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# 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. First published March 21, 2013; doi:10.1152/japplphysiol.00099.2013.—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 pneumophila*, *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.

bacteria; breath analysis; lung infection; SESI-MS; VOC

LOWER RESPIRATORY INFECTIONS, including both community and hospital acquired infections (HAIs), are the leading burden of disease in the world, and the third leading cause of mortality (29). In the United States, ventilator-associated pneumonia is responsible for ~15% of all HAIs, and 36% of HAI-related deaths (22), at an estimated annual cost of $0.78–1.50 billion in this country alone (41). The high morbidity and mortality of pneumonia is due in part to the lack of effective diagnostics. The typical culture-based methods for identifying pneumonia etiologies are slow, requiring days for pathogen identification, with a success rate at ~20% (16). Even with the most sophisticated and thorough molecular-based analyses at in-patient care facilities, nearly half of pneumonia etiologies cannot be identified (14, 16, 49). Treatment choices, such as the administration of antibiotics, should be based on the pathogen causing the pneumonia, but until more accurate and faster diagnostics are developed, treatment decisions will continue to be partly speculative (2).

Molecular diagnostics (e.g., genomic and protein-based methods) are vast improvements over traditional culture-based methods; however, these protocols still rely on recovering pathogen material from the infection site (25). This presents a significant obstacle for diagnosing lower respiratory infections, particularly in young children, because they do not reliably produce sputum for clinical analysis (10, 40). Therefore, rapid, noninvasive methods for determining the etiology of lung infections in situ could significantly improve diagnosis success rates and treatment outcomes for lower respiratory infections. Breath-based diagnostics eliminate the need for sputum production and are under development. For instance, electronic nose sensors have been used to monitor the expired breath gases of ventilated patients and can detect the presence of pneumonia and respiratory tract infections (17, 18). In addition, others have demonstrated that breath may be used to diagnose the cause of lung infections because *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, and *Aspergillus fumigates* infections can be differentiated from uninfected controls using breath analyses (8, 15, 31, 32, 37, 40, 43). However, all of these studies have focused on the presence vs. absence of a single pathogen, or in the case of electronic nose sensors, on the presence vs. absence of disease without etiological data.

The ultimate goal in developing a new diagnostic tool is to identify unknown causes of disease, and diagnosing the etiologies of lung infections directly from breath will require a robust and unique breathprint for each infectious species. We have previously explored the utility of secondary electrospray ionization-mass spectrometry (SESI-MS) for differentiating two common opportunistic lung pathogens in situ in a murine infection model (51). SESI-MS is a technique that can rapidly characterize volatile mixtures, separating the components by their mass-to-charge (*m/z*) ratio, yielding a mass spectral fingerprint of the mixtures (3, 26). We have found that the SESI-MS fingerprints of breath, also known as breathprints, of mice infected with *Staphylococcus aureus* and *P. aeruginosa* are unique and reproducible, and that the breath can be used to differentiate strains of *P. aeruginosa* in situ (51). In the experiments described herein, we aim to further prove the utility of SESI-MS breathprints for diagnosis by analyzing the breath of mice with lung infections caused by one of seven different bacterial lung pathogens: *Haemophilus influenzae*, *Klebsiella pneumoniae, Legionella pneumophila*, *Moraxella catarrhalis*, *P. aeruginosa*, *S. aureus*, and *Streptococcus pneumoniae,* representing the primary causes of bacterial pneumonia worldwide (14, 20, 34, 36, 49). We observe that in addition to markers that may be used to distinguish infected lungs from healthy controls, the SESI-MS breathprints from all seven lung infections are unique, moving the concept of breath-based diagnostics another step closer to practical application.

