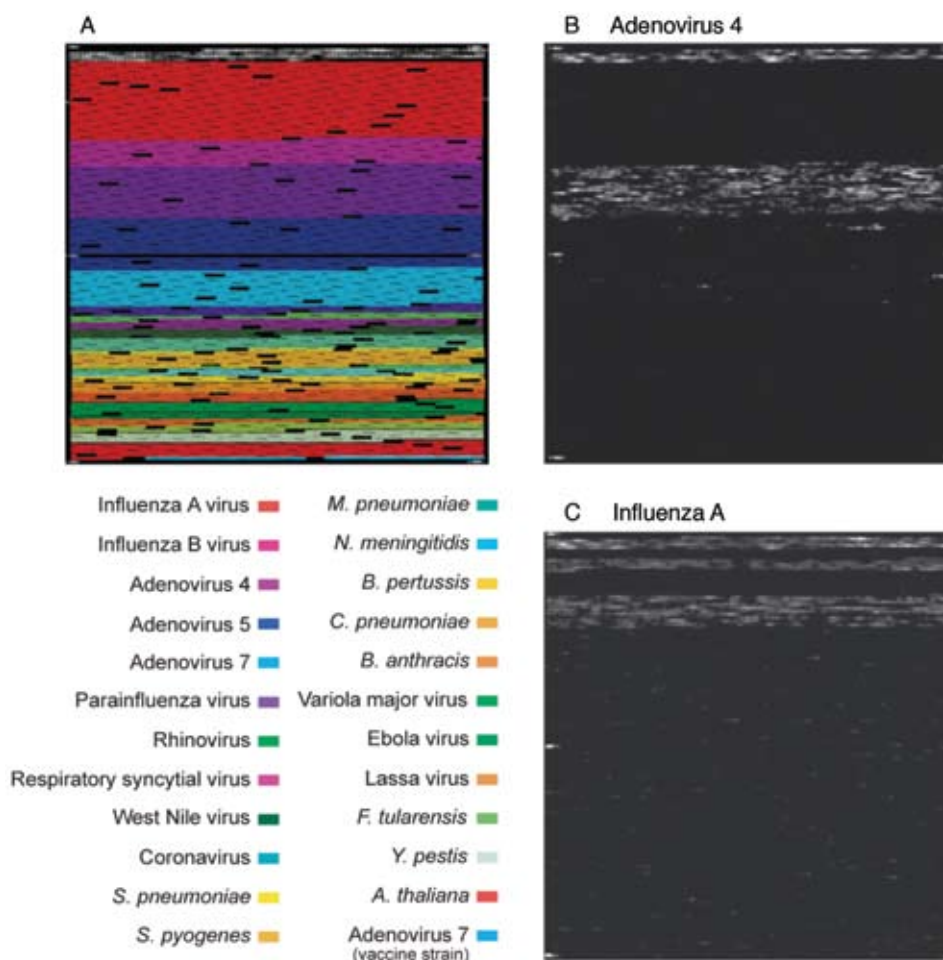


FIGURE 2

Images of RPM v.1 microarray. Panel A is a false color image showing the regions of the chip for different pathogens. Panels B and C are images of a sample positive for Adenovirus 4 and Influenza A, respectively.

military, retired military, and their families) who had a temperature >100.4 deg Fahrenheit and any flu-related symptom(s) could provide consent and donate sample. After the study, individual symptoms were compared to culture and microarray results; there was no statistical correlation between pathogen identity and any individual symptom. This confirms the requirement for a diagnostic test to differentiate between pathogens causing flu-like symptoms.

A total of 565 patients met all study criteria and donated nasal wash; 33 also donated nasal swabs. Samples were tested using the standard random amplification protocol, and the results reported to COHORT. Of these samples, 450 were sent to Naval Health Research Center (NHRC) for culture and PCR characterizations. Hard-to-culture pathogens of interest (*Coronavirus*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Bordetella pertussis*) were characterized using PCR.

