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Analysis of urban aerosols in major US cities - implications for asthmatic airways.

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Abstract

The objective of the proposed study was to compare seasonal aerosol samples collected during Spring, Summer, Fall and Winter from 3 US cities with high prevalence of asthma, Boston, Philadelphia and Detroit, to determine the effect of seasonal variation on bacterial urban aerosol composition. In addition comparisons were made between aerosol bacterial communities from these three cities and those present in San Francisco in the Spring. To determine if there were any similarities between aerosol and airway bacterial communities comparisons were also made between aerosol and respiratory samples from asthmatic patients (collected in a separate multi-center US trial). Finally, analysis of commonalities and distinctions in urban aerosols in East coast versus West coast samples were examined to determine whether geographic location impacted the bacterial community present at these sites. Bacterial community composition varied across sites but appeared, at least in the samples analyzed, to be more associated with specific geographic location than by seasonality. Phyla detected in these samples represented a vast diversity of bacteria and included many members of the Firmicutes and Bacteroidetes, two of the primary phyla colonizing the human gastrointestinal tract as well as members of the Sphingobacteria and Actinobacteria that have previously been detected in the respiratory tract of patients with inflammatory airway disease. A comparison of bacterial communities from Boston, Philadelphia and Detroit (collected in the Spring) to those detected in the same season in San Francisco revealed a core of bacteria common to all samples. Nonetheless, geographic-based differences in aerosol community composition were detected between San Franciscan and East Coast samples, suggesting that habitation at a specific site may result in distinct bacterial exposures and, potentially contribute to geographic-specific differences in airway disease prevalence. Comparative analysis of communities detected in urban aerosols and those found in the airways of asthmatics demonstrated a large overlap in these communities, supporting the notion that bacterial species present in environmental aerosols may serve as an inoculum for human respiratory mucosal surfaces in patients predisposed to colonization at these sites and contribute to airway disease prevalence at geographically distinct sites.

Executive Summary

Background.

Recently, using a high-density 16S rRNA phylogenetic microarray it has been demonstrated that urban aerosols in two cities in Texas harbored highly diverse communities of bacterial taxa. These populations were so complex that they approximated the richness of bacterial diversity detected in some soil microbial populations (approximately 1,800 bacterial phylotypes [1]). More recently, we have demonstrated that polymicrobial communities composed of multiple pathogens exist in the airways of patients with a variety of respiratory disorders including ventilator-associated pneumonia [2], cystic fibrosis (CF; Cox *et al*, In review, PLoS One;[1]), chronic obstructive pulmonary disease (COPD;[2]) and asthma (In review; AJCCRM). We hypothesize that inhaled aerosolized microbial diversity seeds the airways of patients predisposed to colonization (e.g. asthmatics, CF patients) with organisms which may contribute to incidence and severity of pulmonary pathogenesis in these vulnerable patient populations.

In a survey conducted in 2006 by the CDC (http://www.cdc.gov/nchs/data/ad/ad381.pdf), Massachusetts had the highest prevalence of asthma amongst children aged 0-17 years old (12.1%), Pennsylvania and Michigan also had relatively high rates of 9.9 and 9.4% respectively. However, the rate of asthma prevalence in this age group was only 7.1% in California. The factors that underlie these rates and the increasing prevalence of asthma in the developed world are undoubtedly multi-faceted. However, we hypothesize that in addition to host genetic factors, environmental aspects such as the microbial composition of the air may also contribute to differential rates of asthma prevalence at geographically distinct sites.

Methods.

Bacterial community composition of aerosol (collected in 2003 – 2004 through a Homeland security bioterrorism program) and asthmatic airway respiratory samples (collected in an NIH-supported multi-center trial) was determined using a recently designed novel high-density 16S rRNA microarray, the PhyloChip [3, 4]. This tool uses amplified 16S rRNA genes from a mixed bacterial population, which is assayed in parallel by the microarray

that contains 500,000 probes and can detect approximately 8,500 bacterial taxa (defined as clusters of bacterial 16S rRNA signature sequences sharing at least 99% sequence identity). This tool can detect low abundance species (0.01% of the community) even when the community is dominated by a small number of highly abundant organisms.

