

Microbial Volatiles as Diagnostic Biomarkers of Bacterial Lung Infection in Mechanically Ventilated Patients

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Background. Early and accurate recognition of respiratory pathogens is crucial to prevent increased risk of mortality in critically ill patients. Microbial-derived volatile organic compounds (mVOCs) in exhaled breath could be used as noninvasive biomarkers of infection to support clinical diagnosis.

Methods. In this study, we investigated the diagnostic potential of in vitro–confirmed mVOCs in the exhaled breath of patients under mechanical ventilation from the BreathDx study. Samples were analyzed by thermal desorption–gas chromatography–mass spectrometry.

Results. Pathogens from bronchoalveolar lavage (BAL) cultures were identified in 45 of 89 patients and *Staphylococcus aureus* was the most commonly identified pathogen ($n = 15$). Of 19 mVOCs detected in the in vitro culture headspace of 4 common respiratory pathogens (*S. aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*), 14 were found in exhaled breath samples. Higher concentrations of 2 mVOCs were found in the exhaled breath of patients infected with *S. aureus* compared to those without (3-methylbutanal: $P < .01$, area under the receiver operating characteristic curve [AUROC] = 0.81–0.87; and 3-methylbutanoic acid: $P = .01$, AUROC = 0.79–0.80). In addition, bacteria identified from BAL cultures that are known to metabolize tryptophan (*E. coli*, *Klebsiella oxytoca*, and *Haemophilus influenzae*) were grouped and found to produce higher concentrations of indole compared to breath samples with culture-negative ($P = .034$) and other pathogen-positive ($P = .049$) samples.

Conclusions. This study demonstrates the capability of using mVOCs to detect the presence of specific pathogen groups with potential to support clinical diagnosis. Although not all mVOCs were found in patient samples within this small pilot study, further targeted and qualitative investigation is warranted using multicenter clinical studies.

Keywords. hospital-acquired infection; exhaled volatile organic compounds; gas chromatography-mass spectrometry.

Ventilator-associated pneumonia (VAP) is the most frequent nosocomial infection in critically ill patients worldwide [1]. Rapid and accurate identification of the causative pathogen is required for administration of optimal, narrow-spectrum

antimicrobial therapy. Currently empiric, broad-spectrum antimicrobial therapy is recommended to avoid delays in treatment, which are associated with worse clinical outcomes [2]. However, overuse of broad-spectrum antimicrobial therapy is associated with drug-induced toxicity, microbiome dysbiosis, and emergence of antimicrobial-resistant strains [3–6].

A diverse range of bacteria is typically found in critically ill patients with respiratory infections (such as VAP), including gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*) and gram-negative bacteria (*Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Haemophilus influenzae*, *Escherichia coli*, and *Klebsiella pneumoniae*) [7]. Current diagnostic strategies usually rely on microbiological culture of respiratory samples, which is often time-consuming (approximately 3 days), invasive, and infrequently carried out following clinical suspicion of VAP [8]. A possible solution is to monitor molecular phenotype changes in host or bacterial metabolism [9]. There is substantial

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interest in the analysis of volatile organic compounds (VOCs) for the early detection of infection, primarily due to the benefits of noninvasive sample collection, adaptability of technologies, and applicability to a wide range of diseases [10–12]. Several studies have reported changes in the exhaled breath metabolome of patients with infection [13–15]. However, translational research unifying *in vitro* and *in vivo* studies, the important next step in biomarker validation, is lacking.

Microbial volatile organic compounds (mVOCs) are a diverse set of metabolites that originate from microbial biochemical processes and are representative of cellular signaling and metabolism [16]. Although mVOCs associated with core metabolic pathways are consistently produced, the volatilome of a microbe differs between various taxa and are highly influenced by the surrounding environment (nutrients, pH, and CO₂/O₂ levels). This makes it difficult to pinpoint the causative pathogen in a study designed to focus solely on a type of infection, rather than commensal resident microbes, particularly in VAP where several causative pathogens are routinely identified. Instead, volatile metabolites are related to infection pathogenesis as a whole, which may include physiological and host response factors, as demonstrated by previous untargeted studies, which advocate a high sensitivity or “rule-out” test method [17–19]. Alternatively, a limited number of studies have focused solely on mVOCs for VAP and invasive aspergillosis in critically ill patients [20–22].