In addition to the results obtained from the standard protocol, further experiments were performed on a subset to examine other modifications. First, 464 replicate patient samples were amplified by multiplex PCR using a specific primer cocktail (UberMix) rather than random amplification. A second set of experiments involved re-analysis of sequence calls using a “relaxed” algorithm in which targets were identified by associating the top sequence similarity ranking instead of satisfying an arbitrary value for goodness of fit. Table 1 show the collective number of positive results obtained using culture, standard protocol (random amplification), the “relaxed criteria” for the sequence calls in the standard protocols, and “relaxed criteria” multiplexed PCR (UberMix). As seen in this table, culture successfully detected Influenza A, Influenza B, *S. pneumoniae* and *S. pyogenes* (Group A Strep), and some Adenovirus from nasal wash specimens. Coronavirus is very difficult to culture; therefore, the positives were determined by PCR. Influenza (A and B) virus was the most commonly identified respiratory pathogen by all methods. Of the 598 samples reported to COHORT, 102 (17%) were identified as positive for influenza when using the Silent Guardian standard protocol. Cultures lasting up to 42 days were performed on 450 of the samples and revealed that 213 (47%) were positive for influenza by culture. Additional pathogens were identified in low incidence.

Three hundred fifty-one “matched” experiments for which there were data for culture, random amplification, and multiplexed amplification were analyzed with the relaxed algorithm. Of the 351 matched experiments, 30-day culture detected Influenza A in 147 (42%) of the specimens. Used in combination with the relaxed algorithm, the random amplification and UberMix amplification protocols resulted in

a total of 114 unique positive results for 32% of the total “matched” sets. Overall, modifications to the protocol allowed increased detection of a variety of additional respiratory pathogens without false detection of BT agent genetic sequences. As expected, the use of multiplexed primers (UberMix) increased both the number and types of pathogens detected relative to the standard protocol. Especially noticeable was the large increase in calls for Adenovirus and the bacterial pathogens *N. meningitidis* and *S. pneumoniae*, both of which are known to colonize the nasal passage in healthy individuals. Also observed were the increased detection of *S. pyogenes* and respiratory syncytial viruses A and B. Only this amplification technique gave detection of erythromycin resistance gene markers (plasmid conferred) in 19 individuals.

For standard comparisons of resequencing microarray results to those obtained with culture, the FDA recommendation of using an overall percent agreement (defined as the proportion of sample where both the new test and the imperfect reference test agree) was used. Based on these values, the overall percent agreement for samples tested by both methods was 70%. Additional determinations of agreement for positive and negative samples showed 33% positive agreement and 93% negative agreement. When similar calculations using the “relaxed” data were done, FDA analysis method indicated 70% overall percent agreement. There was 66% agreement for positive analyses and 92% agreement for negative analyses, suggesting that improvements in the resequencing microarray assays and analytical software would be required to meet the clinical thresholds in the future. [NOTE: Recent progress has led to significantly improved sensitivity with clinical agreement of over 90% for a set of over 100 samples we have received from NHRC for Influenza and Adenovirus. Retesting of the samples collected from Silent Guardian is now in progress.]

BLIND B. ANTHRACIS SPIKE TESTS

To demonstrate the detection capability for bioterrorism agents and to test the notification system, 11 test samples containing spikes of **certified, inactivated** *Bacillus anthracis* (1 containing Ames strain and 10 containing Sterne vaccine strain) in pooled normal nasal wash. These samples were included with clinical samples through standard sample processing without prior knowledge of the technical staff. Samples were put into the normal patient specimen jars with a special LIMS barcode that appeared to be from an MTF with no indication that they were not clinical samples. Once the sample was analyzed as anthrax-positive, the designated senior staff member at NRL for that shift initiated testing with a standard rapid testing protocol

Table 1 — Positive Results for All Methods

| Positives for | Culture/PCR | Standard | Standard “Relaxed” | UberMix “Relaxed” |
|-------------------------------|-------------|------------|--------------------|-------------------|
| Influenza A | 184 | 91 | 108 | 104 |
| Influenza B | 29 | 11 | 13 | 15 |
| Adenovirus | 2 | 0 | 0 | 17 |
| Coronavirus | 33 | 4 | 7 | 2 |
| Rhinovirus | 0 | 1 | 3 | 0 |
| Neisseria meningitides | 0 | 0 | 0 | 38 |
| Streptococcus pneumoniae | 10 | 0 | 0 | 21 |
| Streptococcus pyogenes | 9 | 0 | 0 | 3 |
| Respiratory Syncytial Virus A | 0 | 0 | 0 | 1 |
| Respiratory Syncytial Virus B | 0 | 0 | 1 | 4 |
| Erythromycin resistance | NA | NA | 0 | 19 |
| Total | 450 | 598 | 598 | 464 |

to verify the microarray results. Samples that tested positive for anthrax in both tests were reported directly to the on-duty Air Force Silent Guardian officer. Notifications of spike positives were received by the Air Force Silent Guardian senior staff within 10 minutes of sample analyses. Time from initial receipt of sample in Room A to the staff notification ranged from 17.4 hours to 21.3 hours. All 11 samples showed up as positives for *B. anthracis*.

