ORIGINAL ARTICLE

Determination of serum IgG antibodies to *Bacillus anthracis* protective antigen in environmental sampling workers using a fluorescent covalent microsphere immunoassay

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Aims: To evaluate potential exposure to *Bacillis anthracis* (*Ba*) spores in sampling/decontamination workers in the aftermath of an anthrax terror attack.

Methods: Fifty six serum samples were obtained from workers involved in environmental sampling for *Ba* spores at the American Media, Inc. (AMI) building in Boca Raton, FL after the anthrax attack there in October 2001. Nineteen sera were drawn from individuals both pre-entry and several weeks after entrance into the building. Nine sera each were drawn from unique individuals at the pre-entry and follow up blood draws. Thirteen donor control sera were also evaluated. Individuals were surveyed for *Ba* exposure by measurement of serum *Ba* anti-protective antigen (PA) specific IgG antibodies using a newly developed fluorescent covalent microsphere immunoassay (FCMIA).

Results: Four sera gave positive anti-PA IgG results (defined as anti-PA IgG concentrations ⩾ the mean μg/ml anti-PA IgG from donor control sera (n = 13 plus 2 SD which were also inhibited ⩾ 85% when the serum was pre-adsorbed with PA). The positive sera were the pre-entry and follow up samples of two workers who had received their last dose of anthrax vaccine in 2000.

Conclusion: It appears that the sampling/decontamination workers of the present study either had insufficient exposure to *Ba* spores to cause the production of anti-PA IgG antibodies or they were exposed to anthrax spores without producing antibody. The FCMIA appears to be a fast, sensitive, accurate, and precise method for the measurement of anti-PA IgG antibodies.

Anthrax is mainly a zoonotic disease of herbivores. Before the anthrax attacks in 2001, modern experience with inhalation anthrax was limited to an epidemic in Sverdlovsk, Russia in 1979 following an unintentional release of *Bacillus anthracis* (*Ba*) spores from a Soviet bioweapons factory, and to 18 occupational exposure cases in the United States during the 20th century.[1] In response to the *Ba* attack at American Media, Inc. (AMI), Boca Raton, Florida, on 20 October 2001, the Federal Bureau of Investigation (FBI) granted permission to the Environmental Protection Agency (EPA) and the Florida Department of Health (FDOH) to begin sampling and analysis. Members of the United States Coast Guard (USCG) Gulf Strike Team, the Florida National Guard 44th Civil Support Team (CST), workers from the IT Corporation, the National Institute for Occupational Safety and Health (NIOSH), and the EPA conducted environmental sampling for *Ba* at the AMI building as well as several post offices in Boca Raton. Samples (n = 460) were collected from the first, second, and third floors of the building. Surface wipe samples were collected from random surfaces (desk tops, counter tops, fax machines, keyboards, etc), return air vents, and exhaust vents on the roof of the building. High efficiency particulate air (HEPA) vacuum samples were also collected. Air sampling using 25 mm mixed cellulose ester filters and pumps were performed on the 1st and 2nd floors. Samples were analysed in a temporary laboratory by personnel from United States Army Medical Research Institute of Infectious Diseases (USAMRIID) and the Centers for Disease Control and Prevention (CDC). By 9 November 2001 sampling operations were complete. Eighty four samples tested positive for anthrax. Positive *Ba* results were found on all three floors of the building. Anthrax contamination was most prevalent on the first floor where positive results were found in carpet and floor samples. Positive results were also found in the second floor carpet and on surfaces on the third floor.[2] One or more mailed letters or packages have been identified as the likely source of exposure, leading to one fatality.[3] Several environmental specimens from regional and local postal centres that provided mail services to the work site were also culture positive for *Ba*.[2]

Recently it has been shown that viable *Ba* spores from a terror attack can be reaerosolised under semiquiescent and simulated active office conditions and that breathing zone samples of workers sampling for *Ba* could be as high as ~85 CFU/m^3.[4] Although the environmental investigation workers of the present study were protected from exposure to *Ba* by the use of personal protective equipment (PPE), prophylactic antibiotics, and in some cases vaccination with anthrax vaccine adsorbed (AVA, BioThraxJ, BioPort Corp., Lansing, MI), they were still at risk for exposure. Personal protective equipment is not 100% protective, individual work practices might lead to exposure, breaches in PPE and environmental controls might occur, and some breaches might go unrecognised. Neither the infective dose for development of inhalation anthrax in humans nor the level of exposure to *Ba* during remediation activities has been characterised adequately.[5] No validated methods exist for monitoring a person's exposure to *Ba*, although a greater than fourfold increase in levels of serum IgG to PA was shown in a confirmed case of inhalation anthrax at the AMI building using an enzyme linked immunosorbent assay (ELISA).[6 7] In