In this study, the diagnostic potential of measuring mVOCs was evaluated. Culture headspace experiments of bacterial species associated with VAP informed which mVOCs to target in patient breath samples. Compounds detected in breath were then compared in culture-negative (<10⁴ colony-forming units [CFU]/mL⁻¹) and other pathogen-positive (all other samples with pathogens identified in bronchoalveolar lavage [BAL] culture apart from the pathogen of interest for statistical comparison) groups. The outcome of this study demonstrated the feasibility of mVOC biomarkers to detect the presence of a known pathogen and potentially guide early and appropriate antimicrobial treatment.

METHODS

Headspace Sample Collection

The reference strains *S. aureus* ATCC 29213, *P. aeruginosa* PAO1, *K. pneumoniae* ATCC 13887, and *E. coli* ATCC 25922 were used in this study. All strains were cultured in nutrient broth (Sigma-Aldrich, St Louis, Missouri). For headspace samples, axenic cultures were standardized to optical density at 600 nm (OD₆₀₀) 0.01 to a total volume of 2 mL (~1 × 10⁶ to 1 × 10⁷ CFU mL⁻¹) were crimp-sealed in 20 mL glass vials with a PTFE septum and incubated for 24 hours at 37°C under shaking conditions. Two headspace gas sampling methods were used to capture mVOCs, as described previously [23]. Further details are provided in the [Supplementary Material](#).

Patient Sample Collection

Exhaled breath sample data from the BreathDx study were used (UKCRN ID 19086, May 2015). During this study, 1200 mL exhaled breath from ventilator circuit tubing was sampled into Tenax-GR sorbent tubes (flow rate of 200 mL min⁻¹) as previously described [24]. Eighty-nine patients from the intensive care unit (ICU) departments of 4 university hospitals—Salford Royal NHS Foundation Trust (n = 29), Academic Medical Centre Amsterdam (n = 42), Central Manchester NHS Foundation Trust (n = 16), and Wythenshawe Hospital NHS Foundation Trust (n = 2)—were recruited over a 2-year period from February 2016 to February 2018. Semi-quantitative BAL cultures were used as the standard reference for pathogen identification with a cutoff of ≥10⁴ CFU mL⁻¹ to define a positive culture. Exhaled breath and BAL were sampled within 24 hours of clinical suspicion of VAP and before antimicrobial treatment.

Analysis by Thermal Desorption–Gas Chromatography Mass Spectrometry

Culture headspace and breath samples were analyzed by thermal desorption–gas chromatography mass spectrometry (TD-GC/MS) using the method described previously [24]. For further details, see the [Supplementary Material](#).

Data Preprocessing

Initial tests were performed on full scan data that were previously preprocessed for the preliminary BreathDx untargeted analysis described in van Oort et al [19]. This data set, comprising 1247 MS features, was tested for differences between pathogen-positive and pathogen-negative groups. A batch search method was then created in Masshunter Quantitative analysis software (B.07.00, Agilent, Cheadle, United Kingdom) based on chromatograms from culture headspace samples. Peaks were selected if they were present in at least 1 pathogen across all culture replicates. Features that were present in culture versus media control samples were not assessed. The batch method was then used to determine breath analytes. Mass spectra and retention indices of external standards were used to support identification (ie, selection of appropriate compound quantifier and qualifier ions, and retention time windows), adhering to the metabolomics standards initiative [25, 26]. Missing values were allocated based on the determination of qualifier ions. An *n* × *p* data matrix was created for subsequent data treatment and statistical analyses. Estimated VOC concentrations were determined from a standard curve created using a calibration standards loading rig (Markes International, Bridgend, United Kingdom).

Statistical Analysis

Statistical analyses were carried out in GraphPad Prism (version 9.1) and R (version 4.1.1) software. All sample intensities were normalized using a spiked internal standard (4-bromofluorobenzene). Volcano plots were made from the

untargeted dataset, and significant features were listed. Peak intensities from the in vitro culture study were mean centered and hierarchical cluster analysis was carried out on all in the R package *phreatmap* generating a heatmap for visual comparisons. For breath sample data, missing values were imputed with a random value between zero and the limit of detection ($3 \times \sigma$ of background samples) for each compound. Intraclass correlation coefficients were calculated between repeat breath samples from the same patient.