## 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. pneumophila* ATCC 33152, *S. pneumoniae* ATCC 6301, *M. catarrhalis* ATCC 43628, and *K. pneu-*

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Table 1. *Infection doses and end-point bacterial cell counts, six mice per group were tested in this study*

| Bacteria | Infection dose (CFU/lung) | Infection time (h) | Lung harvest bacterial counts (CFU/lung) | SE |
|---|---|---|---|---|
| *Moraxella catarrhalis* | $1.0 \times 10^8$ | 3 | $2.4 \times 10^6$ | $7.0 \times 10^4$ |
| *Klebsiella pneumoniae* | $1.0 \times 10^7$ | 24 | $7.8 \times 10^4$ | $2.2 \times 10^3$ |
| *Pseudomonas aeruginosa* | $1.0 \times 10^7$ | 24 | $3.5 \times 10^5$ | $5.6 \times 10^3$ |
| *Staphylococcus aureus* | $1.0 \times 10^8$ | 24 | $1.6 \times 10^6$ | $1.1 \times 10^5$ |
| *Streptococcus pneumoniae* | $5.0 \times 10^6$ | 24 | $2.0 \times 10^5$ | $1.1 \times 10^4$ |
| *Haemophilus influenzae* | $1.0 \times 10^8$ | 48 | $5.4 \times 10^5$ | $4.6 \times 10^3$ |
| *Legionella pneumophila* | $2.5 \times 10^6$ | 48 | $1.7 \times 10^4$ | $2.0 \times 10^2$ |

*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 $>10^9$ 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* for 1 min, washed twice with PBS, and resuspended in 40 μl PBS to give the desired concentration of bacteria (listed in Table 1). Mice were briefly anesthetized (isoflurane by inhalation) and infected by oropharyngeal aspiration as described previously (1, 48). Additional mice were exposed to 40 μl PBS as a negative control. Six mice per group were exposed and tests were conducted over several days to ensure data reproducibility.

*Mice ventilation and breath sample collection.* At 3 h, 24 h, or 48 h post infection (Table 1), the mice were anesthetized with pentobarbital and their tracheas were cannulated. The mice were placed on the ventilator and paralyzed with intraperitoneal pancuronium bromide (0.5 mg/kg), and an electrocardiogram was used to monitor heart rate to ensure proper anesthesia. Breath coming out of the ventilator was collected in 5-liter Tedlar bags (SKC, Eighty Four, PA) at 180 breaths/min with a positive end-expiratory pressure of 3 $cmH_2O$ for 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, Terrytown, NY). Then, BALF cells were fixed onto glass slides ($2 \times 10^4$ 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 $CO_2$ (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 $CO_2$ 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

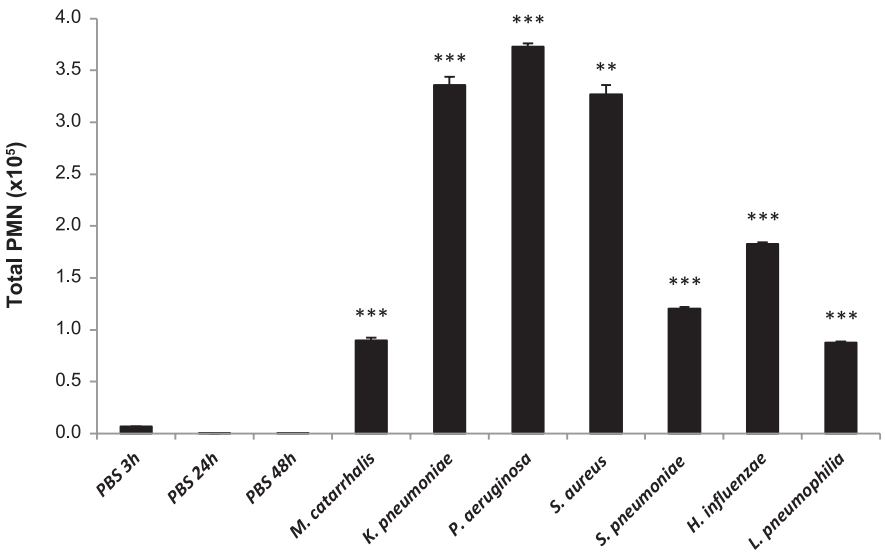


Fig. 1. Total number of polymorphonuclear neutrophils (PMNs) in bronchoalveolar lavage fluid (BALF). Statistical significance determined by *t*-test (3-h infection) or one-way ANOVA (24- and 48-h infections); $***P < 0.0001$, $**P < 0.001$ compared with the corresponding PBS-treated mice (control) as per Table 1. Values represent mean ± SE of all replicates in each group.