Abbreviations: AMI, American Media, Inc; AVA, anthrax vaccine adsorbed; *Ba*, *Bacillus anthracis*; FCMIA, fluorescent covalent microsphere immunoassay; MDC, minimum detectable concentration; MFI, median fluorescence intensity; PA, protective antigen; PPE, personal protective equipment; RDL, reliable detection limit

Main messages

- A fluorescent covalent microsphere immunoassay (FCMIA) method was developed to evaluate potential exposure to *Bacillus anthracis* (*Ba*) in sampling/decontamination workers in the aftermath of the anthrax terror attacks of 2001.
- Anti-anthrax protective antigen (PA) IgG antibody levels were used as a biological monitoring tool. No apparent exposure related increases in anti-Ba PA IgG were seen; however, workers who had been recently vaccinated with anthrax vaccine adsorbed (AVA) were identified.
- The FCMIA is faster, appears to have enhanced sensitivity, equivalent or better accuracy and precision, and the potential to multiplex analytes (that is, measure numerous analytes in the same assay simultaneously) compared to ELISA.

the present work we describe the results of a new anti-PA IgG fluorescent covalent microsphere immunoassay (FCMIA) as a biological monitoring tool to evaluate exposure to *Ba*.

MATERIALS AND METHODS

Serum samples

This study was performed as a NIOSH Health Hazard Evaluation in response to a request from the EPA. Informed consent was obtained from all participants. In November 2001, we were provided with a list of 45 persons who performed environmental sampling for *Ba* at the AMI site. Blood had already been drawn from 28 workers prior to their entry into the AMI building by either the local health department or the USCG. Several weeks after work was completed, 28 workers had blood drawn by their local medical facility. Nineteen workers participated in both blood draws. Nine blood samples each were drawn from unique individuals at the pre-entry and follow up blood draws. Standard human anti-AVA sera (AVR414, 170.1 μg/ml and AVR733, 198.0 μg/ml anti-*Ba* PA IgG) were obtained from the National Center for Infectious Diseases, Division of Bacterial and Mycotic Diseases (Atlanta, GA). AVR414 was prepared by plasmapheresis of healthy adult CDC volunteers who had received at least four subcutaneous injections of AVA with the licensed regimen (0, 2, and 4 weeks; 6, 12, and 18 months; and yearly boosters). Serum AVR733 contained 198 μg/ml anti-PA IgG and was from a case of clinically confirmed anthrax. AVR414 was diluted to produce standard curves (n = 7), while AVR733 was used in conjunction with AVR414 for dilution recovery investigations.

Control sera were randomly selected (13 sera) from a pool of blood donor sera (Indiana Blood Center, Indianapolis, IN and Cincinnati, OH), not known to have been vaccinated with AVA. All sera were supplied to the Biological Monitoring Laboratory Section of NIOSH in Cincinnati, OH without knowledge of their identity, and were stored frozen at −20℃ until used.

Antigen

Recombinant PA, produced in an avirulent, non-capsulated, sporulation suppressed *Ba* host was obtained from List Biological Laboratories, Inc. (Campbell, CA). The PA migrated as a single major band with an apparent molecular weight of 83 000 daltons on 10% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS). The PA was reconstituted in distilled water and stored aliquoted at −20℃. Before use, individual aliquots were thawed and used immediately.