The nonparametric Kruskal-Wallis test was applied to screen for statistical differences between groups. This was followed by a post hoc Dunn multiple comparison test between 2 independent groups. Individual *P* values were reported ($\alpha = .05$) and no correction was made for multiple comparisons. For the in vitro culture study, differences between sampling methods were assessed. For breath samples, differences between pathogen-positive, other pathogen-positive, and culture-negative groups were assessed. The untargeted dataset was analyzed using the univariate Mann-Whitney *U* test between pathogen-positive and pathogen-negative groups where statistical significance and fold change were illustrated using volcano plots. To explore the diagnostic performance of any potential VOC markers, the area under the receiver operating characteristic curve (AUROC) was calculated.

RESULTS

Patient Characteristics

Patient characteristics, stratified for positive and negative BAL cultures, are summarized in Table 1. Differences were found between admission type (medical compared to nonmedical) and the Clinical Pulmonary Infection Score between culture-positive and culture-negative patients.

Microbial VOCs From Culture Headspace

A total of 19 mVOCs were detected from the headspace of axenic cultures. Compounds detected in control samples, including culture media, sampling equipment, and the instrument background, were not included. Compounds that could not be confidently detected due to coelution or were outside the predefined criteria were not analyzed further. The heatmap in Figure 1 highlights the differences in mVOC abundance across microbes and sampling methods.

Based on volatile profiles alone, all sample replicates clustered by microorganism, regardless of the headspace sampling method used. Acetone, dimethyl disulfide, and dimethyl trisulfide were found in the headspace of all bacteria. No other mVOCs were found to be consistently produced in all microbes investigated in this study. Some mVOCs were only observed in 1 microbial species compared to others. These included 1-undecene, methyl thiocyanate, dimethyl sulfide, and 2-aminoacetophenone from *P. aeruginosa* cultures; ethyl acetate,

2-heptanone, and 2-nonanone from *K. pneumoniae* cultures; and benzaldehyde and indole from *E. coli* cultures. Several mVOCs, such as 3-methyl-1-butanol, 3-methylbutanal, and 3-methylbutanoic acid, were found across 2 or more species and were higher in *K. pneumoniae* and *S. aureus* but were not detected in *P. aeruginosa* and *E. coli*, with the exception of 3-methylbutanal for *E. coli*.

Differences between the 2 sampling methods employed were also observed. Methyl thiocyanate and dimethyl sulfide, produced by *P. aeruginosa*, were detected using active sampling only. Conversely, compounds detected solely by passive sampling included 2-aminoacetophenone from *P. aeruginosa* ($P = .036$) and 3-methylbutanoic acid ($P = .007$) and furfuryl formate ($P < .001$) from *S. aureus*. A significant increase in dimethyl disulfide from *P. aeruginosa* was demonstrated

Table 1. Patient Clinical Characteristics

Characteristic	Positive BAL Culture n = 45 (50.6%)	Negative BAL Culture n = 44 (49.4%)	<i>P</i> Value
Age, y, median (IQR)	58.5 (39.25–68.75)	59 (46–66)	.788 ^a
Sex			
Male	27 (60)	30 (68.2)	.509 ^b
Female	18 (40)	14 (31.8)	
BMI, kg/m ² , median (IQR)	24.4 (20.9–31.0)	26.2 (22.9–32.0)	.290 ^a
Admission type ^c			
Medical	15 (33.3)	27 (61.4)	.011 ^b
Planned surgical	16 (35.6)	6 (13.6)	.364 ^{b, d}
Emergency surgical	14 (31.1)	10 (22.7)	
Admission characteristics			
Trauma	18 (40)	9 (20.5)	.065 ^b
Neurosurgery	15 (33.3)	8 (18.2)	.146 ^b
Clinical score, median (IQR)			
SOFA lung	4 (2–4)	3 (2–4)	.118 ^a
APACHE II	17 (10–23)	20 (14.3–23.8)	.389 ^a
CPIS	7 (5.5–7)	5 (4–6)	<.001 ^a
ICU LOS, d, median (IQR)	21 (15–32)	21 (13–33)	.926 ^a
ICU mortality	7 (15.6)	11 (25)	.302 ^b
Pathogen isolated ^e			
<i>Staphylococcus aureus</i>	15 (33.3)	...	
<i>Pseudomonas aeruginosa</i>	8 (17.8)	...	
<i>Escherichia coli</i>	2 (4.4)	...	
<i>Klebsiella pneumoniae</i>	2 (4.4)	...	
Other	24 (53.3)	...	
Indole producing ^f	9 (20.0)	...	

