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Note

Identifying methicillin-resistant *Staphylococcus aureus* (MRSA) lung infections in mice via breath analysis using secondary electrospray ionization-mass spectrometry (SESI-MS)

Heather D Bean[1,3], Jiangjiang Zhu[2,3], Jackson C Sengle[1] and Jane E Hill[1]

[1] Thayer School of Engineering, Dartmouth College, 14 Engineering Drive, Hanover, NH 03755, USA
[2] Northwest Metabolomics Research Center, University of Washington School of Medicine, 850 Republican Street, Room S140, Seattle, WA 98109, USA

E-mail: jane.hill@dartmouth.edu

Abstract

Invasive methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a serious health threat, causing an estimated 11 000 deaths per year in the United States. MRSA pneumonias account for 16% of invasive infections, and can be difficult to detect as the current state-of-the-art diagnostics require that bacterial DNA is recovered from the infection site. Because 60% of patients with invasive infections die within 7 d of culturing positive for MRSA, earlier detection of the pathogen may significantly reduce mortality. We aim to develop breath-based diagnostics that can detect *Staphylococcal* lung infections rapidly and non-invasively, and discriminate MRSA and methicillin-sensitive *S. aureus* (MSSA), *in situ*. Using a murine lung infection model, we have demonstrated that secondary electrospray ionization-mass spectrometry (SESI-MS) breathprinting can be used to robustly identify isogenic strains of MRSA and MSSA in the lung 24 h after bacterial inoculation. Principal components analysis (PCA) separates MRSA and MSSA breathprints using only the first component ($p < 0.001$). The predominant separation in the PCA is driven by shared peaks, low-abundance peaks, and rare peaks, supporting the use of biomarker panels to enhance the sensitivity and specificity of breath-based diagnostics.

Keywords: MRSA, lung infections, diagnostics, SESI-MS, breath

S Online supplementary data available from stacks.iop.org/JBR/8/041001/mmedia

(Some figures may appear in colour only in the online journal)

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[3] These authors contributed equally to this work.

1. Introduction

In 2013 the Centers for Disease Control and Prevention (CDC) listed methicillin-resistant *S. aureus* (MRSA) as a serious antibiotic-resistant microbial threat in the United States, which is the second-highest threat level used by the CDC [1]. Although MRSA typically causes skin infections, there were an estimated 80 000 invasive MRSA infections in the US in 2011, resulting in approximately 11 000 deaths [2]. Pneumonias (hospital-associated or community-acquired) represent 16% of the invasive MRSA infections annually [2, 3], and MRSA is also a growing threat in chronic lung infections, such as those associated with cystic fibrosis (CF) [3, 4]. Not only are chronic MRSA infections becoming more prevalent in CF, but co-infections of MRSA and *P. aeruginosa* are correlated with more rapid lung function decline than those caused by methicillin-sensitive *S. aureus* (MSSA) co-infections with *P. aeruginosa* [5, 6]. Overall, MRSA infections cost the US economy billions of dollars annually in direct costs alone [7–11], and are correlated with increased morbidity [9, 12, 13] and short-term and long-term mortality [2, 10–15].

In acute invasive MRSA infections, 60% of patients die within 7 d of culturing positive for the pathogen [2], highlighting the need for very sensitive and rapid diagnostics that enable early detection [3]. Over the past several years, new polymerase chain reaction (PCR) methods have been developed that can identify MRSA in clinical specimens within a few hours [16, 17], which is a significant improvement over previous culture-based methods that took days to produce a diagnosis. However, PCR-based diagnostics require samples of bacterial DNA from the infection site [17], which is difficult to obtain from lower respiratory tract infections of some patients–particularly children [18]. Therefore, the driving force behind our work is to develop rapid and sensitive mass spectrometry-based diagnostics that can detect bacterial and host metabolites arising from lower respiratory tract infections, and exhaled on the patient's breath. We have demonstrated previously that secondary electrospray ionization-mass spectrometry (SESI-MS) breathprinting can be used to identify seven different genera of lung pathogens *in situ*, including *S. aureus* [19], and we have observed *in vitro* that we can differentiate two strains of *P. aeruginosa* using SESI-MS [20]. Two studies, by others, used gas chromatography-mass spectrometry (GC-MS) to demonstrate that non-isogenic MSSA and MRSA strains produce different suites of volatiles *in vitro* [21, 22]. Based on these *in vitro* results, and on our previous *in vitro* and *in vivo* SESI-MS analyses, we hypothesized that we would be able to discriminate MRSA and MSSA lung infections *in vivo* using SESI-MS breathprinting.