Preparation of PA coupled microspheres

Briefly, after washing twice with 80 μl activation buffer (0.1 M NaH_2PO_4, pH 6.2), the carboxylated microspheres (2.5×10^6, Luminex Corp., Austin, TX) were pelleted in 1.5 ml centrifuge tubes using a microcentrifuge (Eppendorf, Hamburg, Germany). The microspheres were resuspended (all resuspensions were performed using sonication (mini sonicator, Cole Parmer, Vernon Hills, IL), and gentle vortexing (VWR, Intl., West Chester, PA)) in 80 μl activation buffer to which 10 μl activation buffer containing 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, Pierce Chemical Company, Rockford, IL), and 10 μl of activation buffer containing 50 mg/ml N-hydroxysulphosuccinimide, sodium salt (sulfo-NHS, Pierce Chemical Company) were added. The mixture was allowed to incubate for 20 minutes at room temperature. The microspheres were then washed twice in 500 μl coupling buffer (0.05 M 2-[N-morpholino]ethanesulfonic acid, MES, Sigma Chemical Co., pH 5.0) and a solution of PA (40 μg/ml) in 500 μl coupling buffer was added and incubated for two hours at room temperature. The coupled microspheres were then washed twice in 1 ml wash buffer (PBS containing 0.05% Tween 20 (Sigma #3563)) and stored in 0.5 ml storage buffer (PBS, 1% BSA (Sigma #3688), 0.05% Na Azide (EM Sciences, Cherry Hill, NJ), pH 7.4). Microsphere concentrations were determined using a haemacytometer (Bright Line, VWR, Intl.).

Anti-PA IgG measurements using PA coupled microspheres

A suspension of microspheres (100 microspheres/μl) was incubated for one hour at 37℃ in blocking buffer (PBS (Sigma #3813), 5% dried skim milk (Difco, Sparks, MD), 0.05% Na azide, pH 7.4) to minimise non-specific binding during subsequent steps. An aliquot (50 μl) of this suspension (approximately 5000 microspheres) was added to the wells of a 1.2 μm filter membrane microtitre plate (Millipore Corp., Part #MABVN1250, Bedford, MA) and the liquid aspirated by use of a vacuum manifold filtration system (Millipore, Part #MAVM09601). The microspheres were then washed three times with 200 μl wash buffer, each wash followed by vacuum aspiration. To run an experiment, 50 μl (in duplicate) of diluted (1/50 in dilution buffer; PBS (Sigma #3813), 5% dried skim milk (Difco, Sparks, MD), 0.05% Na azide, 1% Tween 20, pH 7.4) sample, control sera, standards prepared from AVR414 (0.017, 0.057, 0.170, 0.567, 1.70, and 3.40 μg/ml anti-PA IgG) or reagent blank were added to the microspheres in the wells of the filter membrane microtitre plate and incubated for 30 minutes at 37℃ with shaking. Standard curves prepared from sera diluted in human sera depleted of IgG, IgA, and IgM (GAM, Sigma #S5393) gave similar responses to those prepared with dilution buffer. The liquid was vacuum aspirated and the wells were again washed three times with 200 μl wash buffer. Fifty μl of a 2 μg/ml solution of detection antibody (mouse anti-human IgG-R-phycoerythrin, clone HP6043, BIOTREND Chemicals, Inc., Destin, FL) in dilution buffer was added to the wells of the plate and incubated for 15 minutes at 37℃. The wells were again washed three times with 200 μl wash buffer and resuspended in 100 μl wash buffer. The plate was shaken vigorously for approximately one minute to disperse the microspheres and was placed into the autosampler platform of the Luminex 100 instrument (Luminex 100 flow analyser, coupled with a 96 well plate autosampler, Luminex XYP, Luminex, Austin, TX) using software, calibration microspheres, and sheath fluid supplied by the manufacturer. The

instrument was programmed to collect data from100 microspheres (classified by their internal fluorescence ratio) and acquire the median fluorescence intensity (MFI) of the microsphere-PA-IgG-anti-IgG-R-PE complex.

Competitive inhibition

To determine the specificity of measurements performed by FCMIA, pre-incubation of positive sera, control sera, negative worker sera, and a dilution of AVR414 with PA (competitive inhibition) was performed. Sera (130 µl final volume) were treated with either 80 µg/ml PA (final concentration) or dilution buffer and incubated overnight at 4°C. The sera were then centrifuged and the supernatants analysed as outlined above. Inhibition was calculated by the following expression:

$$\% \text{ inhibition} = \left(\frac{(\text{non-inhibited serum MFI}) - \text{inhibited serum MFI}}{\text{non-inhibited serum MFI}} \right) \times 100$$