Data are presented as No. (%) unless otherwise indicated.

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; BAL, bronchoalveolar lavage; BMI, body mass index; CPIS, Clinical Pulmonary Infection Score; ICU, intensive care unit; IQR, interquartile range; LOS, length of stay; SOFA, Sequential Organ Failure Assessment.

^aMann-Whitney *U* test.

^bFisher exact test.

^cOne patient with no information in the negative culture group.

^dComparison between planned and emergency surgical admission type.

^ePotentially >1 cultured pathogen per patient.

^fPathogens that are known to produce indoles: *Escherichia coli* (n=2), *Haemophilus influenzae* (n=5), and *Klebsiella oxytoca* (n=2).

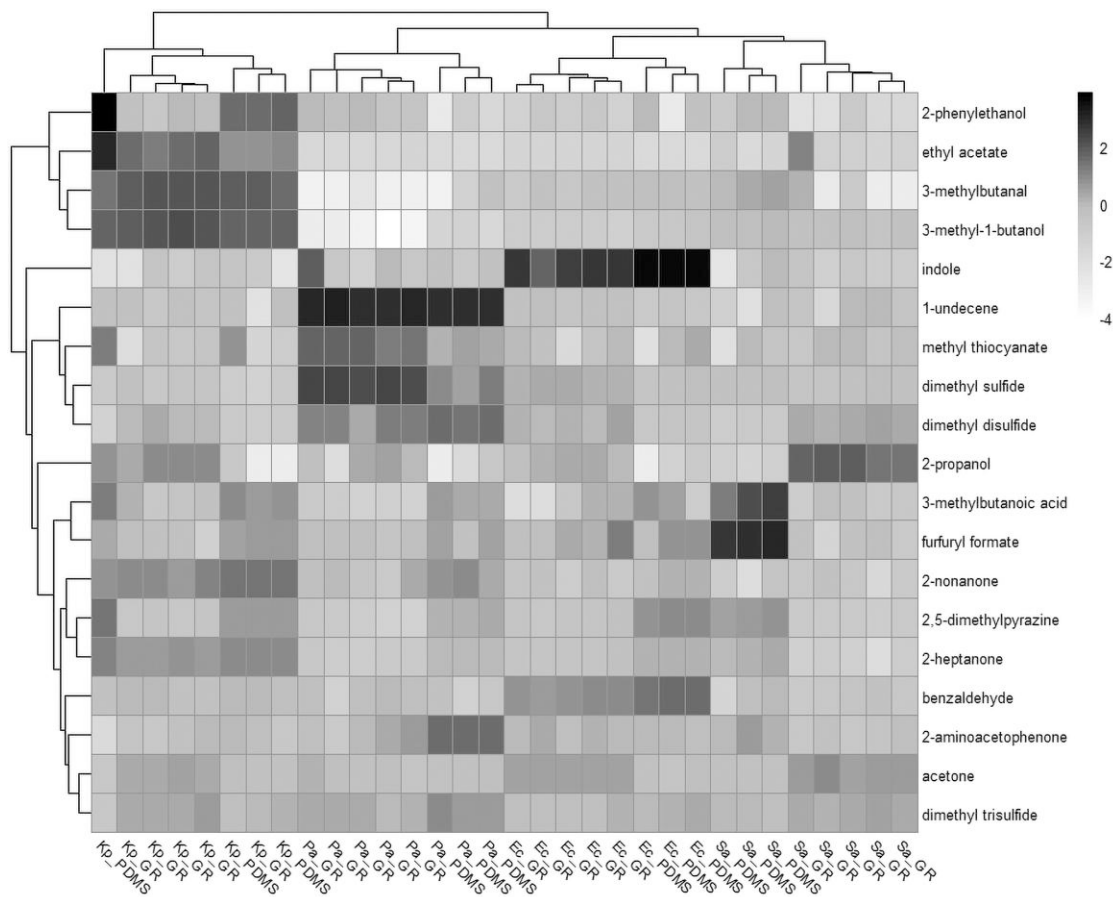


Figure 1. Heatmap ordered by hierarchical cluster analysis for microbial volatile organic compounds (VOCs) and individual samples produced by different pathogens and sampling methods (n = 5 replicates for active sampling, n = 3 replicates for passive sampling). The color scale (white to black) represents VOC peak intensity z-scores from axenic culture headspace. Sampling methods and microorganisms are annotated below the heatmap. Abbreviation: Pa, *P. aeruginosa*; Sa, *Staphylococcus aureus*; Kp, *Klebsiella pneumoniae*; Ec, *Escherichia coli*; PDMS, polydimethylsiloxane (passive sampling); GR, Tenax GR (active sampling).

(*K. pneumoniae* $P < .001$, *E. coli* $P = .003$, *S. aureus* $P < .001$) using the same passive method.

Microbial VOCs in Patient Exhaled Breath Samples From the Ventilator Circuit

Initial tests with full scan data previously analyzed in the preliminary BreathDx study [19] revealed significant differences between pathogen-positive and pathogen-negative cultures, as shown in the volcano plots (Supplementary Figure 1). Several significant features ($P < .05$) were found for *S. aureus*, *P. aeruginosa*, *Klebsiella* species, and indole-producing pathogens when compared to culture-negative samples (n = 24, n = 21, n = 10, and n = 143, respectively).

To explore mVOCs produced by pathogens, a search of mVOCs detected in culture headspace was carried out on patient breath samples. Five mVOCs were not confidently measured in breath (eg, due to coelution or because they were outside predefined criteria) and were therefore excluded from further statistical analysis, namely 2-propanol, methyl thiocyanate, furfuryl formate, 2,5-dimethylpyrazine, and dimethyl trisulfide.

