Secondary electrospray ionization-mass spectrometry (SESI-MS) breathprinting of multiple bacterial lung pathogens, a mouse model study

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Zhu J, Bean HD, Jiménez-Díaz J, Hill JE. Secondary electrospray ionization-mass spectrometry (SESI-MS) breathprinting of multiple bacterial lung pathogens, a mouse model study. J Appl Physiol 114: 1544–1549, 2013.—Bacterial pneumonia is one of the leading causes of disease-related morbidity and mortality in the world, in part because the diagnostic tools for pneumonia are slow and ineffective. To improve the diagnosis success rates and treatment outcomes for bacterial lung infections, we are exploring the use of secondary electrospray ionization-mass spectrometry (SESI-MS) breath analysis as a rapid, noninvasive method for determining the etiology of lung infections in situ. Using a murine lung infection model, we demonstrate that SESI-MS breathprints can be used to distinguish mice that are infected with one of seven lung pathogens: Haemophilus influenzae, Klebsiella pneumoniae, Legionella pneumophilia, Moraxella catarrhalis, Pseudomonas aeruginosa, Staphylococcus aureus, and Streptococcus pneumoniae, representing the primary causes of bacterial pneumonia worldwide. After applying principal components analysis, we observed that with the first three principal components (primarily comprised of data from 14 peaks), all infections were separable via SESI-MS breathprinting ($P < 0.0001$). Therefore, we have shown the potential of this SESI-MS approach for rapidly detecting and identifying acute bacterial lung infections in situ via breath analysis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study were H. influenzae ATCC 51907, P. aeruginosa PAO1-UW, S. aureus RN450 (courtesy of Prof. G. L. Archer, Virginia Commonwealth University, Richmond, VA), L. pneumophilia ATCC 33152, S. pneumoniae ATCC 6301, M. catarrhalis ATCC 43628, and K. pne-
moniae ATCC 13883. Before the bacteria were inoculated into the mice airways, strains were incubated aerobically in tryptic soy broth (16 h, 37°C; final cell counts >10^8 CFU/ml). After breath collection, the lungs were harvested and homogenized in 1 ml PBS, and lung bacterial cell counts were obtained by plating.

**Mice and microbial airway exposure protocols.** Six- to 8-wk-old male C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME). The protocols for animal infection and respiratory physiology measurements were approved by the Institutional Animal Care and Use Committee, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility at the University of Vermont (Burlington, VT). Overnight cultures of bacteria were measured for optical density, centrifuged at 13,000 g, and resuspended in 40 μl PBS as a negative control. Six mice per group were exposed to 40–60 min.

**Bronchoalveolar lavage fluid: hematology and lung damage assays.** After breath collection, 1 ml of cold PBS with 5% fetal bovine serum (FBS) was instilled into the lungs and the bronchoalveolar lavage fluid (BALF) was collected through the cannula installed previously for ventilation. BALF cells were pelleted and immediately resuspended in the same solution (PBS + 5% FBS). Total cells were counted using an ADVIA cell counter (Bayer, Tarrytown, NY). Then, BALF cells were fixed onto glass slides (2 × 10^4 cells/slide), stained with Hema-3 (Biochemical Sciences, Swedesboro, NJ), and the leukocytes were counted (300/slide) and categorized as macrophages, eosinophils, polymorphonuclear neutrophils (PMNs), or lymphocytes on the basis of characteristic morphology and staining.

In vivo lung tissue damage was determined by measuring lactose dehydrogenase activity (LDH) in BALF samples using the CytoTox 96 NonRadioactive Cytotoxicity Assay (Promega, Madison, WI), according to the manufacturer’s instructions.

**Secondary electrospray ionization–mass spectrometry (SESI-MS) and breath sampling.** SESI-MS breath analysis was performed in positive-ion mode within 1 h of breath collection, as previously described (27, 50) on a modified SCIEX API 3000 mass spectrometer (Concord, ON, Canada; for a detailed schematic of the SESI-MS system, see Ref. 3). The breath sample was introduced into the reaction chamber for 30 s at a flow rate of 3 liters/min, and supplemented with 2 liters/min CO₂ (99.99%) at ambient temperature. Formic acid [0.1% (v/v)] was used as the electrospray solution, delivered at a flow rate of 5 nl/s through a nonconductive silica capillary (40 μm i.d.). The operation voltage was ~3.5 kV, and the declustering, focusing, and entrance potentials for the mass spectrometer were set to 5 V, 350 V, and 2 V, respectively. Spectra were collected for 30 s as an accumulation of 10 scans. The system was flushed with CO₂ between samples until the spectrum returned to background levels.