Results

The objective of the proposed study was to compare seasonal aerosol samples collected during Spring, Summer, Fall and Winter from 3 US cities with high prevalence of asthma, Boston, Philadelphia and Detroit, to determine the effect of seasonal variation on bacterial urban aerosol composition. Bacterial community composition varied across sites but appeared to be more associated with specific geographic location than by seasonality. The primary phyla detected in these samples represented a vast diversity of bacteria and included a number of phyla, members of which are common inhabitants of human niches, including the respiratory tract of patients predisposed to airway colonization. A comparison of bacterial communities from these cities (collected in the Spring) to those detected in the same season in San Francisco revealed a core of bacteria common to all samples. Nonetheless, geographic-based differences in aerosol community composition were detected between San Franciscan and East Coast samples, suggesting that habitation at a specific site may result in exposure to a distinct community of bacteria and, potentially contribute to geographic-specific differences in airway disease prevalence. Comparative analysis of communities detected in urban aerosols and those found in the airways of asthmatics demonstrated a large overlap in these communities, supporting the notion that bacterial species present in environmental aerosols may serve as an inoculum for human respiratory surfaces.

Conclusions.

The diversity of bacteria detected in urban aerosols appears to be location-dependent rather than season, potentially acting as a source of geographic-specific microbial exposures. The large overlap in aerosol and airway bacterial communities, suggest that bacteria in inhaled air may serve as a reservoir for human inoculation, particularly for individuals predisposed to airway colonization due to underlying disease.

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Final Report.

Introduction.

The objective of the proposed study was to compare seasonal aerosol samples collected during Spring, Summer, Fall and Winter from 3 US cities with high prevalence of asthma, Boston, Philadelphia and Detroit, to determine the effect of seasonal variation on bacterial urban aerosol composition. In addition we compared bacterial communities from these cities in the Spring to those detected in the same season in San Francisco. Finally, comparisons were made between the communities detected in urban aerosols and those found in the airways of asthmatics to determine whether any overlap existed between these two niches.

Materials and Methods.

Sample collection

Samples collected during the course of a DOE-funded homeland security study to detect biowarfare agents in urban aerosols were used for this study. Air samples were collected using an air filtration collection system under vacuum located at sites in San Francisco, Philadelphia, Boston and Detroit. Approximately 10 liters of air per minute were collected on a Celanex polyethylene terephthalate, 1.0-μm filter (Calanese, Dallas, TX). Samples were collected daily over a 24-h period. Sample filters were washed in 10 ml buffer (0.1 M sodium phosphate/10 mM EDTA, pH 7.4/0.01% Tween-20), and the suspension was stored frozen until extracted. Samples were collected from 4 May to 29 August 2003. Sample dates were divided according to a 52-week calendar year starting January 1, 2003, with each Monday-to-Sunday cycle constituting a full week. Samples from four randomly chosen days within each sample week were extracted.

DNA Extraction and 16S rRNA Gene Amplification.

Pooled samples for each city for each week were centrifuged at 16,000 × *g* for 25 min, and the pellets were resuspended in 400 μl of 100 mM sodium phosphate buffer (pH 8). DNA

extraction was performed as described in DeSantis *et al.* (29), but only a single bead-beating velocity and duration was used (6.5 $m \cdot s^{-1}$ for 45 s). DNA was quantified by using a PicoGreen fluorescence assay according to the manufacturer's recommended protocol (Invitrogen, Carlsbad, CA). 16S rRNA gene amplification was performed according to standard procedures as previously described [5].

PhyloChip Processing, Scanning, Probe Set Scoring, and Normalization.

The pooled PCR product was spiked with known concentrations of synthetic 16S rRNA gene fragments and non-16S rRNA gene fragments as internal standards for normalization with quantities ranging from 5.02×10^8 and 7.29×10^{10} molecules applied to the final hybridization mix. Target fragmentation, biotin labeling, PhyloChip hybridization, scanning, and staining were as described by Brodie *et al.* (30), and background subtraction, noise calculation, and detection and quantification criteria were as reported in Brodie *et al.* (30). Data sets were conservatively filtered, with taxa determined as present if ≥ 90% of probes in a probe set (for an individual taxon) were positive. Changes in probe-set fluorescence intensity are equivalent to changes in taxon relative abundance between samples. For taxa determined to be present in at least one sample, PhyloChip probe-set fluorescence intensity data was log transformed prior to analysis using packages in the R statistical environment [6].

