Autonomous Detection of Aerosolized *Bacillus anthracis* and *Yersinia pestis*

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We have developed and tested a fully autonomous pathogen detection system (APDS) capable of continuously monitoring the environment for airborne biological threat agents. The system is designed to provide early warning to civilians in the event of a terrorist attack. The final APDS will be completely automated, offering aerosol sampling, in-line sample preparation fluidics, multiplexed detection and identification immunoassays, and orthogonal, multiplexed PCR (nucleic acid) amplification and detection. The system performance (current capabilities include aerosol collection, multiplexed immunoassays, sample archiving, data reporting, and alarming) was evaluated in a field test conducted in a Biosafety Level 3 facility, where the system was challenged with, and detected, a series of aerosolized releases containing two live, virulent biological threat agents (*Bacillus anthracis* and *Yersinia pestis*). Results presented here represent the first autonomous, simultaneous measurement of these agents.

The anthrax attacks that occurred in 2001 demonstrated the ease with which biological warfare agents can be disseminated, particularly when dispersed as aerosols, and underscore the critical need for aerosol detection systems that can operate in civilian settings. Real-time characterization of bioaerosols has been achieved by measuring particle aerodynamic size and intrinsic fluorescence.[1−7] Such measurements can be used to differentiate particles of biological origin from nonbiological particles (e.g., dust, diesel exhaust), but they cannot be used to *identify* organisms, and they are highly susceptible to false alarms caused by operation in dirty environments, particulate perturbation associated with routine activities, and naturally occurring background variations.

An ideal aerosol monitoring system would be capable of detecting and identifying multiple pathogens in real or near-real time. The system should run unattended for long periods of time, require infrequent maintenance, and be inexpensive to operate. The platform must exhibit detection limits such that life-threatening doses of airborne pathogens[8] can be detected and have sufficient selectivity to virtually eliminate false positives. Many research groups are directing their efforts toward the development of biological detection and identification technologies that meet these criteria,[9−14] and several reports describe autonomous aerosol collection followed by in-line sample processing with identification and quantification.[15−18]

We have developed, built, and tested the autonomous pathogen detection system (APDS), a stand-alone instrument capable of continuous, fully autonomous monitoring for multiple airborne biological threat agents.[19] The APDS is intended for use in domestic applications (e.g., office complexes, transportation terminals, convention centers) where the public is at high risk of exposure to covert releases of bioagents and as part of an integrated network of biosensors for wide-area monitoring of urban areas and major gatherings (e.g., inaugurations, Olympics). Multiplexed antibody-