Significant changes between pathogen-positive, other pathogen-positive, and culture-negative samples were assessed for mVOCs detected in breath samples (Table 2). Breath sample reproducibility was assessed by calculating intra-class correlation coefficient between sequential samples from the same patient and ranged from 0.49 to 0.98 for mVOCs detected in patients' breath (Supplementary Table 1).

Acetone and benzaldehyde were detected in all breath samples. Acetone was also significantly lower in *P. aeruginosa*-positive compared to other pathogen-positive samples ($P = .007$). A lower abundance of 3-methylbutanal was also observed for *P. aeruginosa* compared to other pathogen-positive samples ($P = .012$). In samples with positive culture identification for *S. aureus*, both 3-methylbutanal and 3-methylbutanoic acid were significantly higher compared to culture-negative samples ($P < .001$ and $P = .001$, respectively). When compared to other pathogen-positive samples, 3-methylbutanal and 3-methylbutanoic acid were also significantly higher in *S. aureus*-positive samples ($P < .001$ and $P = .001$, respectively). Importantly, no significant differences were found for either compound between other

Table 2. Microbial Volatiles Detected in Patient Samples With a Comparison of Pathogen-Positive Samples

Compound Name ^a	CAS ^b	Synonym	<i>m/z</i>			Detected in Breath Samples, No.	Relative Concentration and <i>P</i> Value	
			Quantifier Ion	Qualifier Ion(s)	Retention Index ^c		Pathogen Positive/Other Pathogen Positive	Pathogen Positive/Culture Negative
Acetone	67-64-1	Propanone	58	...	505	89	↓ .007 (<i>Pseudomonas aeruginosa</i>)	...
Dimethyl sulfide	75-18-3	Dimethyl thioether	47	62	566	70
Ethyl acetate	141-78-6	Acetic acid, ethyl ester	43	88	607	73
3-methylbutanal	590-86-3	Isovaleraldehyde	58	71, 86	647	59	↓ .012 (<i>P. aeruginosa</i>) ↑ < .001 (<i>Staphylococcus aureus</i>)	↑ < .001 (<i>S. aureus</i>)
3-methyl-1-butanol	123-51-3	Isopentyl alcohol	55	70	730	59
Dimethyl disulfide	624-92-0	(Methyldithio)methane	94	45, 79	769	57
3-methylbutanoic acid	503-74-2	Isovaleric acid	60	87	858	74	↑ .001 (<i>S. aureus</i>)	↑ .001 (<i>S. aureus</i>)
2-heptanone	110-43-0	Heptan-2-one	58	71	896	73
Benzaldehyde	100-52-7	Benzoic aldehyde	77	106	969	89
2-nonanone	821-55-6	Nonan-2-one	58	71, 142	1088	82
1-undecene	821-95-4	Undec-1-ene	55	70, 83, 97, 154	1101	26
2-phenylethanol	60-12-8	Phenylethyl alcohol	91	91.9, 122	1122	71
Indole	120-72-9	Benzopyrrole	90	117	1278	84
2-aminoacetophenone	551-93-9	Ethanone, 1-(2-aminophenyl)-	120	92, 135	1312	37