In the study described herein we tested two isogenic strains of *S. aureus*—RN450 (MSSA) and 450M (MRSA), differing only by the presence of the Staphylococcal Cassette Chromosome *mec* (SCC*mec* [23]) Type I in the latter—in a murine lung infection model. We found that the SESI-MS breathprints of mice with MRSA and MSSA lung infections, unchallenged by antibiotics, are different 24 h after initial pathogen exposure, and that principal components analysis (PCA) will robustly separate the MRSA and MSSA breathprints using only the first component ($p < 0.001$). Evaluating the features of the breathprints that classify the infection groups in PCA, we found that the peaks driving separation are just as likely to be shared by both MRSA and MSSA infections as they are to be unique to one strain. In addition, many of the high-loading score peaks that are unique to an infection type appear only rarely amongst the biological replicates in the group. These findings support the use of biomarker panels that include shared peaks, rather than single compounds that are unique to an infection, to identify clinically important phenotypes, such as antibiotic resistance, using breath-based diagnostics.

2. Materials and methods

2.1. Bacteria, airway exposure, sample collection and analysis

The isogenic strains of *S. aureus* used in this study were *S. aureus* RN450 (methicillin-sensitive; MSSA) and *S. aureus* 450M (methicillin-resistant; MRSA), both courtesy of Professor G L Archer, Virginia Commonwealth University. Methicillin resistance is conferred in 450M by a Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Type I, containing the *mecA* gene encoding penicillin-binding protein 2a (PBP2a [23]). To prepare the lung inoculum, the bacteria were cultured aerobically in tryptic soy broth (TSB) at 37 °C for 16 h to cell concentrations >10^9 CFU mL^{-1}.

Six- to eight-week-old male C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Overnight bacterial cultures of *S. aureus* RN450 and 450M were measured for optical density (OD$_{600}$), then centrifuged at 13 000 rpm for 1 min, washed twice with phosphate buffered saline (PBS), and resuspended in PBS. Groups of six mice each were infected by oropharyngeal aspiration with 40 μL of PBS containing 1×10^8 CFU of MSSA, 6×10^7 CFU of MRSA, or no cells (PBS negative control), as previously described [24–26]. The six samples in each treatment group were split over 2 d of analysis in order to account for inter-day variances in the breathprints. The infection and breath collection procedures were each completed within 1 h for all mice in a testing cohort, and the schedules for the two test days were the same, thereby reducing any circadian influences on the breathprints. One mouse in the MSSA group died before breath collection, and therefore only five biological replicates are presented for this test group. 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 (AAALAC) guidelines. All mice were housed in the AAALAC–accredited animal facility at the University of Vermont (Burlington, VT).