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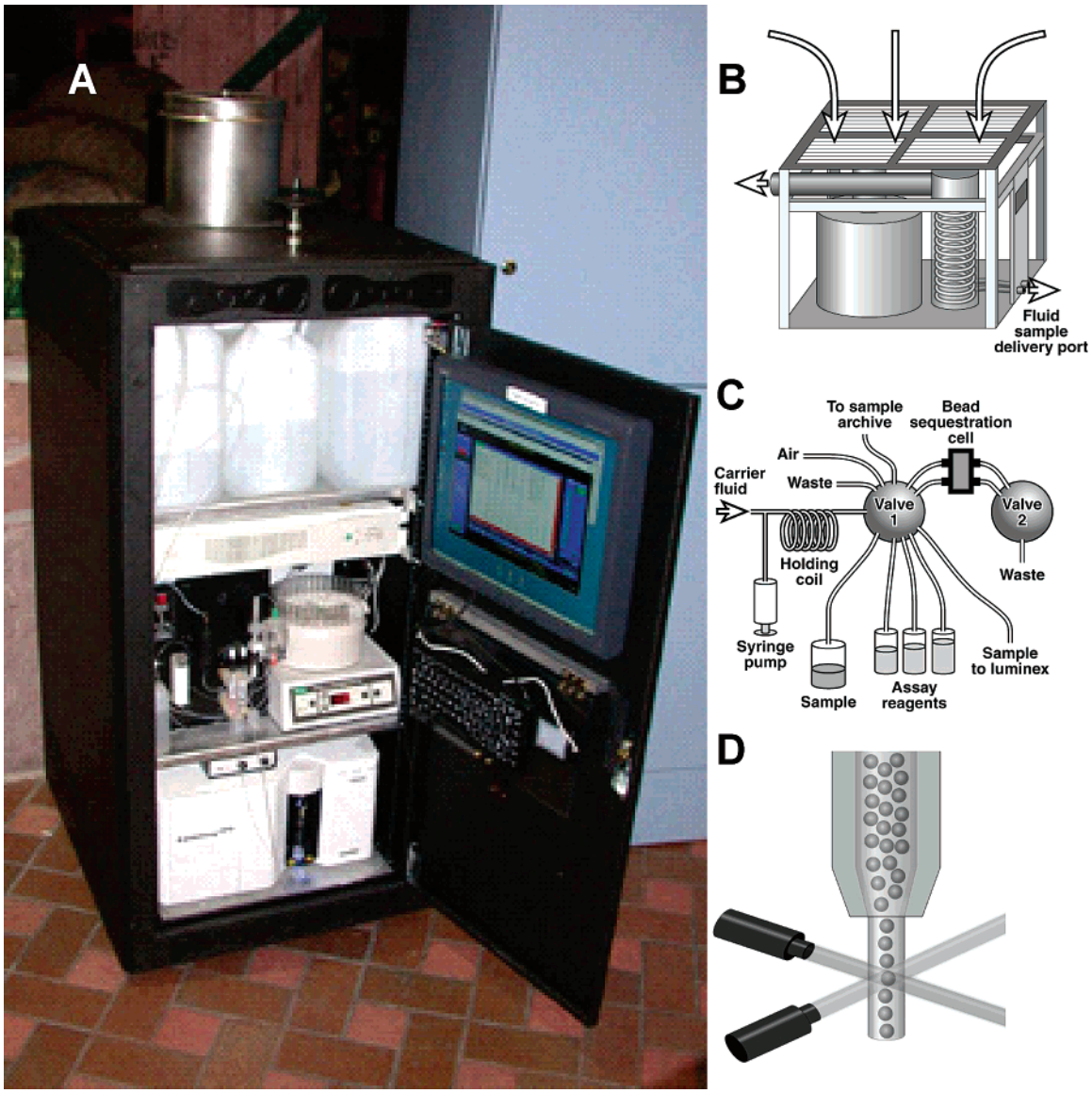

Figure 1. (A) Autonomous Pathogen Detection System (APDS): comprises several independent, integrated modules (i.e., aerosol collector, sample preparation fluidics, and multiplexed immunoassay flow cytometer detector). Modules, fluid containers, sample archiving fraction collector, and the GUI display panel are contained within a rugged, mobile chassis. The stainless steel aerosol "stack cap" and cellular communication transmitter extend from the top of the chassis. (B) Schematic of the aerosol collector (stack not shown). The particle fractionator (four-paneled screen) screens incoming particles (white arrows). The virtual impactor (cylinder, lower left) separates particles based on size and is tuned to select particles in the 1−10-μm range. A wetted wall cyclone (cylinder, lower right) captures particles in fluid; at prescribed intervals the fluid is dispensed to the fluidics module. (C) Schematic of the fluidics module. The sample preparation fluidics module (black box, shelf 2) conducts automated immunoassays. Upon completion of the assay, the sample is moved to the detector (white box, bottom shelf) for analysis. (D) Schematic of the detector flow path. The sample (100 μL) contains several thousand beads comprising multiple bead classes. After analysis, results are displayed on the GUI panel (chassis door).

based tests (immunoassays) allow the detector to respond to broad classes of bioagents, including those without nucleic acids such as protein toxins, and are used to reduce reagent costs, making long-term monitoring operations possible. Nucleic acid assays (PCR) allow much more sensitive detection, reducing the number of sensors needed to protect a given area. This unique, orthogonal detection approach combines antibody-based and nucleic acid-based assays, reducing false positives to a very low level. The fully autonomous aerosol collection and sample preparation capabilities limit maintenance requirements and make integration into a central security or monitoring network possible.