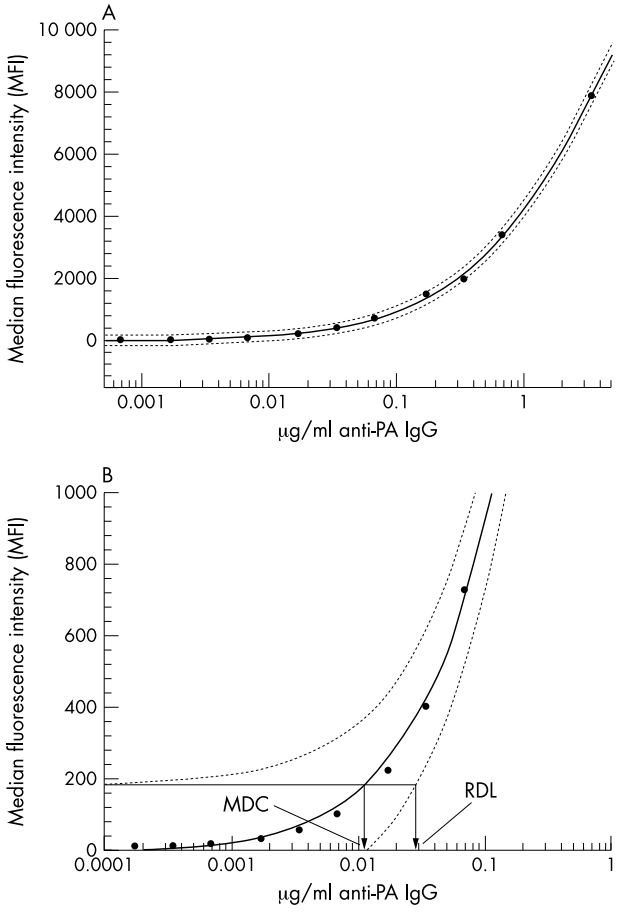

Figure 1 (A) Representative FCMIA four-parameter logistic log fit (solid line) of µg/ml anti-PA IgG *v* MFI. The dotted lines represent the upper and lower 95% CI of the regression. The regression coefficient was $R^2 = 1.000$ ($p<0.001$). (B) Magnified view of (A) showing a graphic representation of MDC and RDL. The MDC is the concentration of anti-protective antigen IgG corresponding to the interpolated intersection of the lower asymptote of the upper 95% CI with the four-parameter logistic log fit of the standard curve data. The RDL is the concentration of anti-PA antibody corresponding to the interpolated intersection of the lower asymptote of the upper 95% CI with the lower 95% CI of the standard's data (modified from Quinn *et al*[7]).

Data analysis

Standard curves were constructed from a four-parameter logistic log fit (4-PL) of MFI versus µg/ml anti-PA IgG standard prepared from diluted AVR414 reference serum (SigmaPlot, SPSS, Chicago, IL). Duplicate results from individual samples were averaged and concentrations interpolated from the standard curve. The quality of the curve fit was evaluated by back calculating the known diluted standard sera antibody concentrations using the parameters of the 4-PL fits and comparing them to the actual concentrations expected from diluting the sera and evaluating by the formula:

$$\% \text{ recovery} = \left(\frac{\text{observed concentration from 4-PL fit}}{\text{expected concentration from dilution}} \right) \times 100$$

The method's minimum detectable concentration (MDC) was calculated from the intersection of the asymptote of the regression's 95% confidence interval (CI) with the regression line, while the reliable detection limit (RDL) was evaluated from the interpolated intersection of the upper 95% CI asymptote with the lower 95% CI of the standards data as previously described[7] (see fig 1 for graphical detail). A positive anti-PA IgG result for a sample was defined as results $\geq$ the mean µg/ml anti-PA IgG from donor control sera plus 2 standard deviations (SD) which was also inhibited $\geq$85% when the serum was pre-adsorbed with PA. An averaging method[8] was employed to minimise bias in cases where sera had results below the limit of detection of the assay. Differences in anti-PA IgG concentrations for paired pre-entry and follow up sera were investigated using a Wilcoxon signed rank test (SPSS, SPSS, Chicago, IL). Probability values ≤ 0.05 were considered statistically significant.