**Data analysis and statistics.** Analyst 1.4.2 software (Applied Biosystems, Foster City, CA) was used for spectra collection and raw data processing. The mass spectra shown are the average spectra of all replicates in each group.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Infection dose (CFU/lung)</th>
<th>Infection time (h)</th>
<th>Lung harvest bacterial counts (CFU/lung)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moraxella catarrhalis</td>
<td>1.0 × 10^6</td>
<td>3</td>
<td>2.4 × 10^6</td>
<td>7.0 × 10^4</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>1.0 × 10^7</td>
<td>24</td>
<td>7.8 × 10^4</td>
<td>2.2 × 10^5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1.0 × 10^3</td>
<td>24</td>
<td>3.5 × 10^5</td>
<td>5.6 × 10^5</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1.0 × 10^4</td>
<td>24</td>
<td>1.6 × 10^6</td>
<td>1.1 × 10^5</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>5.0 × 10^5</td>
<td>24</td>
<td>2.0 × 10^5</td>
<td>1.1 × 10^6</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>1.0 × 10^3</td>
<td>48</td>
<td>5.4 × 10^5</td>
<td>4.6 × 10^5</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>2.5 × 10^6</td>
<td>48</td>
<td>1.7 × 10^4</td>
<td>2.0 × 10^5</td>
</tr>
</tbody>
</table>

Fig. 1. Total number of polymorphonuclear neutrophils (PMNs) in bronchoalveolar lavage fluid (BALF). Statistical significance determined by t-test (3-h infection) or one-way ANOVA (24- and 48-h infections); ***P < 0.0001, **P < 0.001 compared with the corresponding PBS-treated mice (control) as per Table 1. Values represent mean ± SE of all replicates in each group.
The reproducibility of SESI-MS. For six out of seven infection biological replicate breathprints within a single group to assess the average Spearman correlation coefficients between groups, coupled with high intragroup reproducibility. We calculated the average Spearman correlation coefficients between biological replicate breathprints within a single group to assess the reproducibility of SESI-MS. For six out of seven infection groups and all three PBS control groups, the reproducibility of the breathprints is high, ranging from 0.81 to 0.94 (standard deviation ±0.09). The exception is *M. catarrhalis* (0.64 ± 0.14), possibly because of its quick clearance rate (typically less than 4 h) coupled with the short time scale (3 h) between infecting inoculation and breath measurement (4, 12).

The SESI-MS breathprints for infected mice show unique patterns for each bacterium (Fig. 2). To compare and contrast the details of the breathprint patterns from the seven bacterial species involved in this study, we list the peaks from each breathprint in Table 2. Comparing the presence and absence of peaks across these seven infection groups (Table 2) and the uninfected controls (data not shown), we find that *M. catarrhalis* has two unique peaks (m/z = 54 and 92), *K. pneumoniae* has two unique peaks (m/z = 145 and 183), *S. aureus* has one (m/z = 81), and *S. pneumoniae* has three (m/z = 46, 59, and 74), contributing distinguishing markers in the breathprints for these infections. Beyond the unique peaks for individual species, the intensities of the common peaks in the spectra also carry information, as observed by the patterns generated in Table 2. For example, peak m/z = 61 can be measured from all seven bacterial infections, with intensities varying by an order of magnitude.

**RESULTS**

We employed a murine lung infection model using seven different bacteria, establishing a 3-h infection with *M. catarrhalis*; 24-h infections with *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *S. pneumoniae*; and 48-h infections with *H. influenzae* and *L. pneumophila* (Table 1). Bacterial cell counts from lung homogenates indicate that bacteria are present in the lungs at the time of breath collection. The data also show that there is a clearing of bacteria from the initial lung inoculum, as is expected for these doses and infection times (4, 11, 13, 19, 24). To confirm the establishment of infection, the BALF leukocyte cell count, PMN total count, and LDH activity were measured. We observed that the BALF leukocytes were significantly increased in the infection groups vs. controls in most cases (data not shown), which is consistent with previous mouse infection models for each pathogen in this study (5, 21, 23, 38, 39, 47, 48). PMN infiltration is one of the most important steps during the innate immune response against bacterial infections (35), and we observed that the total PMN count was significantly different from that of the control groups in all instances (Fig. 1), with *P* < 0.001 (*t*-test or one-way ANOVA). Further evidence of infection can be ascertained by the presence of lung damage, measured by extracellular LDH activity in BALF (45). We report here that LDH levels were higher in the BALF of all infected mice compared with uninfected controls (*P* < 0.05), except for *S. pneumoniae*. Taken together, the leukocyte cell counts, total PMN counts, and LDH activity indicate that infections were established for all bacteria.