Abbreviations: CAS, Chemical Abstracts Service.

^aMicrobial volatile organic compounds were identified using the mass spectra and retention times of analogous external standards run on the same thermal desorption–gas chromatography mass spectrometry (ie, Metabolomics Standards Initiative level 1).

^bCAS registry number.

^cCalculated Kováts retention index using an alkane ladder (C₆–C₁₅) on a standard nonpolar column.

pathogen-positive samples or between culture-positive versus culture-negative samples.

Diagnostic performance was assessed for *S. aureus*–positive samples against culture-negative or other pathogen-positive groups, which resulted in an AUROC of 0.87 (95% confidence interval [CI], .75–.99; *P* < .001) and 0.81 (95% CI, .66–.96; *P* < .001), respectively, for 3-methylbutanal. For 3-methylbutanoic acid, *S. aureus*–positive versus culture-negative and other pathogen-positive resulted in AUROCs of 0.79 (95% CI, .65–.93; *P* = .001) and 0.80 (95% CI, .64–.95; *P* = .002), respectively. Pearson correlation coefficient showed a weak positive correlation (*r* = 0.21, *P* = .045) between the 2 compounds. Results for 3-methylbutanal and 3-methylbutanoic acid are shown in Figure 2. Due to the low number of *E. coli*–positive (*n* = 2) and *K. pneumoniae*–positive (*n* = 2) cultures, these samples were excluded from further statistical analysis. Although not confirmed using culture headspace in this study, grouping breath samples with bacteria that can metabolize tryptophan (*n* = 9), *Klebsiella oxytoca*, *Haemophilus influenzae*, and *E. coli* displayed higher abundance of indole in breath samples (against other pathogen-positive *P* = .049 and culture-negative *P* = .034). No significant differences were found for *Klebsiella* species alone (*n* = 5) when compared to other pathogen-positive or culture-negative samples.

DISCUSSION

Exhaled breath biomarker discovery for detecting microbial infection in critically ill patients remains a challenge. This study investigates the value of targeting mVOCs as diagnostic markers of bacterial infection and we have presented evidence for noninvasive detection of *S. aureus* infection and indole-producing pathogens from in a multicenter ICU study.

We screened patient breath sample data in an untargeted approach that revealed several significantly different mass spectral features. These features are indicative of bacterial infection including host response and not mVOCs produced by a specific pathogen and group of pathogens. We then developed a target list of mVOCs based on culture headspace data and searched for them in exhaled breath samples collected noninvasively from the ventilator circuit. The most significant result was higher abundance of 3-methylbutanal (syn. isovaleraldehyde) and 3-methylbutanoic acid (syn. isovaleric acid) produced by *S. aureus* in vitro and in exhaled breath from patients with confirmed *S. aureus* infection. Importantly, production of both compounds was higher compared to both other pathogen-positive and culture-negative samples. Previous studies have also detected 3-methylbutanal and/or 3-methylbutanoic acid associated with *S. aureus* from culture headspace [27–35] and ventilator

