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Robust detection of *P. aeruginosa* and *S. aureus* acute lung infections by secondary electrospray ionization-mass spectrometry (SESI-MS) breathprinting: From initial infection to clearance

Jiangjiang Zhu[1], **Jaime Jiménez-Díaz**[1], **Heather D. Bean**[1], **Nirav A. Dapthary**[2], **Minara I. Aliyeva**[2], **Lennart K. A. Lundblad**[2], and **Jane E. Hill**[1]

[1]School of Engineering, University of Vermont, Burlington, VT 05405, USA

[2]The Vermont Lung Center, Department of Medicine, University of Vermont College of Medicine, Burlington, VT 05405, USA

Abstract

Before breath-based diagnostics for lung infections can be implemented in the clinic, it is necessary to understand how the breath volatiles change during the course of infection, and, ideally, to identify a core set of breath markers that can be used to identify the pathogen at any point during the infection. In the study presented here, we use secondary electrospray ionization-mass spectrometry (SESI-MS) to characterize the breathprint of *P. aeruginosa* and *S. aureus* lung infections in a murine model over a period of 120 h, with a total of 86 mice in the study. Using partial least squares-discriminant analysis (PLS-DA) to evaluate the time-course data, we were able to show that SESI-MS breathprinting can be used to robustly classify acute *P. aeruginosa* and *S. aureus* mouse lung infections at any time during the 120 h infection/clearance process. The variable importance plot from PLS indicates that multiple peaks from the SESI-MS breathprints are required for discriminating the bacterial infections. Therefore, by utilizing the entire breathprint rather than single biomarkers, infectious agents can be diagnosed by SESI-MS independent of when during the infection breath is tested.

Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are two problematic opportunistic pathogens causing lung infections in various diseases such as ventilator-associated pneumonia, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) [1-3]. Current plate-based cultivation or PCR approaches for detecting *P. aeruginosa* and *S. aureus* infections require the isolation of bacteria or their genetic material, respectively. This may necessitate difficult or invasive sample collection methods (e.g., sputum induction or bronchoalveolar lavage), which can be challenging for the very young or the elderly, and ultimately under-sample the bacterial population in the patient's lung. If bacterial sample collection is successful, the diagnosis is slow, taking hours to days [4, 5]. Therefore, the

Correspondence and requests for reprints should be addressed to Jane E. Hill, Ph. D., School of Engineering, University of Vermont, Burlington, VT 05405, USA, Jane.hill@uvm.edu, Tel: 802-656-9854.

development of a non-invasive, sensitive, accurate, and rapid detection method for early identification of lung infections would be of great utility to clinicians and patients.

During the past decade, detecting and monitoring the breath volatiles of patients has been advanced as a diagnostic tool for bacterial lung infections [6-10]. Our own work using SESI-MS has demonstrated that bacteria produce unique volatile signatures that can be used for species identification [11, 12]; in a murine model, *Pseudomonas aeruginosa* and *Staphylococcus aureus* lung infections produce robust breath volatile fingerprints (or breathprints) at 24 h post-infection, which can be used to differentiate the infecting strain [13]. SESI-MS (also known as extractive electrospray ionization-mass spectrometry, EESI-MS) [11, 12, 14-17], is a direct gas sampling analytical tool that can rapidly, and in real-time, characterize volatile mixtures based on the mass-to-charge ratios (*m/z*) of the chemical constituents. In various applications such as the detection of explosives, drug vapor, and breath volatiles, SESI-MS has detection sensitivities as low as pats-per-trillion without the need for any sample preparation [18-20], which gives it the potential for high-throughput sample analysis.