Hierarchical cluster analysis (HCA) was performed on a Bray-Curtis dissimilarity matrix generated from PhyloChip fluorescence intensity data using the *vegan* package [7], followed by average linkage clustering. A two-tailed Welch's T-test was used to identify taxa that were significantly altered in relative abundance in specific groups and adjusted for false discovery using the *qvalue* package as previously described. Significance was assigned with a p-value ≤ 0.05, q-value of 0.057.

Asthmatic subjects

Asthmatic subjects enrolled at multiple centers across the continental US, exhibited suboptimal disease control on daily, low dose inhaled fluticasone, as defined by a

threshold Juniper Asthma Control Questionnaire (ACQ) score of $\geq$ 1.25. All subjects were administered an 8 week course of once daily corticosteroid, prior to obtaining airway bronchial brushing samples for PhyloChip analysis. Triplicate bronchial brushings were processed for DNA and RNA extraction and profiled for bacterial community composition using the PhyoChip as described above.

Results and Discussion.

To date, we have analyzed by 16S rRNA PhyloChip, temporal parallel aerosol samples collected in Boston, Detroit and Philadelphia in weeks 17 (Spring), 18, 19, 20 (Summer), 32, 33, 34 (Fall) and 46 (Winter) of the parent DOE funded urban aerosol study. A total of 2,842 bacterial taxa were detected. Detection of so many taxa is partly due to application of high concentrations of 16S rRNA PCR product to the microarray. The numbers of bacterial sub-families detected in individual samples ranged from approximately 170 to 360 (Fig. 1). Many of the sub-families detected contain known or suspected pathogens; however susceptibility to infection has recently been shown, at least in part, to be due to the structure and composition of the host native microbiome, suggesting that the same aerosol may elicit a very different effect on two individuals, depending on the nature of their personal microbiota. Total richness (the number of types of bacteria in a given sample) detected across samples represented 727 bacterial sub-families, which is comparable to that detected in urban aerosols in Texan cities in the parent study [1] and many of these sub-families contain known pathogens or close relatives of known pathogenic species. Comparison of sample richness across

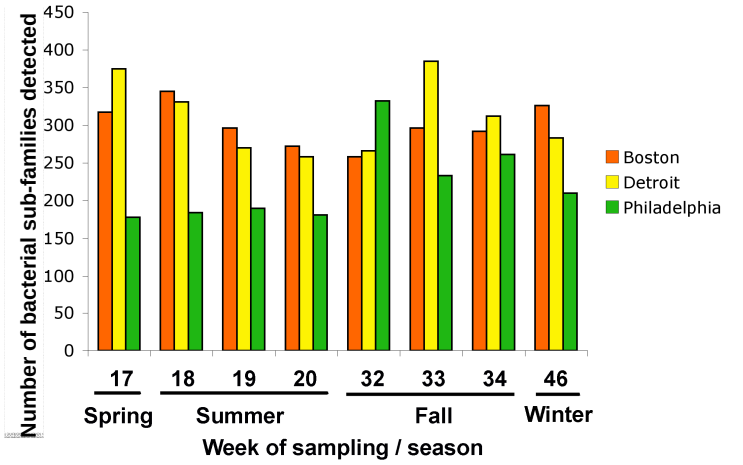

Fig. 1. Number of bacterial sub-families detected by 16S rRNA PhyloChip in urban aerosol samples collected across all four seasons in Boston, Detroit and Philadelphia.

geographical divides demonstrated substantially less sub-family level richness in Philadelphia aerosols compared with that detected in Boston or Detroit in all weeks examined except week 32 (Fig. 1). Interestingly, bacterial diversity of Boston and Detroit aerosols, although from geographically distinct locations, were comparable and broadly followed the same pattern of community richness across all weeks sampled (Fig. 1). This potentially suggests that proximity to water and associated prevailing weather conditions may play a role in increasing urban aerosol richness and hence environmental exposure to a greater breadth of microbial diversity at these locations.

Comparison of the relative distribution of phyla present in parallel aerosol samples collected in Boston, Detroit and Philadelphia during Spring, Summer, Autumn and Winter of the parent study demonstrated that despite detection of reduced diversity in the Philadelphia samples, the distribution of phyla within aerosols at all three sites was relatively similar. Communities were typically dominated by Proteobacteria and Firmicutes with members of the Actinobacteria, Bacteroides and Cyanobacteria representing the majority of other taxa detected at each site. As has previously been reported for urban aerosols in Texas, the most commonly detected class of bacteria in these samples were the Bacilli which belong to the Phylum Firmicutes.