Currently, the APDS (Figure 1) is capable of collecting aerosol samples, performing multiplexed immunoassays at 30−60-min intervals, sample archiving, data reporting, and alarming with continuous unattended operation benchmarked at 8 days; we are integrating and testing PCR.[20,21] The system is contained in a rugged, mobile chassis so the only utilities required are ac power and a network connection for remote communication. A Labview-based system controls instrument functions, and a custom soft-

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ware system has been developed for data acquisition, real-time data analysis, and result reporting via a graphical user interface. We evaluated the performance of the fully integrated APDS in a chamber test where the system was challenged with known concentrations of bioaerosols of two live, virulent biological threat agents (*Bacillus anthracis* and *Yersinia pestis*). The results of the chamber tests, representing the first fully autonomous measurements of those agents, are presented here.

Bioagent aerosols were released into the chamber, collected, processed, analyzed, and identified in-line by the system operating in a completely autonomous mode. Collected samples were compared to benchtop calibration curves to approximate concentrations and to evaluate the sensitivity and specificity of the fully integrated system. A mixed-analyte release was conducted to demonstrate the ability of the system to simultaneously detect two very different pathogen classes (bacterial spore, vegetative bacterial cell) within a single sample. The multiplexed assay data demonstrate the specificity of the system: for each release, samples were analyzed within the context of specially designed internal sample controls and appropriate antigen response was exhibited in each case with no evidence of any cross-reactivity or nonspecific binding.

EXPERIMENTAL SECTION

System Description. Adopting a modular approach to the development of the APDS provided maximum flexibility to develop and optimize each module before integration into the final autonomous system. Modules are briefly described below.

(A) Sample Collection: Aerosol Collector. Because aerosol collectors containing dry matrix-type filters are difficult to couple to autonomous systems and are relatively nonselective in the types and sizes of particles collected, we developed a two-stage aerosol collector that utilizes an LLNL-designed virtual impactor preconcentration stage in front of a commercial wetted-wall cyclone collector (Research International SASS 2000). To be effective weapons, biological agents must be disseminated as aerosols consisting of particles 0.5−5 μm in diameter.[22] The virtual impactor has been optimized to capture particles in the 1−10-μm size range, and the wetted-wall cyclone traps the particles in fluid. The APDS/SASS 2000 hybrid collector samples up to 2300 L of air/min (lpm), allowing many more particles to be collected over a shorter period (the SASS 2000 samples ~275 lpm). Particle collection, fractionation, and concentration are nearly continuous in autonomous testing. At prescribed intervals, the collector pauses and a peristaltic pump delivers the collected fluid to the sample preparation module. Aerosol collection resumes immediately, and at the same time, sample processing begins.

(B) Sample Preparation: Fluidics Module. The sample preparation (fluidics) module utilizes a powerful, highly flexible technique called sequential injection analysis (SIA).[23] The commercially available SIA system (Flo-Pro, Global FIA, Gig Harbor, WA) reproduces functions routinely performed by laboratory personnel on the bench: moving the sample from the aerosol collector, preparing the sample (mixing, filtering, incubation, etc.), and delivering the sample reaction volume to the immunoassay detector. The fluidics module consists of a bidirectional, 1-mL, stepper motor-driven syringe pump, two multiport selection valves, a holding coil, reagent and sample reservoirs, and a bead sequestration cell. Although 4 mL of sample is delivered from the aerosol collector to the fluidics module, the immunoassay requires only 100 μL of sample. The remaining sample is archived using a simple fraction collector with a carousel assembly and can be easily retrieved for additional analysis including PCR, culturing, and possible forensic analysis.