While the progress that we and others have made toward breath analysis is promising, there are very limited data available for the variation of breath volatiles over the course of an acute infection – from initial infection to clearance. As Thorn and colleagues conclude in their latest review of microbial volatiles [21], time course analysis may be one of the most under-researched areas in the clinical application of microbial volatiles. Understanding how breath volatiles change over time through the infection and healing process may provide additional value to breath-based diagnostics, such as being able to track the success or failure of infection treatment. In addition, identifying any breathprint information that is conserved for the entire acute bacterial infection makes it possible to create a diagnostic method that will succeed whether the patient's breath is analyzed on the first day or fourth day of an infection. Hence, the goal of this study was to establish a murine infection and clearance model of acute *Pseudomonas aeruginosa* and *Staphylococcus aureus* lung infections, and determine if the SESI-MS breathprints from these infections are robustly distinguishable, independent of infection duration, for up to 120 h. Our overarching hypothesis is that while some portion of SESI-MS breathprints of the infection groups changes during the course of infection and clearance, a core set of breathprint markers for each treatment group persists over time, making it possible to distinguish between *P. aeruginosa*, *S. aureus*, or uninfected controls, independent of the length of the infection.

2. Materials and method

2.1 Bacterial strains and growth condition

Pseudomonas aeruginosa PAO1-UW and *Staphylococcus aureus* RN450 (courtesy of Professor G. L. Archer, Virginia Commonwealth University) were cultured aerobically in 5 mL tryptic soy broth (TSB; 16 h; 37°C; final cell counts $>10^9$ CFU/mL) before the bacteria were inoculated into the mice airways. After ventilation and breath collection, the mice lungs were harvested and homogenized, and the lung bacterial cell counts were obtained by plating on *Pseudomonas* isolation agar or Chapman stone medium (BD Diagnostics, Franklin Lakes, NJ, USA).

2.2 Mice and microbial airway exposure protocol

Six- to eight-week-old male C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME). The animal care protocol, animal infection and respiratory physiology measurements protocol, and microbial airway exposure protocol applied in this study are the same as previously described [22]. Additional groups of mice were exposed to 40 μL PBS as a negative control for each time point. Five mice per group were exposed and tests were conducted on different days to measure data reproducibility.

2.3 Mice ventilation and breath sample collection

At six time points (6 h, 12 h, 24 h, 48 h, 72 h, and 120 h) after the infection or control treatments, groups of mice were anesthetized with pentobarbital and their tracheas cannulated. The mice were placed on the ventilator (Flexivent, SCIREQ, Montreal, QC, Canada), paralyzed with intraperitoneal pancuronium bromide (0.5 mg/kg), and an ECG was applied to monitor heart rate to ensure proper anesthesia. Breath from the ventilator was collected in 5 L Tedlar bags (SKC, Eighty Four, PA) at 180 breaths/min with a positive end-expiratory pressure (PEEP) of 3 cm H_2O for 40-60 min. Breath samples were analyzed within one hour of collection by SESI-MS in positive ion mode.

2.4 Bronchoalveolar lavage fluid: Hematology and lung damage assays

After the ventilation process, 1 mL of cold PBS with 5% Fetal Bovine Serum (FBS) was injected into the lungs and the bronchoalveolar lavage fluid (BALF) was collected through the cannula that was installed previously for ventilation. BALF cells were enumerated using an ADVIA cell counter (Bayer, Terrytown, NY) and were then fixed onto glass slides (2×10^4 cells/slide), stained with Hema-3 (Biochemical Sciences, Swedesboro, NJ), then categorized and counted (300/slide) as macrophages, eosinophils, neutrophils, or lymphocytes based on characteristic morphology and staining. Lactose dehydrogenase activity (LDH) in BALF samples was measured to determine *in vivo* lung tissue damage (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI, USA).

2.5 Secondary electrospray ionization mass spectrometry (SESI-MS) for breath analysis

SESI-MS breath analysis and measurement was performed as previously described [12, 16]. Briefly, a stainless steel SESI reaction chamber is used to replace the original electrospray ionization source on a commercially available API 3000 mass spectrometer (SCIEX, Concord, ON, Canada; for a detailed schematic of the SESI-MS system, please see reference [11]). A mechanical pump was used to drive a gas flow of 5L/min to pass through the SESI-MS reaction chamber. The breath sample was introduced into the reaction chamber for 30 s at a flow rate of 3 L/min, supplemented with 2 L/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 non-conductive silica capillary (40 μm ID) with a sharpened needle tip. The electrospray operation voltage was ~ 3.5 kV. The declustering, focusing, and entrance potentials for the mass spectrometer were set to 5 V, 350 V, and 2 V, respectively, for efficient molecular ion generation. 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.