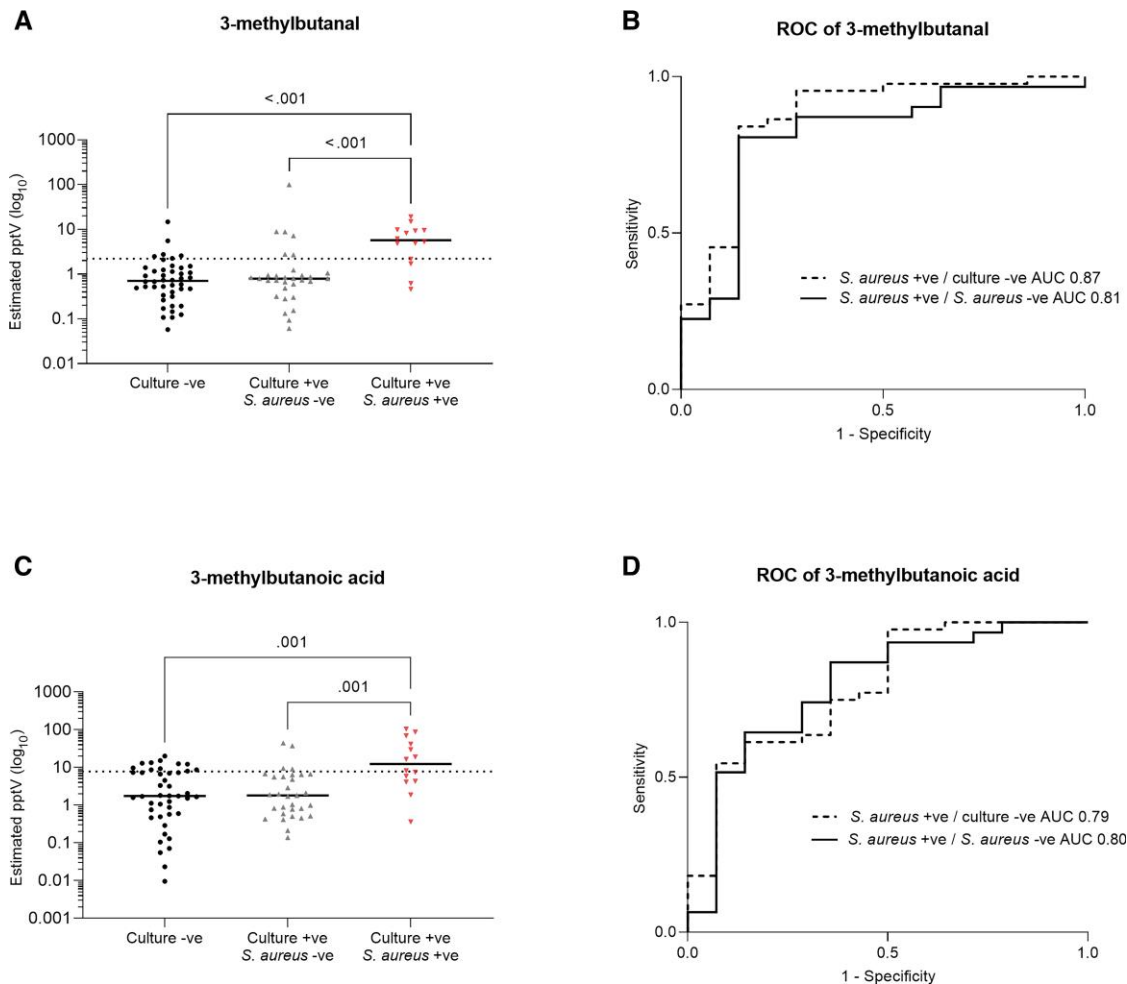


Figure 2. Scatter plots of estimated volatile organic compound concentrations and receiver operating characteristic curves of 3-methylbutanal (A and B) and 3-methylbutanoic acid (C and D). Scatter plots are annotated with the group median (black line), estimated limit of detection (dotted line), and *P* values. Comparisons were made between *Staphylococcus aureus*-positive and other pathogen-positive or culture-negative samples. Abbreviations: -ve, negative; +ve, positive; AUC, area under the curve; pptV, parts per trillion by volume; ROC, receiver operating characteristic.

exhaled breath [21], irrespective of culture conditions, strain type, and sampling methods. As both compounds were found to be correlated with each other, it is likely they share the same metabolic pathway. Branched chain fatty acids (eg, 3-methylbutanoic acid), and their precursors (eg, 3-methylbutanal) from leucine metabolism are important for maintaining membrane fluidity in *S. aureus* [36–38]. Indole was also detected in higher abundance in bacteria known to use the enzyme tryptophanase to catabolize the essential amino acid tryptophan [39]. Previous studies have detected indole in the headspace of *H. influenzae* [29] and *K. oxytoca* [28] and we confirmed indole production from *E. coli* cultures in this study. This highlights the importance of linking mVOCs with their metabolic pathways, which may not be species-specific and instead shared between bacterial classifications and intrinsic biochemical processes.