(C) Sample Analysis: Immunoassay Detection. We have developed immunoassays that are rapid, sensitive, and specific and can detect more than one threat agent simultaneously (i.e., multiplexed) from a single sample.[24] The assays have been developed for use with a commercially available flow cytometer, the Luminex LX-100 (Luminex Corp, Austin, TX). The immunoassays utilize polystyrene microbeads. The beads are embedded with precise ratios of red and infrared fluorescent dyes yielding an array of 100 different bead classes, where each class has a unique spectral address (Figure 2). The immunoassays employ a sandwich immunoassay format, where antigen-specific capture antibodies are immobilized on the beads, antigen is introduced and allowed to bind the beads, and the bound analyte is subsequently detected using secondary antibodies labeled with the fluorescent reporter, phycoerythrin (PE). Each optically encoded and fluorescently labeled microbead is then interrogated by the flow cytometer. A classification laser (635 nm) excites the dye molecules inside the beads, and a reporter laser (532 nm) excites the fluorescent molecules bound to the bead surfaces. The flow cytometer is capable of reading hundreds of beads per second; analysis is completed in 60 s. Upon completion of the automated immunoassay, the fluidics module dispenses the sample to the flow cytometer for analysis. After analysis, the sample is pumped to waste and the system is flushed in preparation for the next sample.

Test Design and Setup. Live agent aerosol testing of the fully integrated APDS was conducted at the West Desert Test Center, Dugway Proving Ground, Dugway, UT, in the containment aerosol chamber (CAC). The CAC is a stainless steel glovebox (5 m^3) with HEPA-filtered inlet and outlet air streams (Figure 3). To minimize unnecessary exposure of APDS components to challenge agents, the aerosol collector was removed from the APDS chassis and set inside a custom-built detector challenge chamber, and the fluidics and detector modules were placed outside the CAC. Samples were pumped from the aerosol collector out of the CAC to the fluidics module.

Antibodies. Immunoassays were conducted using a mixture of seven different antibody-coated bead classes (7-plex bead set). The 7-plex bead set consists of three classes of beads designed to screen for bioagents and four bead classes that serve as assay controls. Protein-G purified capture and biotinylated detector antibodies for all three bioagents were purchased from Tetracore (Gaithersburg, MD). Chicken IgG and biotinylated rabbit-anti chicken were purchased from Jackson Immunochemicals (West

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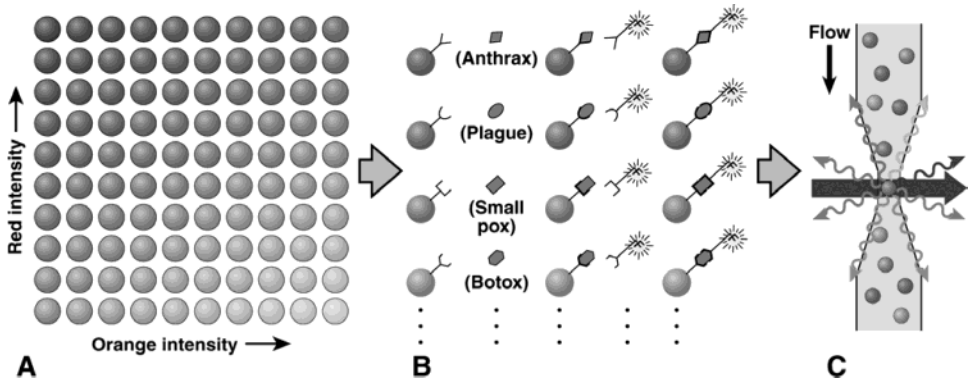

Figure 2. (A) A 100-plex Luminex liquid array generated by intercalating varying ratios of red and infrared dyes into polystyrene latex microspheres. Each optically encoded bead class has a unique spectral address. (B) Bead classes are coated with capture antibodies specific for target antigens. After incubation with the antigens, secondary or detector antibodies are added, followed by addition of the fluorescent reporter, phycoerythrin, to complete the "antigen sandwich". (C) The beads are analyzed in the flow cytometer. Beads are interrogated one at a time. A red laser excites the dye molecules inside the beads, and a green laser excites the fluorescent molecules bound to the bead surfaces. Only those beads with a complete sandwich will fluoresce in the green, and the signal is a function of antigen concentration.