2.6 Data analysis and statistics

The statistical significance of total leukocytes, PMN counts from BAL and LDH activity between infection and control groups were determined by two-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference test. In order to meet the assumption of normal distribution for the ANOVA and t-test, total leukocytes and PMN counts were log transformed before the analysis.

Spectra collection and raw data processing were conducted using Analyst 1.4.2 software (Applied Biosystems, Foster City, CA). Average spectra of all sample replicates in each group are shown in each figure as representative spectra. 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. JMP version 10 (SAS Institute Inc., Cary, NC, USA) was used in this study for all statistical analyses. In order to ensure the reproducibility of experimental results, Spearman correlation coefficients were calculated to compare the similarity of breathprint profiles from each treatment group at each time point.

Partial least squares-discriminant analysis (PLS-DA) was conducted for group classification using spectral intensity data from the breathprints. Peaks between 20 and 200 *m/z* (mass-to-charge ratio) and signal-to-noise ratios greater than two were used as observable variables for PLS-DA in this study. We took all biological replicates into consideration for PLSDA, using leave-one-out cross validation to optimize the results. The first two PLS factors were selected for further data analysis as they explain the largest percentage of the variation (32.13 %) and provide statistically-significant separation of all three groups ($p<0.0001$). Principal components analyses were conducted to evaluate the treatment classification based on a subset of SESI-MS peaks, using peaks in the first two PLS factors that have variable importance plot values greater than 0.8 in the first PCA analysis, and greater than 1.5 in the second. To validate the utility of SESI-MS breathprints in classifying unknown breath samples, two rounds of ten times PLS-DA cross validation tests were performed, first using 90% of randomly selected samples as a training set and the remaining 10% of samples as a testing set, and then using 70% of randomly selected samples as a training set and the remaining 30% of samples as a testing set. The correct classification rates were calculated as the number of correctly classified samples divided by number of samples that were included in the testing set.

Results

1. Murine immunological response and bacterial cell counts during acute bacterial infections and clearance

To confirm the establishment of infection and to track bacterial clearance, we counted the total leukocytes, the total polymorphonuclear neutrophils (PMNs), and measured the lactate dehydrogenase (LDH) activity in bronchoalveolar lavage fluid (BALF) for all mice at each of the six infection and control time points (6, 12, 24, 48, 72, and 120 h). We observed that the total PMNs, the most important leukocyte implicated in innate immune response to bacterial infections [23], was increased in the *P. aeruginosa* and *S. aureus* infection groups

versus controls at all these six time points (Fig. 1), and with statistical significance ($p < 0.05$) for the time points between 12 and 72 h. As can be seen in Fig. 1, total leukocyte counts in BALF and the LDH activity also indicate that on-going bacterial lung infections exist.

We also determined bacterial cell counts from post-breath-collection lung homogenates for all of the mice in this study (Fig. S1). For both the *P. aeruginosa* and *S. aureus* infections, some mice cleared the bacteria slower than others, yielding two measurable bacterial cell counts at 120 h for *P. aeruginosa*, and at 72 h and 120 h for *S. aureus*. The bacterial counts, taken with the total leukocyte PMNs, and LDH activity data indicate that bacterial lung infections were initially established in the test groups, and although the course of the infections were not uniform across the test groups, there was a general trend toward clearance of the acute infections over 120 h.