Breath (1 L) was collected in Tedlar bags 24 h after airway exposure and stored at 37°C prior to secondary electrospray ionization-mass spectrometry (SESI-MS) analysis, which was completed within one hour of breath collection, as previously described [26]. After breath collection, bronchoalveolar lavage (BAL) was performed and the total leukocytes (or white blood cells, WBCs) in the BAL fluid were counted and characterized as macrophages, eosinophils, neutrophils (PMNs), or lymphocytes based on characteristic morphology and staining. Lactate dehydrogenase (LDH) activity in the BAL fluid was
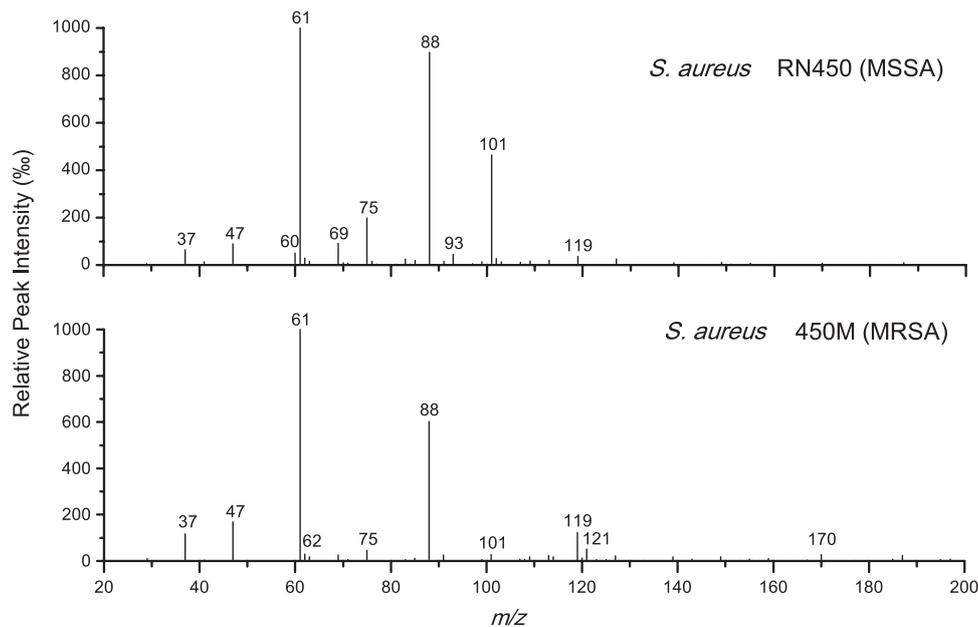
Figure 1. SESI-MS breathprints of mice with methicillin-sensitive (MSSA) or methicillin-resistant (MRSA) *S. aureus* lung infections. The spectra are the averages of five and six biological replicates for MSSA and MRSA, respectively.

quantified. The mouse lungs were harvested and homogenized, and the lung bacterial cell counts were determined by plating on Chapman Stone agar (BD). Full protocols for airway exposure, breath collection, SESI-MS breath analysis, and BAL collection and analysis used in this study can be found in previous publications [19, 26], and summaries are provided in the supplementary information (stacks.iop.org/JBR/8/041001/mmedia).

2.2. Data processing and statistical analyses

Analyst 1.4.2 software (Applied Biosystems) was used for spectra collection and raw data processing. Full scan spectra were blank-subtracted (the blank spectrum was humidified room air collected using the same procedure as for mice breath) and normalized to the peak of greatest intensity. Peaks 20–200 *m/z* (mass-to-charge ratio) and a signal-to-noise (S/N) > 2 were used in subsequent analyses (negative peaks resulting from blank subtraction were set to zero, accounting for 12% of the total peak data we collected). Presence/absence analyses were performed with the criteria that a peak must be observed with S/N > 2 in at least one biological replicate to be considered present in that test group. The relative intensities of shared peaks in MRSA versus MSSA were calculated by dividing the mean peak intensity in MRSA by the mean intensity in MSSA. Independent (unpaired) two-sided Student's *t*-tests were applied to determine whether the mean intensities of the shared peaks are statistically different (H_0: $\mu_{MSSA} = \mu_{MRSA}$). To account for *t*-test biases related to small sample sizes and non-normal distributions, Mann–Whitney *U*-tests were also conducted to validate statistical significance (H_0: $\mu_{MSSA} = \mu_{MRSA}$; $\alpha = 0.05$). One-sided Student's *t*-tests were performed for all peaks that are unique to a single strain (MRSA or MSSA) to determine if the mean peak intensities are statistically different from zero (H_0: $\mu = 0$). Due to small sample sizes, normal distribution was not used as a prerequisite for the *t*-tests.