The number of pathogens identified from BAL culture limited statistical comparisons of several bacterial species (eg, for

E. coli, present in culture from only 2 patients). In addition, previously reported microbial volatiles from literature were not included in this analysis due to methodological differences. Culture headspace samples were analyzed using the same untargeted TD-GC/MS method as in BreathDx, limiting an extensive analysis of all mVOCs. Along with developing an analytical method around known mVOCs, breath sampling methods and influence of bacterial growth phase should be carefully considered in future studies depending on the target analytes. In addition, it is important to state that although commonly reported mVOCs were identified under laboratory growth conditions, they may not represent a ventilated human lung environment exposed to environmental and drug-induced stresses [32, 40, 41]. Additionally, clinical isolates and antimicrobial resistant strains have also shown variation in their volatilomes [42–44]. More investigative work is required in the form of lung infection models and metabolic flux studies [32, 45–47].

Some mVOCs identified from bacteria (eg, ethyl acetate) are also identified in the headspace of fungi [23, 48–50]. Other compounds such as benzaldehyde are known environmental or sorbent artefacts. Acetone is commonly found in almost all breath samples from healthy individuals. Such compounds are challenging to validate as pathogen or disease biomarkers.

Exhaled breath sampling from patients on mechanical ventilation is not without its challenges. It has previously been shown that different ventilator systems influence background VOC profiles differently and vary in the extent to which they dilute VOC recovery [51]. In the BreathDx study, potential confounders including storage time, analysis date, and ventilation parameters were not found to influence breath volatile profiles between patients with or without infection. Samples obtained from 1 of the sites in the study could be separated by principal component analysis (second dimension) and this may be due to site-specific practices, such as preference for anesthetic medication, which could not be controlled [19].

The effect of medication on breath profiles has been observed previously [52], and although patients received anti-infective treatment after breath analysis, effect of other medication across the patient cohort is a potential confounder. For example, anesthetic drugs and their metabolites were detected in chromatograms where propofol administered <24 hours had significantly increased peak area ($P=.009$) compared to patients administered propofol >24 hours (Supplementary Figure 2). Progress has been made in the standardization of breath VOC analysis by preconcentration; however, there are several factors limiting current clinical use such as sample storage and water retention [12, 53–56]. The value of VOCs as diagnostic biomarkers is heavily influenced by the value of the clinical information collected. Due to the highly sensitive nature of VOC analysis, differences between hospital sites, clinical workflows and diagnostic models, ventilator settings, or preferred anti-infective treatment regimen will be reflected in the metabolic phenotype. Although breath was collected on suspicion of VAP and before antibiotic treatment, critically ill patients would already have multiple comorbidities and administered several medications, for example anesthetic and anti-inflammatories, which may likely change the composition of microbiomes [57]. It is widely regarded that the lungs are not a sterile environment [58]; therefore, applying a conservative CFU cutoff value for BAL culture may increase the likelihood of false-positive identifications and impact diagnostic accuracy of measuring mVOCs. Future studies should therefore consider measuring microbiome diversity and abundance and how they correlate to exhaled breath VOCs for those pathogens below the clinical CFU threshold.

Microbial volatiles in exhaled breath may be interpreted as a “gas lavage” of the lungs and representative of systemic infection compared to localized and invasive wash. We have demonstrated using a translational approach that mVOCs have diagnostic utility in detecting pathogens using exhaled breath samples. Further validation studies are required as not all

mVOCs were found in breath samples in our study. A targeted assay of mVOCs may be clinically useful in reducing healthcare-associated infection and minimizing unnecessary use of antimicrobials.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. S. J. F., T. M. E. N., L. D. J. B., M. J. S., H. H. K., P. D., R. G., W. M. A., and T. F. were involved in study design and methodology of the BreathDx study. W. M. A., I. R. W., B. D., P. B., D. F., P. M. P. V. O., and L. D. J. B. were involved in breath and headspace sampling and analysis. W. M. A. wrote the manuscript. All authors approved the final version of the manuscript.

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