2. SESI-MS breathprints can be used to robustly distinguish acute *P. aeruginosa* and *S. aureus* lung infections at all time points from 6–120 h

We hypothesized that the SESI-MS breathprints of the infection groups would change during the course of infection and clearance, but that a core set of breathprint markers for each treatment group would persist over time, making it possible to distinguish between *P. aeruginosa*, *S. aureus*, or uninfected controls, independent of the length of the infection (up to 120 h). The average SESI-MS breathprints from *P. aeruginosa* infections and *S. aureus* infections at each of the six time points are shown in Fig. 2 and 3, and the breathprints within each treatment and time point group are reproducible, as calculated by Spearman correlation coefficients (*P. aeruginosa*: 0.85 – 0.93, SD < 0.12; *S. aureus*: 0.84 – 0.91, SD < 0.05). By visual examination, the breathprints for each bacterial lung infection change noticeably from 6 – 120 h, and there is not an obvious set of peaks that can be used to distinguish between *P. aeruginosa* and *S. aureus* at all time points. In order to identify the commonalities in the SESI-MS breathprint for each test group, we employed partial least squares-discriminant analysis (PLS-DA), which is a supervised classification method that is based on the well-known PLS regression model. PLS is a supervised latent variable-based model that finds a linear model describing some predicted variables in terms of a set of observable variables. In this study, the observable variables (the SESI-MS peak intensities) were compressed into a few latent variables, called PLS factors, and then discriminant analyses were applied using these factors to predict the classification (the bacterial species that infected the mice) of each tested sample [24]. As can be seen in Fig. 4, the three test groups are clearly separated based on their breathprints using only the first two PLS factors (32.13% variation explained, $p < 0.0001$), and all of the replicates for any infection group, regardless of the time point, can be clustered.

The results from the PLS-DA suggest that there are a subset of peaks that were consistently detectable during the 120 h time course of each infection (or control), driving the clustering of each group. Therefore, the contributions of each individual peak to the separation of the groups were examined and are reported in the variable importance plot (VIP; Fig. S2). Using the common 0.8 VIP threshold (a variable having a value of 0.8 or greater is considered a significant contributor to the model) we have 69 breathprint peaks that are primarily

separating the *P. aeruginosa*, *S. aureus*, and uninfected PBS groups. Examining the presence and absence of some of these peaks across the six time points (Table 1), we find that there are 12 peaks, *m/z* = 41, 47, 55, 69, 75, 85, 91, 93, 101, 103 and 149, that were consistently detectable at all six time points for *P. aeruginosa* infections and with VIP > 0.8 [25]. There are also 15 peaks that were measurable in five out of six time points, and 12 peaks that were measurable in four out of six time points (Table 1). Similarly, examining the presence and absence of peaks from *S. aureus*-infected mice breath across the six time points, we find that there are three peaks, *m/z* = 41, 47 and 61, that were always detectable. There are also 10 peaks that were measurable in five out of six time points, and 5 peaks that were measurable in four out of six time points (Table 1). Few of these peaks are unique to either *P. aeruginosa* or *S. aureus*, indicating that it is not the presence vs. absence of these peaks that drive the separation of the SESI-MS breathprints by PLS-DA, but rather it is the difference in peak intensities between the groups that strongly drive the model.

To further validate that SESI-MS breathprints can be used to classify the different treatment groups, two validation processes were utilized in this study. First, we performed two rounds of ten times PLS-DA cross validation tests. When using 90% of the samples as the training set and 10% of the samples as the testing set, we observed a 100% correct classification rate. When using 70% of the samples as the training set and 30% of the samples as the testing set, we observed correct classification for 96 % of the test samples (Table S1). After this validation of the usefulness of the global profile, we extracted two subsets of the SESI-MS breathprint peaks that have strong contributions to the PLS-DA model to apply to the unsupervised principal components analysis (PCA). Based on different published VIP threshold criteria [25, 26]), we performed PCA analysis using peaks with VIP > 0.8, and again using the smaller set of peaks with VIP > 1.5. For the PCA using the peaks with VIP > 0.8, the group separation can be achieved with first three principal components (p-values <0.0001, 47.2% variation covered). Using peaks with VIP > 1.5 for the second PCA, even better separation was observed (p-value <0.0001, Figure S3), and 58.4% variation were covered with the first three principal components; whereas the global profile (PCA using every peak detected by SESI-MS) failed to provide statistically-significant separations.