We also examined the temporal behavior of the two dominant phyla, Proteobacteria and

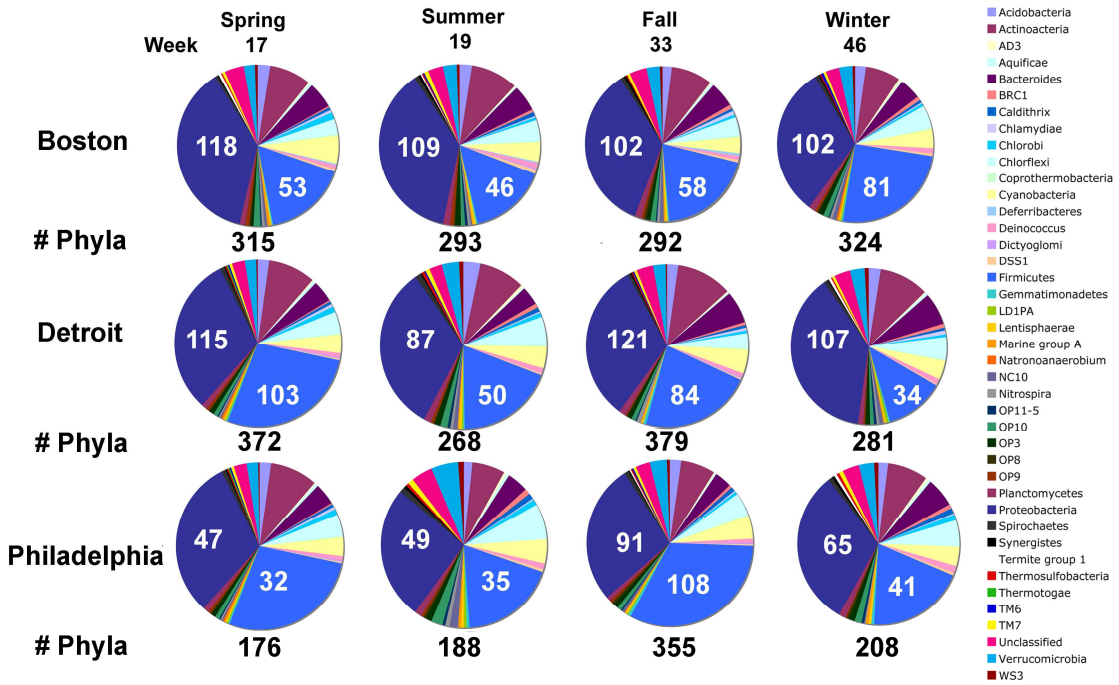

Fig. 2. Distribution of phyla detected in parallel aerosol samples collected in Spring (week 17), Summer (Week 19), Fall (week 33) and Winter (week 46) in Boston, Detroit and Philadelphia. Number of Phyla detected in each sample is provided beneath each pie chart.

Firmicutes in samples from week 17, 19, 33 and 46 to examine seasonal shifts in the abundance of these phyla (Fig. 3). Firmicute relative abundance increased in Fall and Winter relative to Spring and Summer in Boston. In Detroit, the opposite trend was observed; the Firmicutes exhibited the greatest abundance in Spring and least in Winter. In Philadelphia, Firmicute diversity peaked in the Fall, but remained stable in all other seasons. Proteobacteria diversity did not such exhibit dramatic shifts, in Boston a minor decrease in diversity was detected in the Winter, whereas in Detroit and Philadelphia minor increases in diversity were detected in this season. Again these results suggest that environmental conditions proximal to the sample collection point play a key role in defining the bacterial community composition of urban aerosols at that site, a finding that is supported strongly by analysis of Texan urban aerosol samples in the parent study [5]. These observations also suggest that Phyla such as the Firmicutes are highly influenced by seasonal weather conditions but that Proteobacterial diversity appears to remain

relatively stable regardless of seasonal changes. That Firmicutes are more influenced by weather conditions is interesting given that the Bacilli represent the majority of Firmicutes detected in this study. Bacilli are endospore formers and so their presence is highly influenced by both water and heat exposure, characteristics supported by a recent study that demonstrated that the Firmicutes are amongst the phyla most influenced by weather conditions [1]. However, the nature of the study does not permit us to address whether these seasonal variations in aerosol bacterial community composition impact asthma since we do not have any symptomology information available to us for this study. A large-scale study in which asthma symptomology was recorded from patients at specific times of the year in parallel with aerosol sampling performed at geographically relevant sites proximal to these individuals would be necessary to address this question.

