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A RAPID, AUTOMATABLE METHOD TO DETECT AND IDENTIFY THE INFECTIOUS POTENTIAL OF BW AGENTS USING SIGNATURE BIOMARKERS

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ABSTRACT: The rapid detection and identification of classical and genetically engineered biological warfare (BW) agents is crucial; whether the threat is a massive attack or a more subtle terrorist action. For infection, all agents including viruses require living cells which must have an intact polar lipid membrane. Analysis of signature lipid biomarkers (SLB) from these membranes provides a quantitative *in situ* method to detect BW agents (with the exception of prions and some virus particles). This method involves the extraction of membran lipids from any number of matrices including aerosol filter retentates, mud, soils, aqueous filter retentates, vectors, and clinical specimens. Automated high-pressure, high-temperature solvent extraction yields SLB in minutes. SLB analysis involves fractionation, on-line derivatization, and separation and identification using GC/MS or HPLC/ES/MS. Other instrumentation combinations may be useful such as tandem mass spectrometry (MS/MS) for increased selectivity, greater structural information resulting from collisionally induced dissociation's (CID) and greater sensitivity resulting from the reduction of chemical noise; electrospray ionization (ES) for increased speed and sensitivity; HPLC for purification and additional structural conformatior purposes; as well as the use of ion trap mass analyzers for field system portability along with MS^n capabilities. Since SLB's reflect both genotypic and phenotypic BW agent response, SLB's can provide an estimation of the infectious potential of specific agents in complex environmental matrices. The SLB technique has demonstrated the ability to detect the infectious potential of *Cryptosporidium parvum* at the sensitivity of a single oocyst using SIM/MS. The identification, pathogenicity, and drug-resistance of *Mycobacteria* spp. have been determined at sub-femtomolar sensitivities with SLB analysis. Examination of SLB's from aerosol filter retentates has also been utilize to provide quantitative detection and identification of unculturable, but viable anc often infectious indoor air biocontaminants, as well as immune potentiating lipopolysaccharide-endotoxins and mycotoxins.

INTRODUCTION: Over the past 20 years, our laboratory has developed the signature membrane biomarker (SLB) method of analysis for the quantitative definition of the viable biomass, community composition, and nutritional status of microbiota isolated from a wide variety of environmental matrices including air, soil, wounds, and water [1]. In many cases, unique molecules specific to microorganisms that could be useful in detecting, identifying, and determining the infectious potential of life threatening microbial pathogens have been detected.

THE PROCESS:

Collection of the samples: A SLB based microbial testing system for airborne microorganisms based on the collection of particulates on glass fiber filters has been utilized to establish that the standard culturing

ıethods are woefully ineffective in determining the actual number of microbes in indoor air samples [2,3]. The SLB analysis system is quantitative, allows detection of lipopolysaccharide immune potentiators, and through examination of the phospholipid fatty acid (PLFA) profile provides clear evidence of metabolic stress [2,3]. Through this research it became clear that the lipid extraction approach provides an excellent method for the detection and identification of airborne biohazards. The major limiting factors in this technique are the speed of extraction and detection.

Extraction: The most time consuming step in the SLB analysis is the extraction of the lipids. The initial step classically involved a one-phase modified Bligh and Dyer solvent extraction for obtaining phospholipid fatty acid biomarkers used in community analysis. This method, however, is relatively labor intensive and slow, often taking up to 24 hours for the initial extraction.

Our laboratory has explored the utility of supercritical CO_2 extraction as a means of decreasing the time investment for SLB extraction procedures. We have shown that polar phospholipids could not be extracted unless they were prederivatized with trimethylphenylammonium hydroxide (TMPH) to form the methyl esters *in situ* [4]. Super critical fluid extraction, however, did prove remarkably effective in facilitating recovery of DNA from environmental samples[5]. Accelerated solvent extraction (ASE) was used to extract SLB from selected vegetative and/or sporulated biomass as well as from environmental samples collected from water, soil, and air [6]. Dependent on sample type, the automated extraction procedure took approximately 35-45 minutes per sample. Compared to the modified Bligh and Dyer extraction, phospholipid fatty acid (PLFA) lipid yields obtained using the pressurized hot solvent extraction were not significantly different for the vegetative biomass or water and soil samples, but were significantly higher for the spores and the air biomass samples. Initial studies have shown that ASE recovered 3-fold more PLFA from *Bacillus* spores and 2-fold more PLFA from *ıspergillus* spores than the standard one-phase solvent extraction system while significantly reducing extraction time [6]. The system is automated for unattended analysis. The system can be programmed to allow multiple extractions from the same sample so that an automated fractionation can be developed. The increased extraction speed was achieved through a combination of increased pressure and temperature using the Dionex system. The Dionex system can process samples at a temperature of 200°C and pressures of 3,000 psi. with a minimum sample volume of 11 mL (using 18 mL minimum solvent).