Discussion

Clinical breath-based diagnostics for lung infections must be able to account for the intra-individual changes to the breath volatilome that will occur during the course of infection. In this study we have shown that SESI-MS breathprint analysis, combined with PLS-DA, is able to correctly classify acute *P. aeruginosa* and *S. aureus* mouse lung infections from six time points over a 120 h period. The separation of the treatment groups is independent of time, suggesting that infections could be correctly diagnosed at any point in the illness, from early infection through to the beginning of clearance. In addition, breath tests must overcome the high inter-individual differences in human breath that arise from factors such as diet, environment, gut and respiratory tract microbes, and underlying diseases. While the most common approach to creating breath-based diagnostics has been to identify one or two volatile biomarkers of disease [27, 28], Robroeks and colleagues have demonstrated that the specificity and selectivity of diagnostics increases with the inclusion of more breath volatiles [6]. The variable importance plot from PLS indicates that separation of the three treatment

groups by SESI-MS relies on a large portion of the breathprint data, suggesting that our breathprinting approach may be robust enough to overcome the variation in the human breath volatilome. It is not necessary, however, to identify the source of the SESI-MS peaks in order to use them for diagnosis. Similar to electronic nose patterns, which do not contain information on compound ID [29-31], the SESI-MS breathprint pattern can be used to correctly classify the etiology of a bacterial lung infection.

The distinguishing characteristics of breathprints from lung infections likely arise from a combination of bacterial metabolites and the host volatiles that are related to infection and inflammation. The relative contributions of host and pathogen metabolomes to the breathprint may change over the course of the infection, and may also carry information that could be used to predict the outcome of infections. For example, in both the *P. aeruginosa* and the *S. aureus* infection groups there were mice who cleared the infection by the third day, and others that still had quantifiable bacterial cells in their lungs on the fifth day. Using breathprints, it may be possible to identify early in the infection which mice would quickly clear the infection, and which mice would struggle to do so. In human patients, this type of information could be used to prescribe antibiotics more judiciously, reserving them for the patients who will not be able to clear the infection on their own. The SESI-MS technology provides a new, broadly applicable, nearly real-time metabolomics tool for scientists and clinicians in the area of infectious disease diagnostics. To that end, we are utilizing SESI-MS to further understand host and pathogen contributions to the diagnostic breathprint.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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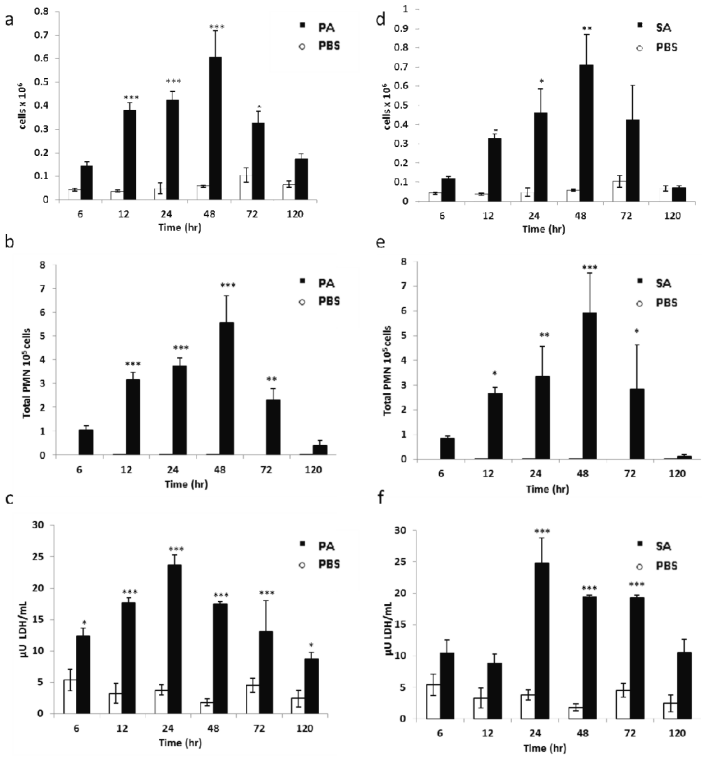

Figure 1.

