Headspace volatile organic compounds from bacteria implicated in ventilator-associated pneumonia analysed by TD-GC/MS

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Abstract

Ventilator-associated pneumonia (VAP) is a nosocomial infection which occurs in ventilated patients in the intensive care unit (ICU) after 48 h of intubation (Hunter 2012). Potential mechanisms of pathogenesis include a switch in bacterial species colonising the upper respiratory tract, upregulation of pro-inflammatory cytokines, and the compromise of mechanical defense systems (the cough reflex, mucociliary clearance and the epithelial barrier) following the introduction of the endotracheal tube into the patient (Safrar et al 2005). As individuals are usually critically ill before mechanical intubation, with their immune systems already burdened and their mechanical defense systems compromised, this provides an ideal environment for pathogens to overwhelm the host and thus culminate in VAP (Safrar et al 2005). Diagnosis of VAP is not straightforward; it usually requires a combination of clinical, radiographic, and microbiological information before a diagnosis can be made (Koenig and Truwit 2006). Microbiological investigation can take up to seven days...
to yield a result and therefore suspected VAP patients are administered with empirical antibiotics, thus risking inappropriate treatment that may result in antibiotic resistance, mortality and morbidity. Diagnosis also often requires an invasive approach in order to collect airway samples for microbiological analysis, often done by means of bronchoscopy (Kalanuria et al 2014).

To overcome these problems, breath analysis is proposed for the purpose of identifying and/or ruling out suspected VAP in patients, which would lead to improved antimicrobial stewardship. Exhaled breath contains volatile organic compounds (VOCs) which originate from exogenous, endogenous, and microbial sources (Boots et al 2015), and it is therefore of interest to study VOCs emitted from bacteria as these could be unique to the pathogen and may act as useful biomarkers for microbial speciation. The microbiology of VAP-associated microbes has been discussed elsewhere (Park 2005, Ahmed et al 2017). VOCs are typically present in breath and headspace samples at trace concentration and their analysis requires sample enrichment, often performed by sorbent tube sampling (Filipiak et al 2012) or solid-phase microextraction (Buszewski et al 2008) followed by thermal desorption. An artificial sputum medium (ASM) was developed by Sriramulu et al to mimic the sputum of cystic fibrosis (CF) patients in which Pseudomonas aeruginosa infection commonly occurs (Sriramulu et al 2005). In this study, P. aeruginosa and other VAP-associated bacteria such as Escherichia coli, Klebsiella pneumoniae, and Staphylococcus aureus were cultured in vitro in ASM and nutrient broth (NB) to identify VOCs emitted by these microbes which may be potential diagnostic markers. NB was used as a reference condition for comparison. By manipulating growth media outside the lung environment, changes to the VOC profile can be observed and thus media essential components can be identified, which may help translate findings to humans.

**Materials and methods**

**Media preparation**

Nutrient agar (NA) (Oxoid, Basingstone, UK), nutrient broth (NB) (Oxoid), and ASM were utilised in this study. NA and NB were prepared according to manufacturer’s specification. Details for preparing ASM can be found in (Sriramulu et al 2005). Briefly, type II mucin (Sigma-Aldrich, UK), salmon sperm DNA (Sigma-Aldrich, UK), diethylenetriaminepentaaetic acid (Sigma-Aldrich, UK), sodium chloride (Sigma-Aldrich, UK), potassium chloride (Fisher scientific, UK), Tris base (Formedium, Hunstanton, UK), egg yolk emulsion (Oxoid), casamino acids (BD, Sparks, USA) were all dissolved in distilled water and subsequently autoclaved. More information is provided in Nature Protocol Exchange (Diraviam Dinesh 2010). M9 minimal medium was also used to grow Escherichia coli strains. Briefly, the minimal medium is a mixture of four solutions: glucose (5 g; Sigma-Aldrich, UK) dissolved in 50 ml distilled water (DW); ammonium chloride (1 g; Fisher Scientific, UK) and magnesium sulfate heptahydrate (0.5 g; AnalaR, UK) dissolved in 800 ml DW; disodium phosphate (3 g; Fluka, Switzerland) and monopotassium phosphate (3 g; Sigma-Aldrich, UK) in 150 ml DW, and iron (II) sulfate heptahydrate (0.1 g; Sigma-Aldrich, UK) and calcium chloride anhydrous solution (0.1 g; Fisher Scientific, UK) dissolved in 100 ml DW. One ml of the latter solution was added to the combined mixture of the other solutions before they were autoclaved at 121 °C for 15 min and combined.