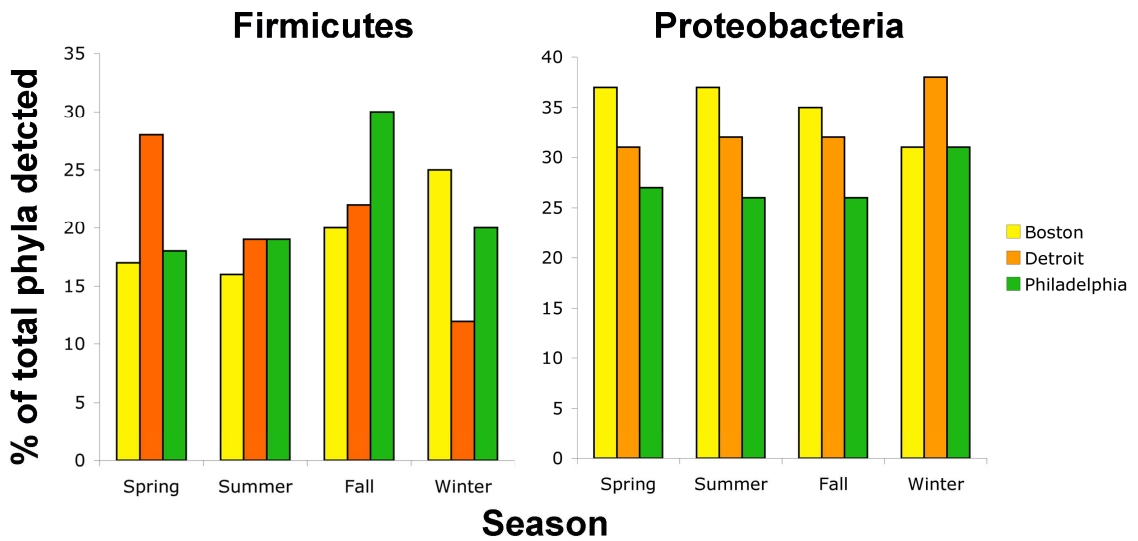

Fig. 3. Seasonal variation in number of phyla detected at each site in this study.