RESULTS

The FCMIA assay yielded an excellent fit for the 4-PL model (fig 1), $R^2 = 1.000$ ($p < 0.001$). Evaluation of the recovery of concentrations based on the parameters of the 4-PL fit yielded mean values of 100.9% $\pm$ 13.8% and 100.7% $\pm$ 12.7%, respectively for AVR414 and AVR733. The assay yielded intra- and inter-assay coefficients of variation of 3.41% and 16.0% ($n = 3$ independent assays), respectively. The method MDC was 0.011 µg/ml anti-PA IgG while the RDL was 0.030 µg/ml anti-PA IgG. When the MDC was multiplied by the 1/50 dilution used to measure sample sera (a dilution selected such as to maximise the number of individual sera results > MDC), the whole serum equivalent MDC was 0.55 µg/ml. Of the 56 worker sera and 13 control sera tested, 33 and five sera, respectively, had concentrations of anti-PA IgG below the method's MDC. The concentrations of anti-PA IgG for these sera were estimated based on an averaging method designed to estimate concentrations in the presence of non-detectable values.[8] The 13 control sera had a mean anti-PA IgG value of 1.8 µg/ml with an SD of $\pm$3.8 µg/ml anti-PA IgG, yielding a positive cutoff of 9.4 µg/ml anti-PA IgG. Using this cutoff, four serum samples were positive for anti-PA IgG: sera 23A, 23B, 32A, and 32B with values of 16.4, 13.8, 20.6, and 16.6, µg/ml anti-PA IgG, respectively (fig 2). These four sera were pre-entry and follow up sera of two workers, both of whom had received anthrax vaccine, with the last dose administered in 2000.

There were no significant differences ($p > 0.05$) in anti-PA IgG concentrations between the 19 paired pre-entry and follow-up sera (mean $\pm$SD, pre-entry = 2.8 $\pm$ 5.4 µg/ml; follow up = 2.6 $\pm$ 5.0 µg/ml). The mean inhibition result

for 16 anti-PA IgG negative worker and two control sera which were pre-incubated with PA was 11.1 ± 21.4% (±SD). Interestingly, one of the control sera had an apparent anti-PA concentration of 14.3 μg/ml; however, on pre-adsorption with PA, an inhibition of only 9% was observed. As such, this serum did not meet our positive result criteria. When a 1/500 dilution of AVR414 was pre-incubated with PA, 98.2% inhibition of the uninhibited FCMIA value was observed. Inhibition results for the positive worker sera were sera 23A, 23B, and 33B, 98%; serum 32A, 96%. These data are shown in fig 3.

DISCUSSION

Environmental sampling of the AMI building revealed extensive *Ba* contamination and implicated one or more mailed letters or packages as the likely source(s) of exposure.[3] It has been recently shown that secondary aerosolisation of *Ba* spores can occur when sampling and simulated office activity occurs after a *Ba* terror attack. From that study it was shown that workers sampling for *Ba* could have exposures as high as ~85 CFU/m^3 in their breathing zones.[4] The environmental sampling workers evaluated in the present study had the potential for exposure to *Ba* spores if their PPE was breached or somehow rendered ineffective. It is known that anti-PA IgG antibody levels are increased by both vaccination with anthrax vaccine[9] and clinical infection with *Ba*.[7] It is unclear whether exposure to *Ba* spores while on prophylactic antibiotic therapy can result in the production of anti-PA IgG, and little information exists on whether antibiotic therapy can suppress the IgG immune response. Preliminary results from individuals exposed during the anthrax attacks on the Capitol suggest that *Ba* exposure, even while on prophylactic antibiotics, can lead to the production of anti-PA antibodies.[10] It has been shown that anti-PA IgG can inhibit anthrax spore germination, suggesting that the spore has surface proteins which are homologous or cross-reactive to PA.[11] While our data indicate that two workers had positive levels of anti-PA IgG during the pre-entry and follow up blood collection, it also suggests that the concentrations of anti-PA IgG did not change between pre-entry and follow up blood collection. In fact, the concentrations of anti-PA IgG were reduced from 16.4 and 20.6 (pre-entry) to 13.8 and 16.6 (post-entry) μg/ml, respectively, for the two workers. The finding that these workers had received AVA in 2000 is consistent with these findings. Two other workers had received AVA in 1995 and 1997 but yielded negative anti-PA IgG results, suggesting a diminishment in immunity over time. This is supported by a recent report by the National Academy of Sciences, Institute of Medicine indicating either non-existent or low levels anti-*Ba* antibodies two years after initial vaccinations with AVA.[12] Although numerous ELISAs have been described for anthrax anti-PA antibodies,[7 13–15] this is the first report of an FCMIA for anti-PA IgG. The diagnostic sensitivity and specificity for clinical anthrax infection cannot be determined from the data of this paper as we did not have access to sera from known positive and negative cases, although the FCMIA as reported here can detect anti-PA IgG in both vaccinees (AVR414) and a clinical anthrax case (AVR733). The MDC for the FCMIA compares favourably with the MDC reported for an ELISA[7] for anti-PA IgG (0.011 *v* 0.06 μg/ml anti-PA IgG) and suggests possible enhanced sensitivity. In a preliminary double masked comparison[16] of the FCMIA versus a validated, sensitive, and specific ELISA[7] for anti-PA IgG (n = 20 sera), a correlation of 0.923 (Pearson, $p < 0.001$) was observed between the methods. When either control sera or sera from anti-PA IgG "negative" workers were pre-adsorbed with PA, a mean inhibition of 11.1% was observed. When the four serum samples from the two positive workers or diluted standard sera AVR414 were pre-incubated with PA, almost complete inhibition (96–98%) of anti-IgG binding was observed. These results strongly support the immunochemical specificity of the FCMIA for anti-PA IgG. The prevalence of false positive anti-PA antibody results in the general population is unknown; however, rates of 2.2% have been reported[7] in relatively large non-anthrax