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sample replicates in each group. Full scan spectra are blank-subtracted (the blank spectrum is humidified room air collected using the same procedure as for mice breath) and normalized to the peak of greatest intensity. MS/MS fragmentation spectra of some high-abundance peaks in the breath sample were also collected and built into a small database using NIST MS search V 2.0 software (National Institute of Standards and Technology, Gaithersburg, MD). The spectral pattern comparison algorithms of the NIST V 2.0 software were used to assess the similarities between the fragmentation patterns, as previously described (9, 28, 42). For this study, peaks from different breath samples that have fragmentation pattern match scores of 700 or greater (≥70% match) are considered identical peaks (i.e., identical variables) for subsequent principal component analysis, described below.

The statistical significance of total leukocytes, PMN counts from BALF, and LDH activity between infection and control groups were determined by a *t*-test (3-h infection) or one-way ANOVA (24- and 48-h infections) using JMP version 10 (SAS Institute, Cary, NC). To meet the assumption of normal distribution for the ANOVA and *t*-test, total leukocytes and PMN counts were log-transformed before the analysis. SAS version 9.2 (SAS Institute) and JMP version 10 were used to conduct spectral principal component analysis (PCA) on absolute intensity spectra, and to determine the statistical significance of observed PCA score differences. Peaks between 20 and 200 *m/z* and signal-to-noise ratios greater than 2 were used as variables for PCA, while all experimental replicates were used as observations.

## 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 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

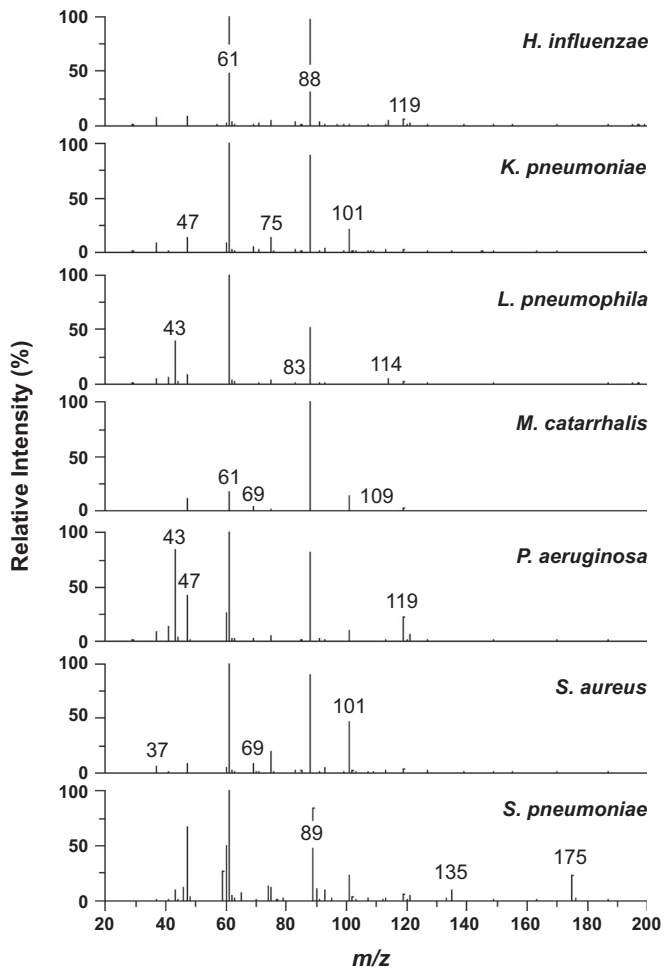


Fig. 2. SESI-MS breathprints from mice with *H. influenzae, K. pneumoniae, L. pneumophila, M. catarrhalis, P. aeruginosa, S. aureus*, or *S. pneumoniae* lung infections. The spectra shown here are representative spectra (average of six replicates from each group).