Proposed Modifications: The next technological step in rapid extraction will come through the utilization of a system capable of higher pressures such as the ISCO SFX 3560 that will drive the reaction at 10,000psi and 150°C and utilize samples of 0.5 ml volume. This increased pressure will significantly increase the solubility of the lipids & lipophilic membrane proteins from the membranes of the organisms and increase rates of *in situ* derivatization for further separation and analyses. Sufficient separation of "signature" membrane biomarkers will occur rapidly with a high performance liquid chromatography system. Once separated the individual lipid classes and proteins will be ionized by an electrospray ionization source and identified by the ion trap mass spectrometer capable of monitoring successive product ion spectra. The patterns of membrane components could be analyzed by an artificial neural network which will then identify the class or specific type of biological threat

Separation & Detection: The literature is rich in methods for the high performance liquid chromatographic (HPLC) separation and purification of lipids [7]. Moreover, polar lipids readily ionize under

electrospray ionization conditions [8]. To facilitate the initial phases of analysis, a C-18 coated open tubular capillary column between the extraction system and the electrospray ionization ion tr mass spectrometry system will provide in-line separation without the need for post-column splitting. This will provide for the class fractionation and quasi-molecular speciation of the polar lipid components. Electrospray ionization, widely regarded as the most versatile and sensitive method of ionization available, operating in the negative ion mode will provide information regarding the nature of the intact phospholipid, the identity of the two fatty acyl constituents, as well as that of the polar head group. As has been previously demonstrated, organic bases will be added at levels so as to enhance the electrospray signal of phospholipids components [9]. However, pH values greater than 8 have been demonstrated to rapidly deteriorate silica based columns.

The type of mass analyzer which possesses both high sensitivity and a reasonableness as a potential fie detector are based on quadrupole based systems. Quadrupole mass spectrometers can be either beam type, wit a linear trajectory for the analyte ions, or ion trap quadrupoles, where the ions are stored in a "trap" and ejected for subsequent detection. The ion trap mass spectrometers (ITMS) have features particularly desirable for fiel instruments such as smaller size and lesser power requirements. The Teledyne ITMS has been chosen by the Army Environmental Center, Aberdeen, MD as its current field portable Tri-service Site Characterization and Analysis Penetrometer System (SCAPS) [10] and seems to be a reasonable choice for the next CBW detector.

Previous investigators have demonstrated the application of fast atom bombardment tandem mass spectrometry to the identification of intact phospholipids. Through a combination of positive ion, negative ion, and constant neutral loss scans, the molecular species of each phospholipid was unambiguously characterized without the need for prior chromatographic purification. Constant neutral loss scans provided assignment of component lipids to their respective phospholipid class (m/z = 141 for phosphatidyl ethanolamine, m/z = 184 f phosphatidylglycerol, etc.). The nature and position of the fatty acyl constituents was determined through daughter scans in the negative ion mode. Preferential formation of the carboxylate anion at the β-position (*sn*-. results in a daughter ion of much greater intensity than the carboxylate anion at γ-position 1(*sn*-1). Whereas, t positive ion mass spectra provides molecular weight information regarding the identity of the molecular ion minus the polar head group $(M-Y)^+$ [11]. Detection limits specific for phosphatidylethanolamine (PE) were found to be below 1 pg from a mixture of phospholipids. This is roughly the quantity of phospholipids present in 10 *E. coli* cells. In the proposed system, the greater ionization efficiency of electrospray ionization was use to identify phospholipids. Electrospray ionization proved to be an extremely sensitive tool (detection limits below 0.1 pg of material) and a structurally informative means for the rapid identification of intact, underivitiz phospholipids. By ramping the cone voltage so as to induce thermal dissociation, fragmentation patterns identical to the tandem collisionally induced techniques were observed in both positive and negative ion mode. The addition of HPLC class and molecular speciation capabilities along with the MS^n features of the ion trap system will result in a rapid, automatable, and field-portable biological warfare detection system [12].