JMP Version 10 (SAS Institute Inc.) was used to conduct principal components analysis (PCA) as well as to assess SESI-MS spectral reproducibility by calculating the Spearman's rank correlation coefficient (ρ) within each exposure group and control. The data were mean centered and range scaled prior to PCA analysis, and the statistical significance of the MSSA–MRSA separations in PCA was calculated using two-sided Student's *t*-tests of the principal component 1 (PC1) scores. For the MSSA–MRSA–PBS principal components analysis, one-way analysis of variance (ANOVA) was used on all 17 PC1 scores to determine if the mean scores are statistically different (H_0: $\mu_{PBS} = \mu_{MSSA} = \mu_{MRSA}$), followed by Tukey's honestly significant difference (HSD) test to confirm pair-wise significance between the groups' PC1 score means. The loading scores in principal components 1–3 were analyzed for each SESI-MS peak, and peaks with at least one loading score with an absolute intensity > 0.7 (|LS| > 0.7) were considered significant drivers of the PCA separation. The correct classification rates for MSSA, MRSA, and uninfected PBS groups (categorical variables) were determined using leave-one-out cross validation, performed using multivariate linear discriminant analysis of the normalized breathprint peak intensities (JMP v.10). A total of 83 peaks were present (S/N > 2) in at least one of the 17 normalized SESI-MS spectra, and their continuous relative intensity values were treated as covariates.

3. Results and discussion

3.1. Isogenic strains of MSSA and MRSA produce unique SESI-MS breathprints in vivo

We established lung infections in a murine model using two isogenic strains of *S. aureus*–RN450, which is methicillin-sensitive (MSSA), and 450 M, which is methicillin-resistant

Table 1. SESI-MS breathprint peaks unique to methicillin-sensitive (MSSA) or methicillin-resistant *S. aureus* (MRSA) lung infections.

m/z	MSSA peak relative intensities (‰)								LS[a]	t-test[b]
	1	2	3	4	5	N/A	Mean	SD		
33	-	-	8	-	6	-	3	4		
60	42	48	56	56	50	-	50	6	✓	*
76	15	14	16	15	14	-	15	1	✓	*
77	-	10	8	-	-	-	4	5		
81	10	-	-	9	-	-	4	5		
84	7	10	-	-	5	-	5	4		
93	43	44	47	45	42	-	44	2	✓	*
97	-	9	8	8	6	-	6	4	✓	*
100	-	-	12	-	8	-	4	6		
102	27	26	29	25	26	-	26	1	✓	*
103	12	15	15	13	14	-	14	1	✓	*
112		-	-	-	6	-	1	3		
136	13	-	-	-	-	-	3	6		
151	-	-	7	8	6	-	4	4		

m/z	MSSA peak relative intensities (‰)								LS[a]	t-test[b]
	1	2	3	4	5	6	Mean	SD		
48	-	-	8	-	-	-	1	3	✓	
114	32	26	32	17	-	-	18	15		*
120	18	37	-	-	-	18	12	15		
121	60	50	68	52	44	42	52	10	✓	*
123	7	-	16	-	-	8	5	6	✓	
141	-	-	8	-	-	-	1	3	✓	
153	8	-	-	-	-	-	1	3	✓	
159	18	18	-	9	9	10	11	7	✓	*
185	-	-	11	-	21	11	7	9		
195	-	-	14	-	11	8	5	6		
197	-	15	-	12	-	18	7	8		

[a] Check marks indicate peaks driving separation: |Loading Score (LS)| > 0.7 in principal component 1, 2, or 3
[b] One-sided *t*-test of null hypothesis, * $p < 0.05$