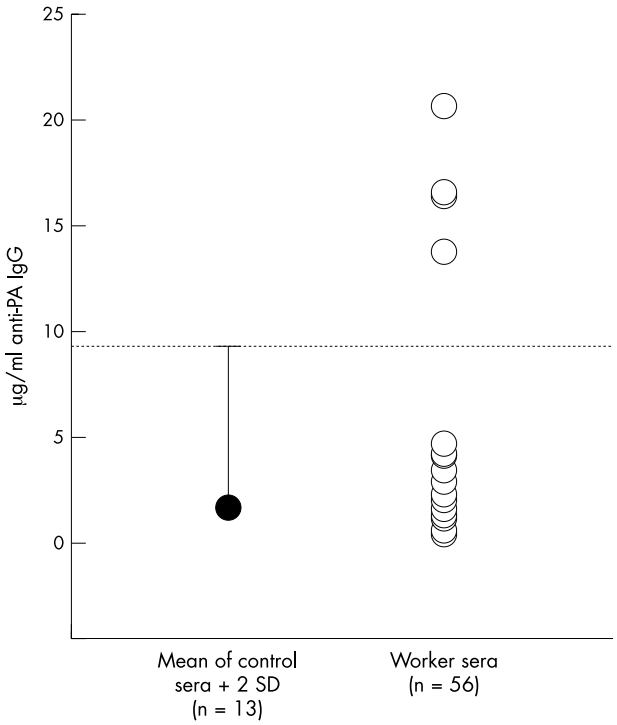

Figure 2 Results of FCMIA. The closed circle represents the mean anti-PA IgG (μg/ml) values for 13 control sera. The error bar is +2 SD. The horizontal dotted line is equivalent to the mean anti-PA IgG +2 SD in μg/ml. The open circles represent individual worker sera concentrations of anti-PA IgG (μg/ml) for 56 worker serum samples. Positive sera (circles above dotted line) had pre-entry values of 16.4 and 20.6 and follow up values of 13.8 and 16.6 μg/ml anti-PA IgG.