The utility of SESI-MS breathprinting relies on high intergroup differences between breathprints from different infections, coupled with high intragroup reproducibility. We calculated the average Spearman correlation coefficients between biological replicate breathprints within a single group to assess the reproducibility of SESI-MS. For six out of seven infection
of magnitude ($10^6$ to $10^7$ cps), whereas peaks $m/z = 41$ and 119 vary by two orders of magnitude. The patterns of peak intensities across the breathprint mass range also confer unique information for bacterial identification.

To determine the statistical difference between groups, we performed principal components analysis with the absolute intensities of the breathprint peaks (Fig. 3). Using the first three principal components (PC), accounting for 44.1% of the total variance, all infections were separable via their SESI-MS breathprints ($P < 0.0001$). In addition, all of the infection breathprints are separated from uninfected controls using the SESI-MS intensities of the breathprint peaks (Fig. 3). Using the first three principal components (PC), accounting for 44.1% of the total variance, all infections were separable via their SESI-MS breathprints ($P < 0.0001$). In addition, all of the infection breathprints are separated from uninfected controls using the SESI-MS intensities of the breathprint peaks (Fig. 3).

### DISCUSSION

MS/MS fragmentation of breath volatiles, which can be used for compound identification and peak verification, is a capability that is afforded by SESI-MS, unlike similar mass spectrometry methods such as selected ion flow tube–mass spectrometry (SIFT-MS) and proton transfer reaction–mass spectrometry (PTR-MS) (6, 7, 46). We conducted more than 200 MS/MS product ion scans to obtain peak fragmentation data on the most abundant peaks from each breath sample, then we used NIST 08 MS software to compare the fragmentation patterns between biological replicates and between bacterial groups. We confirmed that all peaks with the same $m/z$ have similar fragmentation patterns (match score $>700$), and therefore should be recognized as the same compound or group of compounds. Comparing our SESI-MS data to previously published breath analyses, seven peaks listed in Table 2 (peaks $m/z = 101, 103, 107,$
121, 129, 143, and 157), which are observed in the breath of the infected mice in our study, could be tentatively assigned as compounds that have been identified by Peters and colleagues (30, 44), whereas standards tests will be needed before these identifications can be confirmed. The studies by Peters et al. examine inflammation markers (i.e., no infection involved), and therefore, these seven peaks in the breath of infected mice may be markers that are host-derived, rather than pathogen metabolites. We hypothesize that portions of the distinguishing patterns in the SESI-MS breathprints for each pathogen are also host-derived, with the immune system mounting bacterium-specific responses to some infections. We are presently conducting experiments to parse apart the bacterium and host contributions to SESI-MS breathprints.

Translating SESI-MS breathprinting from a mouse model to diagnosing human lung infections is an admittedly large step that we aim to take in the near future. The biggest hurdle will be accommodating the high interindividual variability that exists in the human breath volatilome (33). In developing breath-based diagnostics, it will be necessary to address the influences that genetic, environmental, and behavioral factors have over breath volatiles, which will require many more than six subjects per infection group as was used in these mouse experiments. However, the breathprints of the seven different infections were observed to be highly unique and reproducible, even with the small group size used in this study (six mice per group; \( P < 0.0001 \)), demonstrating the incredible amount of information contained in each breathprint and suggesting that it will be possible to overcome the variability we expect to encounter in human breathprints. In addition, it has been shown that highly specific and sensitive breath tests for human lung infections can be developed when multiple breath volatiles are used for diagnosis (37). Because SESI-MS breathprints measure the relative abundances of many breath volatiles simultaneously, we feel that it holds promise for diagnosing human bacterial lung infections in the future.

To the best of our knowledge, this is the first study to compare and contrast the breath volatile biomarkers from lung infections caused by *H. influenzae*, *K. pneumoniae*, *L. pneumophila*, *M. catarrhalis*, *P. aeruginosa*, *S. aureus*, and *S. pneumoniae*. We have demonstrated that SESI-MS breathprinting can be used to distinguish all seven bacterial infections in situ (\( P < 0.0001 \)), providing evidence that SESI-MS can be a powerful tool for the detection and identification of bacterial lung infections using breath analysis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