(MRSA)—and collected the breath of the mice 24 h post-infection. At the time of breath collection, there was an average of 1.6×10^6 CFU/lung of MSSA and 3.5×10^5 CFU/lung of MRSA, and the host immune response (measured by WBCs, PMNs, LDH; table S1 of the supplementary information) was activated in both infections, in concordance with previous murine lung infections we have studied [19, 26, 27]. The breath samples were analyzed using secondary electrospray ionization-mass spectrometry (SESI-MS), producing a volatile fingerprint, a.k.a. breathprint, for each lung infection (figure 1). The replicate spectra within each group are reproducible; the Spearman's rank correlation coefficients (ρ) for MSSA and MRSA are 0.83 and 0.74, respectively. The MRSA and MSSA breathprints contained a combined total of 62 peaks (S/N > 2), 40% of which are unique to one infection or the other (table 1, and table S2 of the supplementary information). To determine if the breathprints arising from MSSA and MRSA lung infections are statistically different, we performed principal components analysis (PCA; figure 2, and figure S1 of the supplementary information). Using the normalized peak intensities as variables and all experimental replicates as observations in the PCA, we observed that MSSA and MRSA breathprints were separable using only the first principal component ($p < 0.001$), and they are also separable from the breathprints of uninfected controls ($p < 0.0001$; figures S2 and S3 of the supplementary information). In addition, discriminant analysis of the breathprint peaks correctly classified 100% of the samples by MSSA, MRSA, or PBS treatment group membership.
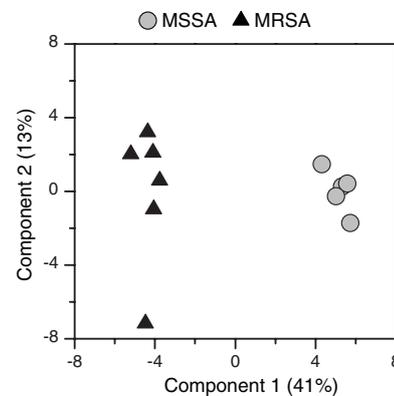
Figure 2. Principal components analysis (PCA) score plot for SESI-MS breathprints of mice with methicillin-sensitive (MSSA; grey circles) or methicillin-resistant (MRSA; black triangles) *S. aureus* lung infections ($p < 0.001$, PC1).

3.2. Unique, shared, dominant, and rare peaks all contribute to identification of MRSA by breathprinting

We were interested in which features of the breathprints drive the strong separation of the MRSA and MSSA infection groups in the PCA, and posited that peaks that are unique to one group or the other would play the most important roles (table 1, and table S2 of the supplementary information). Interestingly, only half (48%) of the 25 unique peaks have significant loading scores (|LS| > 0.7) in any of the first three components of the PCA (table 1), which account for 64% of the
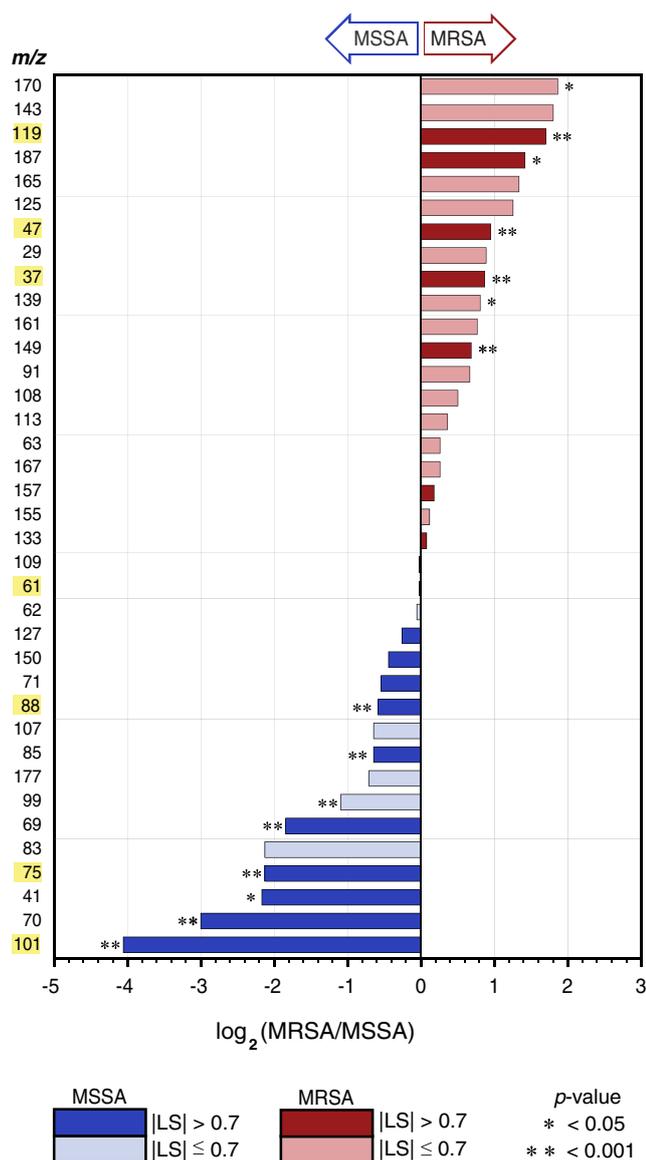
Figure 3. Relative intensities for shared SESI-MS breathprint peaks, expressed as the $\log_2$ fold change in the mean intensities of MRSA/MSSA. Bars marked with asterisks indicate statistically significant differences in the relative peak intensities between the two groups (* $p < 0.05$, ** $p < 0.001$). Dark red and blue bars represent MRSA and MSSA peaks, respectively, that have significant loading scores ($|LS| > 0.7$) in principal components 1, 2, or 3. Peaks (m/z) that are highlighted are among the top 10 most abundant peaks of both strains.