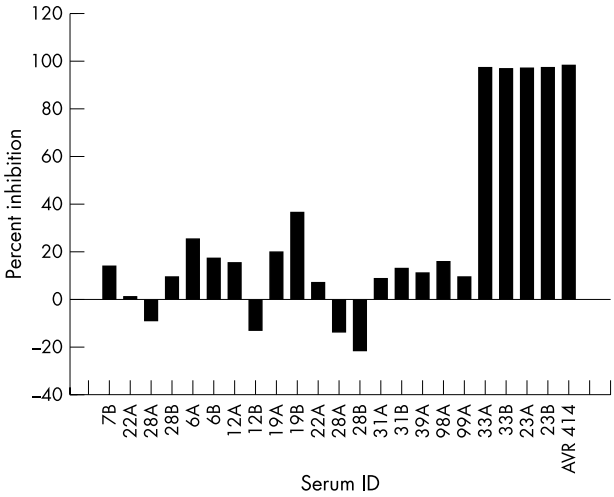

Figure 3 Percentage inhibition from pre-adsorption with 80 μg/ml PA. Sera 7B, 22A, 28A, 28B, 6A, 6B, 12A, 12B, 19A, 19B, 22A, 28A, 28B, 31A, 31B, and 39A were negative worker sera. Sera 99A and 99B were control sera. Sera 99A was a control serum which had 14.3 μg/ml PA, but was only inhibited 9% by PA pre-adsorption. Sera 33A, 33B, 23A, and 23B were positive workers sera, while AVR414 was a 1/500 dilution of pooled standard vaccine sera.

related control sera studies. The apparently high false positive prevalence we observed in the present work (1/13) was 7.7%, presumably due to the small number of control sera (n = 13) tested. When our positive result criteria of ⩾85% inhibition on pre-adsorption with PA was applied to control sera, the false positive prevalence was 0%.

The anti-PA FCMIA has apparent benefits over ELISA methods for the measurement of anti-PA. The FCMIA is faster, has enhanced sensitivity, equivalent or better accuracy and precision, and the potential to multiplex analytes (that is, measure numerous analytes in the same assay simultaneously).[17] This becomes important in the post-bioterrorism incident scenario where multiple chemical and/or biological agents may be used simultaneously or sequentially. This scenario could potentially produce the need for numerous fast, accurate, and precise biological markers of exposure. Multiplexed FCMIA technology is unique in that both chemical and biological assays can be performed simultaneously in one assay with Luminex xMap technology reported on in the present work. Alternative technologies (ELISA, gas liquid chromatography/mass spectrometry) would have to be performed sequentially or simultaneously to measure numerous analytes. As the number of potential analytes were to increase, use of these alternative technologies would present resource and manpower limitations. This technology (FCMIA) also has utility in multiplexed analyses of numerous unrelated analytes such as the detection of pesticides,[17] subclasses of immunoglobulins,[18] virus specific antibodies,[19 20] pneumococcal capsular polysaccharides,[21] analysis of single nucleotide polymorphisms,[22 23] gene expression,[24] and cytokines.[25]

It appears that environmental sampling workers in the present study, protected by PPE, prophylactic antibiotic therapy, and, in some cases, anthrax vaccine, either had insufficient exposure to anthrax spores to cause the production of anti-PA IgG antibodies or they were exposed to anthrax spores without producing antibody. Alternatively, prophylactic antibiotic therapy may have suppressed their immune response,[26] as has been shown in non-human primates where early antibiotic treatment after a known challenge with *Ba* spores abrogates an anti-PA antibody response.[27] Being exposed to *Ba* spores alone does not necessarily cause frank *Ba* infection. For infection to occur, exposure to sufficient numbers of viable spores, germination, and bacterial outgrowth sufficient to overwhelm any pre-existing specific and non-specific immunity is necessary. In order to mount an immune response to PA from *Ba* spore (non-vaccine) exposure, either PA or antigenically similar PA homologous antigen exposure of sufficient intensity and duration has to occur. The number of *Ba* spores necessary to cause frank *Ba* infection in humans is unknown; however, primate studies suggest exposure to only a few spores can result in an infective dose.[28] Whether exposure to sub-infective numbers of *Ba* spores can result in anti-PA immune responses in humans is presently controversial.[11] Due to the uncertainties present in the interpretation of serological tests for anti-PA antibodies in protected sampling and decontamination workers, serologic tests for anthrax exposure should not be used as the sole confirmatory tests for anthrax exposure. The use of paired sampling strategies (that is, pre-entry and follow up), as performed in the present study, should increase the potential to accurately detect exposure.

Conclusion

An FCMIA method was developed to evaluate exposure to *Bacillus anthracis* (*Ba*) in sampling/decontamination workers in the aftermath of the anthrax terror attacks of 2001. Anti-anthrax protective antigen (PA) IgG antibody levels were used as a biological monitoring tool. No apparent exposure related increases in anti-*Ba* PA IgG were seen; however, workers who had been recently vaccinated with AVA were identified. The FCMIA is faster, has enhanced sensitivity, equivalent or better accuracy and precision, and the potential to multiplex analytes (that is, measure numerous analytes in the same assay simultaneously) compared to ELISA.

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