Figure 3. Biosafety-Level 3 containment aerosol chamber (CAC): The CAC is a stainless steel glovebox (5 m^3) with HEPA-filtered inlet and outlet air streams. The APDS can be seen in the foreground. The aerosol collector was removed from the chassis and placed inside the aerosol chamber. Aerosols of highly infectious anthrax spores and plague bacteria were generated using collison nebulizers. Aerosol particles were collected and concentrated in the aerosol collector and the aqueous sample pumped to the fluidics module for immunoassay processing and subsequent analysis.

Grove, PA). The detector antibody cocktail comprised a mixture of four biotinylated antibodies; the three bioagent detector antibodies were each at a final concentration of 3 μg/mL and biotin-rabbit-anti chicken at a final concentration of 0.18 μg/mL.

Buffers and Reagents. All reagents were prepared in phosphate-buffered saline, 0.02% Tween 20, 1% BSA, 0.01% sodium azide pH 7.4 (PBS-TBN). Long-chain biotin-BSA was purchased from Pierce Chemicals (Rockford, IL). Reagent-grade chemicals (BSA, Tween-20, NaN_3) were purchased from Sigma (St. Louis, MO). Streptavidin labeled with R-phycoerythrin (SA-PE; Caltag Laboratories, Burlingame, CA) was used at a concentration of 2.4 μg/mL.

Covalent Coupling of Antibodies to COOH-Microspheres. Different sets of carboxylated fluorescent microspheres were obtained from Luminex Corp. Capture antibodies were covalently coupled to a unique carboxylated bead set (1.25×10^6 microspheres in 100 μL) in accordance with the manufacturer's protocol. Briefly, 1 mL of each bead set was centrifuged for 5 min at 5000 rpm and the supernatant removed. A 500-μL aliquot of Na_2HPO_4 buffer, 0.1 M, pH 6.0, was added to each tube. Aqueous solutions (50 mg/mL) of *N*-hydroxysulfosuccinimide sodium salt (Sulfo-NHS; Pierce Chemicals) and 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride (EDC; Pierce Chemicals) were prepared, and 25 μL of each solution was added to each tube. Tubes were vortexed and incubated at room temperature, in the dark with gentle agitation. After 20 min, beads were washed with 500 μL of PBS, pH 7.4, followed by addition of 500 μL of protein solution (125 μg/mL). Beads were vortexed and incubated at room temperature, in the dark for at least 2 h. Beads were washed in 500 μL of PBS and then resuspended in 500 μL of PBS-TBN pH 7.4, for 30 min to block free carboxylates. Beads were washed, and resuspended in 1 mL of PBS-TBN. Coated beads are stored in the dark at 2−8 °C and are stable for up to 1 year.

Benchtop Titrations: Preparation of Calibration Curves. All antigens (*B. anthracis* spores (*Ba*) the causative agent of anthrax, Ames strain, *Bacillus subtilis* var *niger* (*Bg*) spores, a simulant for *B. anthracis* spores, and *Y. pestis* (*Yp*), the causative agent of pneumonic plague, India 195/p strain cells, were provided by Dugway Proving Ground. Serial dilutions were prepared in PBS at concentrations ranging from 1.0×10^2 to 1.0×10^8 colony forming units (cfu)/mL. Assays were conducted in 96-well filtration plates, pore size 1.2 μm (Millipore, Bedford, MA). A 50-μL aliquot of the bead solution was mixed with 100 μL of sample and incubated for 20 min at ambient temperature. The mixture was vacuum aspirated, washed 2 times with 100 μL of buffer to remove unbound antigen, and resuspended in 100 μL of PBS-TBN. A 50-