total variance in the infection-related breathprints. On the flip side, of all the peaks that do have significant loading scores (29 in all; table S2 of the supplementary information), only 41% are unique peaks, which is the same proportion as the number of unique peaks amongst all of the peaks we observed (table S2 of the supplementary information). Therefore, counter to what we hypothesized, unique peaks are no more likely to drive separation than the peaks that are shared by both infection groups. Another surprising feature of the unique peaks with high loading scores in principal components 2 and 3 is that many of them, particularly in the MRSA breathprints, are rare peaks, detected in no more than half of the replicates in that group (table 1, and table S2 of the supplementary information).

Although a one-tailed *t*-test indicates that the intensity of these rare peaks is not statistically greater than zero (table 1), some still make important contributions to the separation of MSSA and MRSA infections by breathprinting (e.g. $m/z = 123$; figure S1 and table S2 of the supplementary information).

The breathprints for MRSA and MSSA share the majority of their peaks, including 7 of the 10 most intense peaks for each group (figures 1 and 3). Qualitatively, the most notable differences between the two breathprints are in the relative intensities of the dominant peaks they share (e.g. $m/z = 75$, 88, 101, and 119; figures 1 and 3), and therefore we posited that these peaks also make statistically significant contributions to the separation of MSSA and MRSA by PCA. Excluding the base peak $m/z = 61$, which was set to 1000‰ for both strains, and $m/z = 62$, the ^{13}C isotope of this base peak, all of the most intense shared peaks have very strong loading scores ($|LS| > 0.9$) in the first principal component (table S2 of the supplementary information). These peaks are also present in every replicate of MSSA and MRSA lung infections, but two-sided *t*-tests reveal that the relative intensities of these peaks are, indeed, significantly different between the two strains ($p < 0.001$; table S3 of the supplementary information; figure 3). There are an additional 11 lower-intensity shared peaks that have significant loadings on one of the first three components of the PCA, and as we observed for the unique peaks, several of these shared peaks are rare among the replicates of one or both groups ($m/z = 41$, 70, 71, 133, 150, and 157; table S3 of the supplementary information). These findings demonstrate that an individual breath biomarker need not be unique, intense, or ubiquitous to be useful for diagnosis, as long as it is not used in isolation.

4. Conclusions

Breath-based diagnostics can provide rapid, non-invasive methods for identifying bacterial lung infections without requiring microbial samples from the infection site. We found that MRSA and MSSA lung infections in a murine model can be robustly identified *in situ* via SESI-MS breathprinting using only 1 L of breath, and without any sample pretreatment prior to analysis. Evaluating the features of the breathprints that classify MRSA versus MSSA in PCA, we found that the peaks driving separation are just as likely to be shared by both MRSA and MSSA infections as they are to be unique to one strain, and rarely observed peaks also make significant contributions. These findings support the use of biomarker panels that include a wide variety of peaks, rather than single compounds that are unique to an infection, to identify clinically important phenotypes (e.g. antibiotic resistance) *in situ* using breath-based diagnostics.

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