DETECTION AND DISCRIMINATION OF BIO-THREAT AGENTS AND NEAR NEIGHBORS

Because of a mild flu season, a much smaller-than-expected number of patient samples were obtained during Silent Guardian. This provided an excellent opportunity to perform a systematic assessment of the Silent Guardian protocol to: (a) detect sequences of pathogens across large concentration ranges, (b) withstand challenge from a battery of genetically similar organisms, (c) simultaneously screen for the presence of biothreat agents and operationally significant pathogens in clinical matrices, and (d) assess utility for use in a clinical laboratory setting with routine and surge testing.

More than 2,100 analyses were performed on spiked samples and blank controls using random amplification, BTA mix, or UberMix. Using these specific primer mixes, we detected *B. anthracis* sequences from Sterne strain vegetative cells at the lowest number of cells tested (20 colony-forming units, cfu) and from Ames spores at levels as low as 6×10^4 cfu per sample. *F. tularensis* sequences were also detected in two strains

at cell numbers as low as 10 cfu per sample (LVS, *F. tularensis* biovar novicida) and 10^3 cfu per sample (Schu4). *Y. pestis* sequences were detected in samples containing 10–300 cfu of *Y. pestis* target, depending on the source. Sequences specific for Vaccinia virus, a simulant for Variola major virus, were detected at the lowest concentration of spiked Vaccinia tested (10^5 cfu)—while allowing for fine-scale genomic discrimination between Vaccinia and the actual smallpox virus.

CONCLUSIONS

The Silent Guardian Demonstration successfully showed its potential as a bio-surveillance system in the Military District Washington during January and February 2005 for both natural and biothreat pathogens. **Multiple pathogens were identified in clinical samples from the general population in less than 24 hours.** Identifications of >20 naturally occurring pathogens were made at the **strain**, as well as species, level in many cases. None of the sequences from six bioterrorist agents included on the RPM chip were detected in patient samples, although *B. anthracis* sequences from all of the blind samples artificially spiked with anthrax were immediately detected. The results produced a high level of confidence in the reliability of the resequencing approach for bio-surveillance in both military and civilian populations.

Silent Guardian was also a unique demonstration of the capability of the Joint Services to bring research to operation in record time (full operational capability within 6 weeks). It exemplified the amazing synergy possible when individuals with widely diverse capabili-



FIGURE 3

Silent Guardian Demonstration personnel at the Naval Research Laboratory: 1st row: SSgt J. Schuerger, Dr. F. Ligler, Capt F. Lobo-Menendez, Dr. C. Taitt, SSgt P. Chernay, SRA B. Sanchez, Ms L. Shriver-Lake, SSgt(s) A. Lowman, Dr. D. Stenger, SSgt N. Barboza, SSgt S. Cooper, Dr. B. White; 2nd row: SSgt T. Finch, Ms G. Gover, SSgt L. Jones, SSgt V. Lee, TSgt A. Davis, Capt D. Lennon, SSgt A. Krammer, SSgt S. Johnson, Dr. J. Schnur, LtCol S. Harmon, SRA S. Lowman, A1C M. Martinez, Ms. A. Kusterbeck, Dr. B. Lin, Ms. K. Shaffer, Dr. G. Lin; 3rd row: SMSgt C. Alexander, Capt D. Haggerty, TSgt T. Oaks, SSgt R. Tyler, MSgt(s) K. Norton, SSgt C. Stokely, TSgt A. Allison, SSgt R. Sullivan, Dr. W. Dressick. Inset: 1st row: Mr. P. Charles, Dr. A. Malanoski, Dr. B. Ringeis, Mr. M. Dinderman, Mr. J. Bongard; 2nd row: Mr. B. Weslowski, Dr. K. Mueller, CDR B. Oyofa, Dr. M. Ngundi, LT S. Hakspiel, Dr. S. Pollack. Missing: Dr. G. Anderson, Capt W. DelaCruz, CAPT(s) B. Hardas, Dr. J. Jones-Meehan, Dr. T. Leski, ENS K. Mandell, LT J. McCardle, MSgt A. Sinclair, Dr. W. Straube, and CAPT W. Suiter.

ties are committed to a common endeavor. Figure 3 show the Silent Guardian staff at NRL, including NRL scientists and active-duty Air Force personnel.

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[Sponsored by OSD]

Reference

¹ B. Lin, et al., "Broad Spectrum Respiratory Tract Pathogen Identification Using Resequencing DNA Microarrays," *Gen. Res.* (2006), in press. ★