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Table 2. *Absolute intensities of breathprint peaks from mice infected with one of seven lung pathogens*

| Peaks | MC | KP | SA | PA | SP | HI | LP | Peaks | MC | KP | SA | PA | SP | HI | LP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 27† | ++ | | | ++ | | | | 108 | | +++ | ++ | | | | |
| 29 | ++ | +++ | ++ | +++ | | +++ | +++ | 109 | ++ | +++ | +++ | ++ | | ++ | |
| 33 | | ++ | ++ | + | | | | 110 | + | | | | ++ | | |
| 37* | | ++++ | +++ | +++ | +++ | ++++ | +++ | 111 | | ++ | | ++ | | | |
| 39 | + | | | ++ | | | | 112 | + | | ++ | | +++ | | |
| 41* | ++ | +++ | +++ | ++++ | +++ | ++ | ++++ | 113* | | +++ | +++ | +++ | +++ | +++ | |
| 42* | ++ | | | ++ | | | | 114 | | +++ | | | | +++ | +++ |
| 43* | | | | ++++ | ++++ | | ++++ | 119† | +++ | +++ | +++ | ++++ | ++++ | +++ | +++ |
| 44* | | | | +++ | +++ | | +++ | 120 | ++ | | | +++ | +++ | +++ | |
| 46 | | | | | ++++ | | | 121 | | | | +++ | ++++ | +++ | |
| 47 | ++++ | ++++ | +++ | ++++ | +++++ | ++++ | ++++ | 123 | | | | | | | ++ |
| 48 | ++ | ++ | | +++ | +++ | ++ | | 125 | | ++ | ++ | | | ++ | |
| 54 | ++ | | | | | | | 127* | ++ | +++ | +++ | +++ | ++ | +++ | +++ |
| 59 | | | | | ++++ | | | 129 | | ++ | | | | | |
| 60 | | ++++ | +++ | ++++ | +++++ | +++ | | 133 | | | ++ | | +++ | | |
| 61 | ++++ | +++++ | +++++ | ++++ | +++++ | +++++ | +++++ | 135 | | +++ | | | ++++ | | |
| 62 | ++ | +++ | +++ | +++ | ++++ | +++ | +++ | 136 | | ++ | ++ | | ++ | | |
| 63 | | +++ | +++ | +++ | +++ | +++ | +++ | 139 | | +++ | +++ | ++ | | +++ | ++ |
| 65 | + | | | ++ | ++++ | | | 141 | | | | ++ | | | |
| 69 | +++ | +++ | +++ | +++ | | +++ | ++ | 143 | | +++ | ++ | +++ | | | |
| 70 | ++ | ++ | +++ | ++ | +++ | | | 145 | | +++ | | | | | |
| 71 | | +++ | ++ | | | +++ | ++ | 147 | + | | | ++ | | | |
| 74 | | | | | ++++ | | | 149 | ++ | +++ | +++ | +++ | +++ | +++ | ++ |
| 75† | +++ | ++++ | ++++ | +++ | ++++ | +++ | +++ | 150 | + | | ++ | ++ | | | |
| 76 | ++ | +++ | +++ | + | | ++ | | 151 | | | ++ | | | | |
| 77 | ++ | ++ | ++ | ++ | +++ | ++ | | 152 | | | | + | | | |
| 79 | | | | | +++ | | | 153 | | | | ++ | | | |
| 81 | | | ++ | | | | | 155 | | ++ | ++ | ++ | | ++ | ++ |
| 83 | | +++ | +++ | ++ | | +++ | +++ | 157 | | | ++ | ++ | | | |
| 84 | | | ++ | + | | ++ | | 159 | | +++ | | ++ | | ++ | |
| 85 | + | +++ | +++ | +++ | | +++ | ++ | 161 | | | ++ | | | | |
| 88* | +++++ | +++++ | ++++ | ++++ | | +++++ | ++++ | 163 | | +++ | | | +++ | | |
| 89* | | | | | +++++ | | | 165 | | | ++ | | | | |
| 90 | | | | | ++++ | | | 167 | | | ++ | ++ | | ++ | |
| 91 | ++ | +++ | +++ | +++ | +++ | +++ | +++ | 170 | | +++ | ++ | +++ | | +++ | ++ |
| 92 | ++ | | | | | | | 175 | | | | | ++++ | | |
| 93† | ++ | +++ | +++ | +++ | ++++ | +++ | +++ | 176 | | | | | +++ | | |
| 95 | | | | | +++ | | | 177 | | ++ | ++ | ++ | | ++ | |
| 97 | | | ++ | | | | +++ | 183 | | ++ | | | | | |
| 99* | ++ | +++ | +++ | ++ | | +++ | ++ | 185 | | ++ | | ++ | | ++ | |
| 100 | | ++ | ++ | | | | | 187 | | +++ | ++ | +++ | +++ | +++ | +++ |
| 101 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | | 191 | | ++ | | | | ++ | |
| 102 | | +++ | +++ | | ++++ | | | 195 | | | | ++ | | ++ | ++ |
| 103 | | +++ | +++ | ++ | +++ | ++ | | 197 | | ++ | | ++ | | +++ | ++ |
| 107 | | +++ | +++ | ++ | +++ | +++ | | 199 | | +++ | | | | +++ | |