μL aliquot of the biotinylated antibody solution was added to the bead mixture, and the resultant mixture was incubated 15 min. The mixture was vacuum aspirated, washed to remove excess detector antibody, and resuspended in 100 μL of PBS-TBN. A 50-μL sample of SA-PE was added and the reaction mixture incubated 5 min. The mixture was vacuum aspirated, washed, and resuspended in 100 μL of PBS-TBN. The solution was transferred to a microtube, and 50 μL of solution was analyzed in the LX-100 flow analyzer. Data were acquired for 60 s. The limit of detection (LOD) is taken as the analyte concentration at which the value of the median fluorescent intensity (MFI) is background plus three times the standard deviation of the background. For *Ba*, the LOD is 3×10^5 cfu/mL and the LOD for *Yp* is 6×10^3 cfu/mL.

Bioaerosol Challenges. A single six-jet Collison nebulizer was used to disseminate respirable-sized particles from slurry into the detector challenge chamber. For each release, 50 mL of spray suspension was prepared at the desired concentration and added to the Collison nebulizer. Typical nebulizer flow rates were 0.25−0.30 mL/min; bioaerosols were collected at a sampling rate of 1700 lpm. At the end of 50 min, the dissemination was terminated and the chamber flushed with air continuously while additional samples were collected and processed. These "blank" samples helped clean out the aerosol collector and fluidics system and provided an excellent measure of carryover. Blanks were collected until signals returned to baseline.

Safety Considerations. All antigen preparations, titrations, and disseminations utilizing live, virulent *Ba* and *Yp* were conducted within a Biosafety-Level 3 facility by qualified Dugway personnel. Personnel handling antigens wore appropriate personal protective equipment (Raycal hoods, gloves, lab coat). All solutions and consumables (filtration plates, tubes, etc.) were collected in biohazard bags and autoclaved. Waste generated in analysis was treated with 10% bleach and disposed of to the sanitary sewer. Equipment and benchtops were disinfected with a 10% bleach solution after use.

RESULTS AND DISCUSSION

A total of eight releases were conducted in the CAC at the West Desert Test Center, Dugway Proving Ground, Dugway, UT (Figure 3). The flow rate of the Collison nebulizer was calibrated in the pilot release, followed by a release of nonpathogenic *Bg* spores. Three trials were conducted with bioaerosols of *Ba*, and two trials were conducted using *Yp*. We conducted a mixed analyte (*Ba* + *Yp*) release to demonstrate simultaneous detection from a single sample of multiple analytes with very different physical and chemical properties.

System Sensitivity. Calibration curves for *Ba* and *Yp* were generated on the bench using a portion of the stock slurry. APDS-generated assay values were compared to the calibration curves to approximate the concentration of the sample. The immunoassays used as standards are well-characterized and quantifiable.[24] Bioaerosolization experiments, however, are extremely difficult to quantify because the aerosolization process, sampling device, collection methods, and collection media can substantially alter antigen surface structures, impair antigenicity, and drastically affect the viability of the organism in ways that are not well understood. Indeed, because of the biological diversity of airborne microbes, there is a lack of a convincing standard for their qualitative and quantitative determination.[25] Bioaerosolization experiments are subject to many potential sources of error, including product loss,[26−28] but the most likely sources are due to inefficiencies in the dispersal process or in the aerosol collector;[29] to our knowledge, these parameters have not been carefully measured. We employed standard bioaerosol references during the releases (e.g., an aerodynamic particle sizer (APS; TSI, Inc., St. Paul, MN) and all-glass impinger air samplers (AGI-30s, Ace Glass Co., Vineland, NJ). The APS data showed that the concentration of particles inside the chamber was nearly constant throughout the release. A portion of the liquid in each AGI was plated out after the release, and colonies were counted over the course of 24 h. Colonies were observed growing from the *Ba* release fractions, but no colonies grew from the *Yp* release fractions, indicating that the fragile bacteria did not survive dissemination.