White blood cell numbers (panels a and d), total cell counts of polymorphonuclear neutrophils (PMNs; panels b and e), and lactose dehydrogenase (LDH) activity (panels c and f) measured from mouse bronchoalveolar lavage fluid (BALF) samples collected after 6, 12, 24, 48, 72 and 120 h post-exposure to *Pseudomonas aeruginosa* (PA) and *S. aureus* (SA). Statistical significance determined by two-way ANOVA followed by Tukey's Honestly Significant Difference test;***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$ compared to the appropriate PBS treated mice (control) at each exposure time (6, 12, 24, 48, 72 and 120 h). Values represent the mean ± SEM of at least five mice per group.

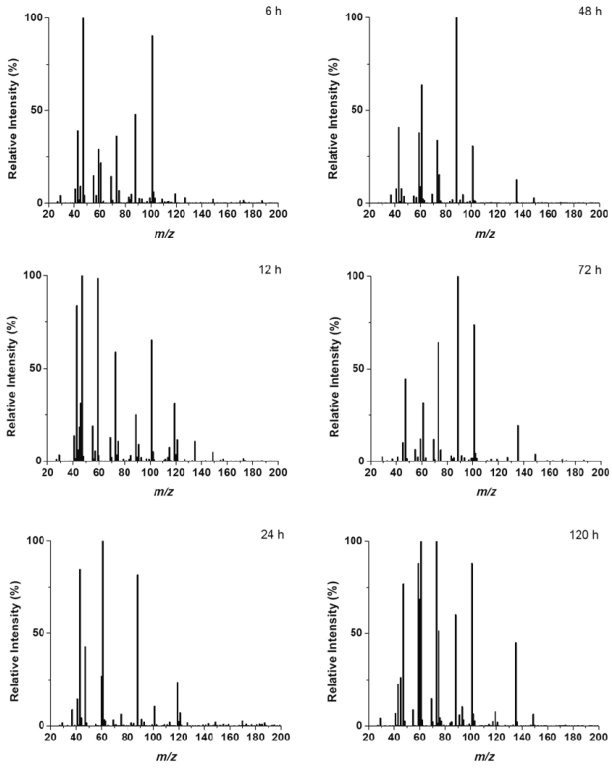

Figure 2.

SESI-MS spectra breathprints of *P. aeruginosa*-infected mice, as they change over 120 h. Each spectrum is the average breathprint from five biological replicates.

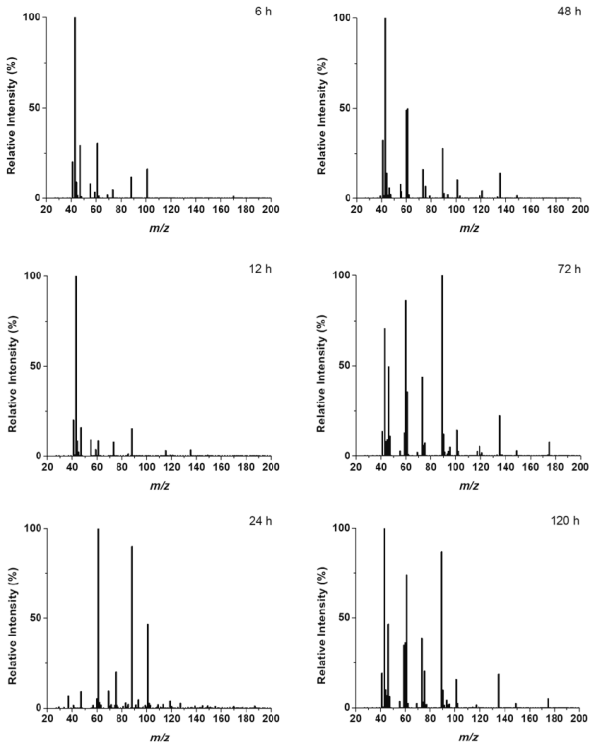

Figure 3.