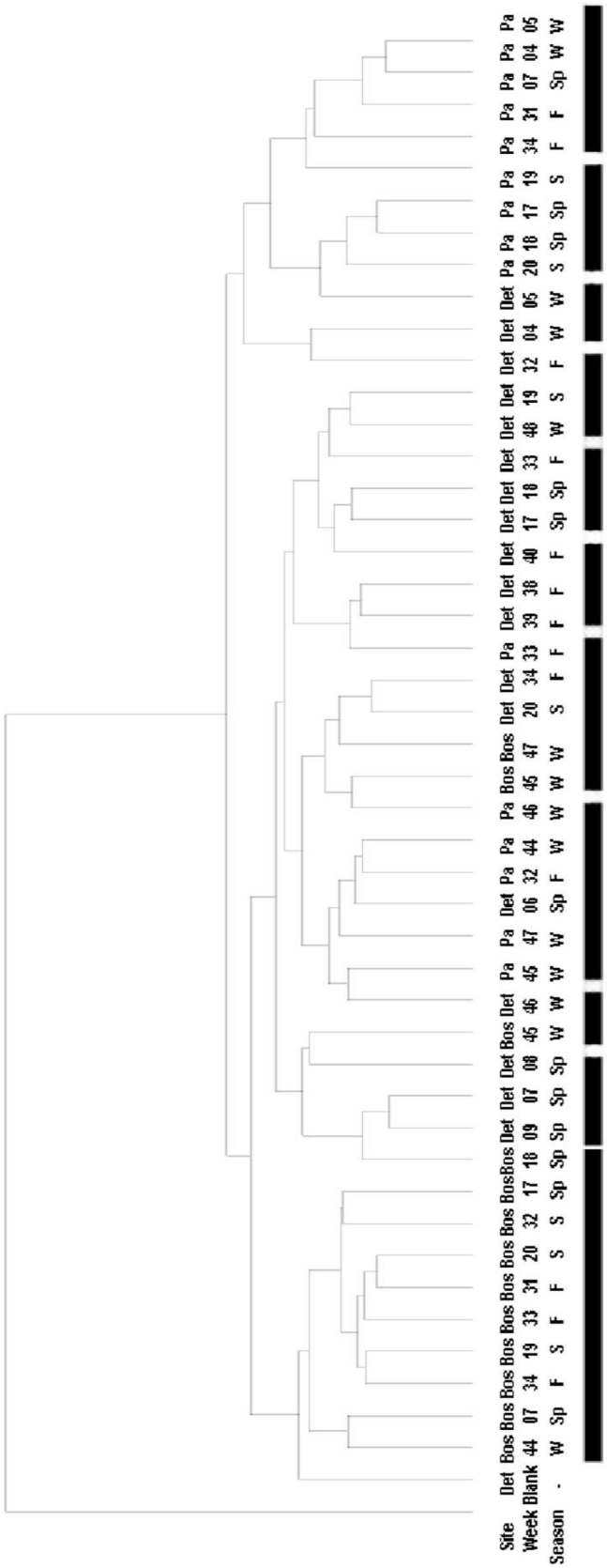

Fig 4. Hierarchical cluster analysis of bacterial communities at the sub-family level demonstrates that samples cluster primarily by geographic location rather than by season.

Samples collected at each of the three sites during various weeks in each of the four seasons at each of the three sites were examined by hierarchical cluster analysis at the sub-family level to determine whether geographical location or seasonal variation had a greater influence on community composition. Figure 4 illustrates sample clustering, and demonstrates that samples typically cluster by geographic region, in addition, samples collected during the same season from a specific site frequently cluster together suggesting that both season and location are two key variables that determine aerosol bacterial community composition.

The datasets used in this study were also analyzed for the presence of specific species previously associated with asthma. The presence and temporal behavior of *Chlamydophila pneumoniae* was initially examined, since atypical intracellular pathogen has been associated with asthmatic airways in a number of relatively recent studies [9-11]. Figure 5 illustrates the relative abundance of this taxon which was detected in all samples examined in this study. Boston possessed the greatest abundance of this taxon (also has the highest incidence of asthma in pediatrics aged 0-17 amongst the cities surveyed in this study) in its aerosols in Spring; relative abundance decreased at this site by Winter, but the decrease was not substantial (less than an order of magnitude). At the other two sites, the relative abundance of *C. pneumoniae* was similar and also did not change dramatically due to seasonal changes.

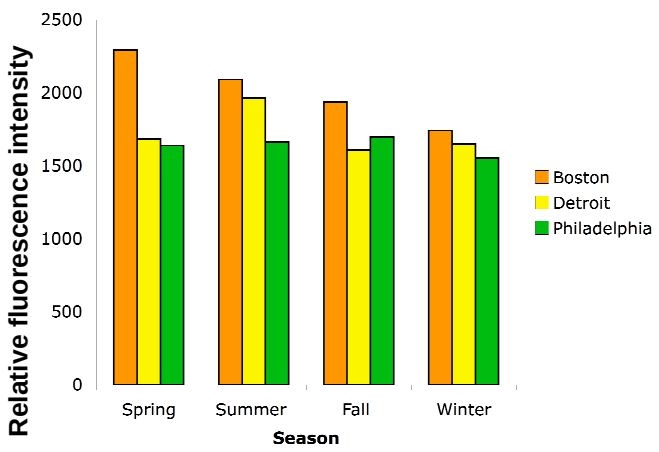

Fig. 5. Relative abundance of *C. pneumoniae* in seasonal samples from Boston, Detroit and Philadelphia demonstrate relatively stable abundance of this taxon in aerosols despite season.

Season-matched comparison of San Francisco vs East coast samples.

Analysis of aerosol bacterial communities collected in the Spring at locations in Boston Detroit, Philadelphia and San Francisco demonstrated that a large number of taxa were present in both West and East coast locations. A total of 1,842 taxa were present in both San Francisco and at least one Eastern US city (Table 1) and included a large number of phyla such as the Gammaproteobacteria, Actinobacteria, Acidobacteria and Sphingobacteria amongst others, which we have previously detected in both the gastrointestinal and respiratory tract of humans (Fig. 6;[2], Huang *et al*, In review, AJCCRM;[8]).