Histograms detailing immunoassay responses for both *Ba* and *Yp* over the course of the entire experiment are shown in Figure 4. Sample cycle is displayed on the *X*-axis and MFI is plotted on the *Y*-axis. The *Y*-axis has been truncated on the *Yp* histogram to keep the plot in scale, since the fluorescent signals were considerably higher for *Yp* than those observed for *Ba*. Black bars highlighted with a star indicate a sample cycle corresponding to a release. The measured responses for each release are above the detection threshold (indicated by the dotted line). Similar to assays conducted on the bench, detection threshold limits for assays performed on the system are taken as the analyte concentration at which the MFI value is background plus three standard deviations of the background. System backgrounds were established based on the average MFI values obtained for 48 individual assays conducted in the absence of antigen. For *Ba*, the mean MFI was 293, with a standard deviation of 29. Thus, we set the detection threshold level (i.e., the lowest MFI value produced on the system that could be called "positive" provided that the assay controls fall within normal parameters) at MFI = 380. For *Yp*, the average MFI of 48 runs was 155, with a standard deviation of 25. Thus, the threshold detection limit was set at an MFI value equal to 230.

Antigen carryover is observed after a release (indicated by the elevated signals obtained in sample cycles following a release), but we note that the carryover does not persist (signals for each subsequent cycle gradually decay back to baseline). We conducted a mixed-analyte release (sample cycle 38) to demonstrate the simultaneous detection of multiple analytes from a single sample. Both *Ba* and *Yp* signals are observed with signal intensities for the mixed release that are comparable to signals obtained in individual releases of each antigen at the same concentrations (*Ba*, cycle 59; *Yp*, cycle 20).

System Specificity. Assays were conducted using a mixture of seven different antibody-coated bead classes (7-plex bead set). The 7-plex bead set consisted of three classes of beads designed

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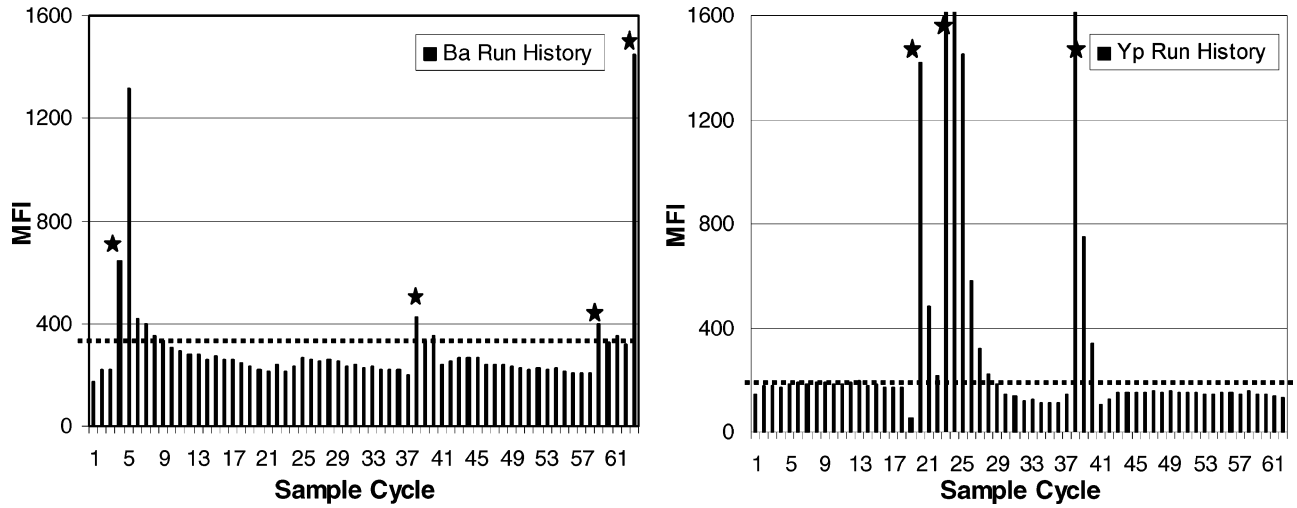

Figure 4. Histograms showing response (MFI) for both *B. anthracis* and *Y. pestis* bead classes over the course of all sample cycles conducted at Dugway. Bars marked with stars indicate the sample cycle corresponding to a release; all release responses are above the detection threshold level (dotted line). Elevated signals observed in subsequent samples cycles indicate antigen carryover. In general, three cycles are required before sample signals return to baseline levels following a release.