MC, *M. catarrhalis;* KP, *K. pneumoniae;* SA, *S. aureus;* PA, *P. aeruginosa;* SP, *S. pneumoniae;* HI, *H. influenzae;* LP, *L. pneumophila.* Order of peak intensity (cps): $+10^3$; $++10^4$; $+++10^5$; $++++10^6$; $+++++10^7$. Peaks that have loadings with an absolute value >0.7 on principal component 1 (*) and 2 (†).

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 first three PCs. Examining the PC loadings for each individual peak, where the closer the loading value is to 1, the higher the peak-to-PC influence, we found that 10 peaks have strong contributions (absolute PC loadings >0.7) to PC1, four peaks have strong contribution to PC2 (Table 2).

## 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,


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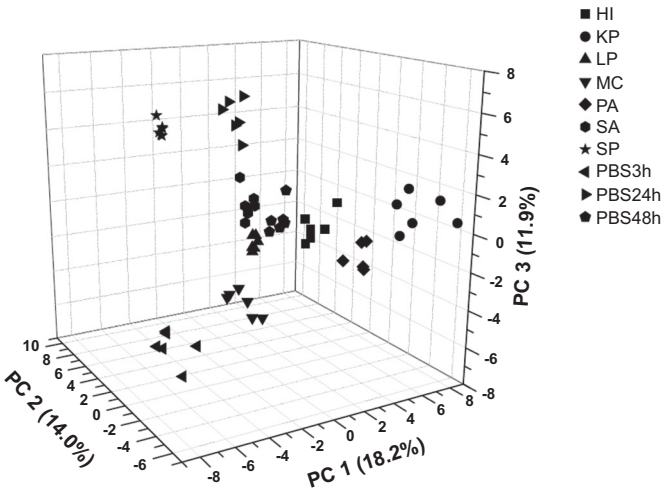


Fig. 3. Principal components analysis of spectral breathprints from mice with lung infections caused by *H. influenzae* (HI), *K. pneumoniae* (KP), *L. pneumophila* (LP), *M. catarrhalis* (MC), *P. aeruginosa* (PA), *S. aureus* (SA), *S. pneumoniae* (SP), and three PBS control groups. Six mice per group were tested in this study.

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.

Transitioning 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

Author contributions: J.Z., H.D.B., and J.E.H. conception and design of research; J.Z., H.D.B., and J.J.-D. performed experiments; J.Z. and J.J.-D. analyzed data; J.Z., H.D.B., and J.J.-D. interpreted results of experiments; J.Z. prepared figures; J.Z. and J.J.-D. drafted manuscript; J.Z., H.D.B., J.J.-D., and J.E.H. edited and revised manuscript; J.Z., H.D.B., J.J.-D., and J.E.H. approved final version of manuscript.

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