Data Interpretation: Signature lipids offer a great advantage over many other molecular probes in that they reflect the phenotypic expression of the cell's genes. The PLFA in Gram-negative heterotrophs show interesting changes. Cells that age and enter the stationary growth phase show increased ratios of cyclopropane PLFA to their monoenoic PLFA precursors. Cells exposed to metals, solvent, or toxicants form *trans* monoenoic PLFA reversibly from the *cis* monoenoic precursors. This can also result in shifts in the

lipopolysaccharide hydroxy fatty acids. The contractor has utilized this phenotypic expression to show infectiousness of *Cryptosporidium parvum* oocysts [13]. Additionally, proteins offer an enormous potential for identification as they are found in all classes of BW agents. The sequencing of proteins through ES/CID/MS^n allows identification of the proteins specific to potential pathogens.

The most sensitive means to predict outcomes from complex lipid analysis patterns or protein sequences has been to apply artificial neural network to the data [14]. Artificial neural network of patterns of neutral lipid wax components of Mycobacteria were used to "train" the artificial neural network so it could successfully predict from the patterns which were slow growers, which were pathogenic, and which were chromogenic [14]. Artificial neural network is much more powerful than principal components analysis as the artificial neural network can utilize non-linear correlations that are either positive or negative and can utilize additional data as experience to train the system to better predict specific outcomes.

Although signature lipids provide explicit biomarkers for identity and physiological status, most of the signature components encode for contextual information. The same is true for most biological constituents as its function is dependent on the presence of other components [15]. From a data analysis point of view, the entangled interdependencies between analytes requires special tools to recover implicit clinical information. Artificial neural networks provide such a solution by emulating natural learning [16]. Artificial neural network models do not require initial mechanistic assumptions about the biological role of analytes, instead encoding the implicit information from examples ("learning from examples"[17]). Consequently, different artificial neural network can be developed to infer specific traits from a chemical signature.

Data analysis by artificial intelligence techniques will be implemented by first building parallel databases of analytical profiles and microbial identity and characteristics. As database size increases, associations between the two data sets will emerge by continuously integrating the new data in the analysis. The emerging association will also identify redundant information; therefore help directing data gathering to more interesting samples, in order to attain predictive capability for predefined target parameters. Standard multivariate statistic tools will also be used to describe and test explicit relationships between analytical profiles and corresponding isolates.

Matlab (Mathworks Inc.) provides an adequate software environment to develop artificial neural network applications, being available both for desktop and mainframe operating systems. Applications developed in this environment can be automatically compiled in C, which can then be incorporated directly into the analytical apparatus and provide an immediate evaluation of a new analysis. This solution would provide an intelligent diagnostic tool with the potential to adapt to new situations as they occur. A faulty diagnosis is incorporated as a source of new information ("learning from errors"[17]). Since artificial learning can be continuously implemented, the next diagnosis will have the benefit of recent information.

SLB of threat organisms: The contractor has established that many threat organisms have sufficiently unique signature lipid patterns that are detectable with signature lipid biomarker analysis. Examples include *Bacillus* sp. spores [18], *Francisella tularensis* [19], 17 species of Mycobacteria [14], Legionella [20], and *Cryptosporidium parvum* [13]. Also, lipid patterns can reflect the phenotypic expression of the membrane lipid metabolic capabilities [21]. Information gained can then be used to relate nutritional/physiological status to infectious potential or drug resistance. For example, in *Mycobacterium tuberculosis,* patterns of micoserosic acids and secondary alcohols derived from the surface waxes correlated with drug resistance when subjected to

artificial neural network analysis[13]. With the protozoal pathogen *C. parvum*, signature lipid biomarker analysis using electrospray ionization mass spectrometric allowed detection at the sensitivity of one oocyte, o *parvum* based on the unusual PLFA, 10-0H 18:0, found at the β-position of phosphatidylethanolamine [22]. T contractor has established that gas chromatography mass spectrometry systems are also effective, although les sensitive, in the signature lipid biomarker analysis. Making use of current state of the art gas chromatography mass spectrometry instrumentation, this method is capable of detecting as little as 10^3 bacteria [1,13, 21].

CONCLUSIONS: The focus of research could focus on the rapid extraction and detection of both lipid and proteins by developing an ultra-rapid membrane extraction/fractionation system for those microbes and spores accumulated on membrane filters. The filters will be subject to rapid extraction separation lipid and protein profiling and artificial neural network identification of the class or specific biological threat.

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