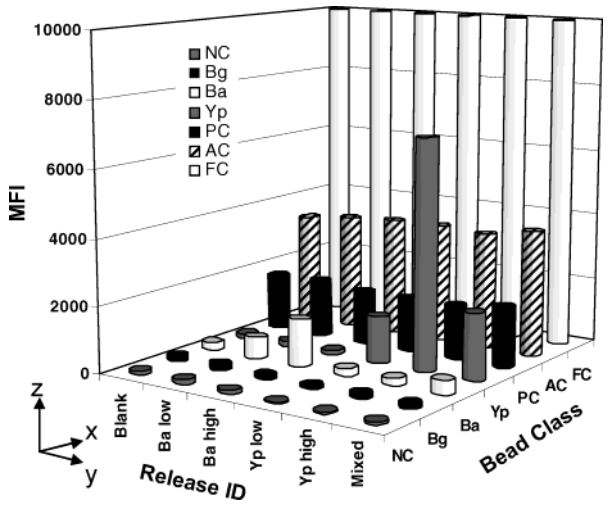

Figure 5. 3-D plot showing the response of each bead class (*X*-axis) in the presence of different bioaerosol challenges (*Y*-axis). Bead classes are coded as per legend. Response is given as the MFI (*Z*-axis). The control beads (NC, PC, AC, FC) exhibit expected responses for each challenge and were very consistent, indicating excellent assay and detector stability and error-free and highly reproducible system performance. The appropriate bead classes(s) respond to the antigen challenges, and no evidence of cross-reactivity or nonspecific binding is observed.

to screen for the biothreat agents (*Ba*, *Yp*, *Bg*) and four bead classes that served as assay controls. The 3-D plot shown in Figure 5 illustrates the specificity of the system. Each of the seven bead classes is shown on the *X*-axis. The *Y*-axis indicates the type of release conducted, and the observed response (MFI) is displayed on the *Z*-axis. Each of the four control bead classes exhibited fluorescent responses of expected intensity in the presence of every release, and the responses remained constant throughout all the releases, indicating error-free and stable system performance. The negative control (NC) did not bind any agent; therefore, the MFI value was low. The instrument control (PC, black bars), designed as a detector diagnostic, exhibited a constant MFI, as expected. Similarly, the antibody control (AC, striped bars) and fluorescent control (FC, white bars), designed to monitor the addition of reagents in the automated system, also exhibited constant MFI values.

Elevated signal was observed for the *Ba* bead class only when *Ba* was released. There was no apparent cross-reactivity or nonspecific binding of *Ba* antigen on the *Bg* bead class, indicating a high degree of specificity between these related organisms. Both *Yp* releases produced the desired results—elevated signals were observed *only* on the *Yp* bead class. The mixed-analyte release was conducted using *Ba* and *Yp* at the same concentrations used for the "*Ba* low" and "*Yp* low" releases. Both antigens were detected, with no apparent inhibition, cross-reactivity, or non-specific binding. The *Ba* signal is slightly lower and the *Yp* signal is slightly higher than expected; we attribute this to the differences in nebulization efficiencies, where bigger, denser spores are nebulized less efficiently than vegetative bacterial cells.

CONCLUSION

We have tested the performance of the fully integrated APDS and have demonstrated that the system can be used to detect and identify two biological threat agents, *Yp* and *Ba*, over a range of concentrations. The system is capable of continuous and unattended operation, and the platform is sensitive and specific, detecting releases with no false positives. Improvements to the APDS represent an active area of research. Our next generation will include automated multiplexed immunoassays followed by a confirmatory, nucleic acid-based test and is expected to be tested against real agents this year. The final APDS prototype, to be completed in 2004, will incorporate a bead-based multiplexed nucleic acid detection capability.

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