Examination of the taxa that discriminated San Francisco aerosols from East coast cities revealed that 216 taxa were detected exclusively in San Franciscan aerosols (Table 2). These taxa represented a number of distinct bacterial families such as the Sphingobacteriaceae, Burkholderiaceae and Moraxellaceae amongst others which have previously been implicated in pulmonary pathogenesis [9-11]. A total of 398 taxa were detected in at least one East coast aerosol sample, but not in San Franciscan aerosols (Table 3). These included members of the Propionibacteriaceae which are normal inhabitants of the skin [12] and members of the Thermoactinomycetaceae which we have recently detected in COPD patient airways [2]. Members of the Thermoactinomycetaceae have previously been associated with COPD in equine populations [16, 17], suggesting a plausible role for these organisms in human airway diseases. Thus it appears while there is a core of shared bacteria in the aerosol samples examined that includes a large diversity of distinct bacterial phyla, specific species, may discriminate geographic locations and many of these families contain members potentially implicated in respiratory disease. Although it is likely a very complex phenomenon, these findings provide evidence for geography-specific microbial exposures which may explain, at least in part, differences in respiratory disease prevalence (including asthma) at these sites.

Comparison of the microbiota of asthmatic vs aerosol samples.

Since many of the taxa detected in urban aerosols have also been detected in respiratory samples, we also examined whether substantial overlap exists between microbiota detected in air and, specifically, asthmatic airways. For this portion of the study, we examined aerosol samples collected from 4 cities (Boston, Detroit, Philadelphia and San Francisco) and compared them to the microbiota detected in protected bronchial brush samples from 38 asthmatics sub-optimally controlled by daily inhalation of a low dose inhaled corticosteroid. Asthmatic samples were collected from 10 sites across the continental USA in a multi-center trial. The hypothesis underlying this aim is that patients predisposed to bacterial colonization of the airways may be inoculated to some extent by the microbiota in inhaled aerosols. Unfortunately, because this portion of the study was predicated on pre-existing data, matching asthmatic samples with urban aerosol samples by season or city was not possible, therefore the data should be interpreted with caution. Nonetheless, comparative analysis of the data sets demonstrated that a total of 1,698 taxa were detected in both aerosol and asthmatic airway samples (Table 4; Fig. 7). This is impressive given that compared to the aerosol samples, the asthmatic samples were assayed at a much lower concentration of 16S rRNA PCR product (1,000 vs 250 ng respectively). This suggests that many of the species colonizing asthmatic respiratory tracts may inocluate the airways through inhalation of environmental aerosols. A large number of taxa (1,144; Table 5) were detected in aerosols but not in asthmatic patient

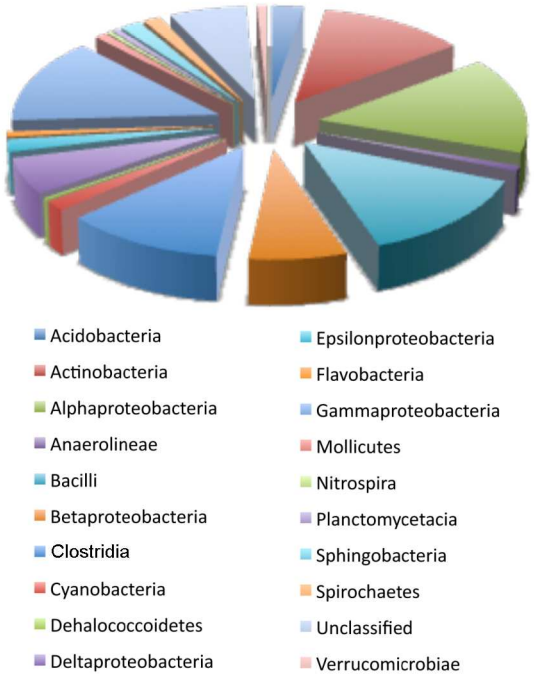

Fig. 6. Major bacterial phyla detected in San Francisco and at least one East coast city (Boston, Philadelphia or Detroit). The width of each slice represents the number of taxa in that phylum detected in both San Francisco and at least one East coast site.

airways, this suggests that these taxa are either poor colonizers of the human respiratory tract or simply below the level of array detection. Interestingly a total of 177 taxa were detected in asthmatic samples exclusively (not identified in aerosol samples; Table 6). Many of these taxa have been identified previously in specific human niches e.g. human subgingival plaque clone CS015 and *Mycoplasma salivarium* str. PG20(T), suggesting that seeding of humans by of these species may be from other sources e.g. water, soil or ingested food.