SESI-MS breathpri ints of *S. aureus*-infected mice, as they change ovver 120 h. Each spectrum is the average breathprint from five biological replicates.

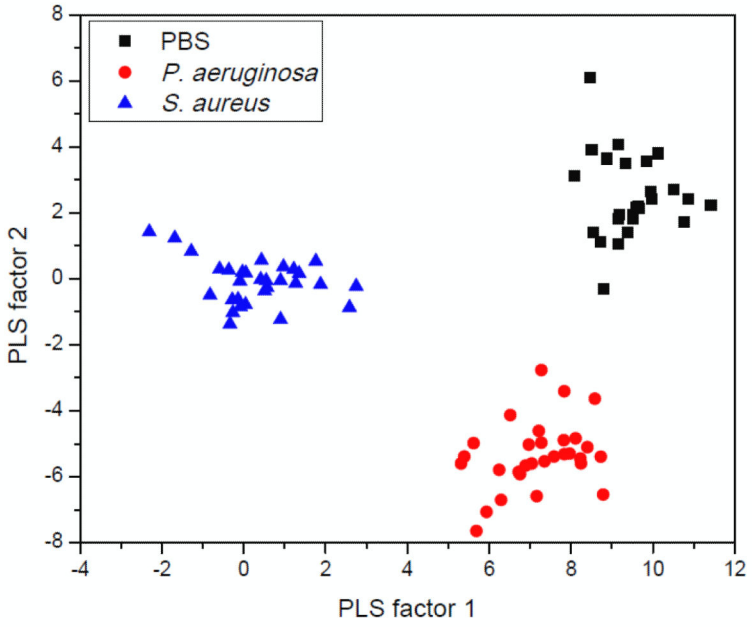

Figure 4.

Discriminant analysis using prediction formulae from partial least squares regression for the separation of breathprints from mice with *P. aeruginosa* and *S. aureus* lung infections, or the uninfected PBS controls. The first two PLS factors explain the largest percentage of the variation (32.13 %), and provide statistically-significant separation of all three groups ($p<0.0001$). All replicates for the six time points for each group were included, resulting in 86 biological replicates in the analysis.

Table 1

Presence (with symbol *)/absence of breathprint peaks from mice with either *P. aeruginosa* or *S. aureus* infections at each of the six time points. Only peaks (*m/z*) that are measurable at four or more time points for one of the two infection groups are listed here.

peaks (m/z)	*P. aeruginosa*						*S. aureus*					
	6 h	12 h	24 h	48 h	72 h	120 h	6 h	12 h	24 h	48 h	72 h	120 h
27	*	*	*			*						
29	*	*	*		*	*						
41	*	*	*	*	*	*	*	*	*	*	*	*
43	*	*	*	*		*	*	*		*	*	*
44	*	*	*	*			*	*		*	*	*
45	*	*		*	*	*	*	*		*	*	*
47	*	*	*	*	*	*	*	*	*	*	*	*
48	*	*	*		*	*	*					
55	*	*	*	*	*	*	*	*		*	*	*
57	*	*		*	*							
59	*	*		*	*	*	*	*			*	*
60		*	*	*		*			*	*	*	*
61	*		*	*	*	*	*	*	*	*	*	*
62	*		*	*	*	*	*		*	*	*	*
63	*		*	*	*				*			
69	*	*	*	*	*	*	*		*		*	*
70	*	*	*		*	*			*			
73	*	*		*	*	*	*	*		*	*	*
75	*	*	*	*	*	*			*	*	*	*
83	*		*	*	*				*			
84	*	*	*		*	*			*			
85	*	*	*	*	*	*		*	*			
88	*		*	*	*	*	*	*	*			
91	*	*	*	*	*	*			*		*	*
93	*	*	*	*	*	*			*	*	*	*
97	*	*		*	*	*						