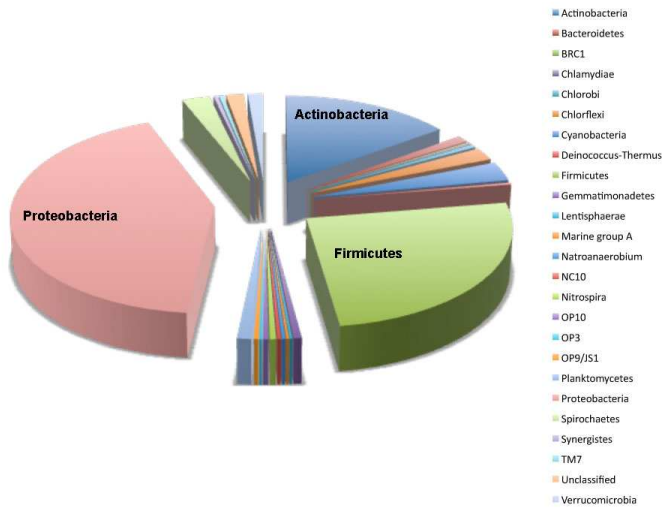

Fig. 7. Phyla detected in both aerosol and asthmatic airway samples. Size of wedge denotes number of taxa detected in that phylum across both sample types.

Conclusion.

This study represents a pilot investigation that took advantage of several on-going studies and as such represents an imperfect study design to address the key questions posed. Nonetheless, examination of seasonal aerosol samples collected in a DOD sponsored study, and airway samples from an NIH-sponsored asthma study, demonstrated several key aspects of aero- and respiratory tract microbiology:

1. The bacterial composition of the air we breathe is influenced by seasonal variation, primarily due to water availability and temperature fluctuations, two of the principal factors that impact bacterial physiology.
2. Aerosol microbiology is distinct at geographically distinct locations (again most likely due to prevailing weather conditions at that site); specific bacterial taxa were detected exclusively in Californian or East coast aerosol samples, suggesting habitation at a specific site may result in distinct microbial exposures.
3. Key phyla in the airway microbiota are inherently sensitive to seasonal variation

and their abundance fluctuates with seasonality, while other phyla appear to be more resilient to these changes.

4. Many of the phyla detected in aerosols in this study are also detected in the gastrointestinal tract of healthy individuals and the respiratory tract of patients with pulmonary disease.
5. A large majority of the taxa detected in aerosols in this study were also detected in the airways of asthmatics, suggesting that inhaled air may act as an inoculum for respiratory mucosa susceptible to microbial colonization.

While we recognize that this pilot study, based on existing samples from on-going studies with specific aims distinct from that of this investigation, does not provide precise answers to a number of questions, it does lay the foundation for future studies to investigate the potential for aerosol microbes to impact pulmonary health. We have demonstrated that diverse bacterial communities exist in aerosols and that locations with higher asthma prevalence have distinct microbial profiles from those with lower prevalence rates. While this data cannot, without further in-depth studies, be concluded to be cause and effect, it does raise the possibility that aerosolized microbes play a role in chronic pulmonary diseases. In healthy individuals recent studies of the lower airways have demonstrated little of no evidence of bacteria [2], this is in stark contrast to the respiratory tracts of patients with chronic airway inflammatory who possess diverse bacterial communities [3, 4]. In this study, we demonstrated a large overlap in bacterial phylogeny detected in both environmental aerosols and asthmatic airways, further supporting the hypothesis that aerosol microbes may play a role in seeding vulnerable airways. Many of the families common to both the respiratory tract of asthmatics and the aerosol samples studies are either known or suspected pathogens, however the factors which govern virulence gene expression in the human host are many and complex, and involve more recently determined factors such as native microbiome composition at the site of pathogen invasion [13]. Hence the detection of an organism at a particular site does not necessarily imply that that organism is active or indeed expressing virulence factors. Thus other studies are

essential to examine the fundamental question of whether inhaled aerosol microbes play a prominent role in pulmonary and, in particular, asthma pathogenesis.

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Glossary of Terms, Abbreviations, and Symbols

16S rRNA PhyloChip: High-density phylogenetic microarray

Hierarchical cluster analysis: Statistical tool to group samples with similar bacterial community composition

COPD: Chronic Obstructive Pulmonary Disease

CF: Cystic fibrosis