**Bacterial culture**

*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13887, *Pseudomonas aeruginosa* PA01 and *Staphylococcus aureus* ATCC 29213 were used in this study since these bacteria are most commonly associated with VAP (Park 2005). Strains were recovered from glycerol frozen stocks, sub-cultured twice on NA plates and incubated overnight at 37 °C to obtain axenic colonies. Single colonies were subsequently transferred into 50 ml liquid media (ASM, NB) in 250 ml conical flasks sealed with sponges and incubated at 37 °C with 200 rounds per min (rpm) shaking.

**Growth curves**

The optical densities (ODs) of overnight liquid cultures were assessed using a spectrophotometer at a wavelength of 600 nm. The overnight liquid cultures were then subsequently aliquoted (200 μl) into a 100 well plate (10 replicates per bacterial species) at an OD of 0.1 and growth was monitored using a Bioscreen C growth curve analysis system (Oy Growth Curves Ab Ltd, Helsinki, Finland) at 37 °C with constant shaking for 24 h.

**Bacterial culture headspace sampling**

The headspace (200 ml) above liquid cultures was actively sampled onto Tenax GR thermal desorption tubes at a flow rate of approximately 100 ml min⁻¹ using an Easy-VOC pump (Markes international, Llantrisant, UK) after incubation at time points that were determined based on culture growth curves as follows: *E. coli* NB (3, 5, and 15 h), *E. coli*-ASM (10, 12 and 24 h), *K. pneumoniae* NB (5, 7, and 15 h), *P. aeruginosa* NB (3, 5, and 15 h), *P. aeruginosa* ASM (5, 7, and 12 h), *S. aureus* NB (3, 5, and 15 h), *S. aureus* ASM (4.5, 6.25, and 15 h). These time points reflect approximately 50%, 75%, and 100% of respective maximum ODs where the cultures had entered the stationary phase. Samples were collected under sterile conditions in a laminar flow hood. All experiments were performed within a week with a minimum of five replicates for each condition and time point.
Headspace analysis
VOC analysis was conducted on a thermal desorption-gas chromatography–time-of-flight mass spectrometer (GC-TOF-MS) platform (Unity II TD with Ultra Autosampler, Markes International, and Micromass GCT Premier, Waters Corp, Manchester, UK). Prior to desorption, 100 μl of an internal standard (IS) (1 ppmV 4-bromofluorobenzene in N2; Thames Restek, Bucks, UK) was loaded onto each tube. This compound was used as an IS due to exhibiting similar chemical properties and vapour pressure to the analytes of interest and also being not naturally found in the samples as recommended in EPA method TO-17 (US Environmental Protection Agency 1999); we also use this in our clinical studies (van Oort et al 2017). VOCs were desorbed from sorbent tubes at 280 °C for 5 min, cryofocussed on a cold trap maintained at 10 °C and desorbed from the cold trap onto the GC (Agilent 6890N) column by flash heating to 280 °C for 3 min with a flow path temperature of 200 °C. The GC column (DB-5MS column, 30 m, 0.25 mm internal diameter, 50 μm film thickness, Agilent) was held at an initial temperature of 40 °C, ramped to 170 °C at 6 °C min⁻¹ and to 190 °C at 15 °C min⁻¹. A post run ramp to 250 °C was held for 2 min. The GC runtime was 23 min with a total TD cycle time of 40 min. The ToF-MS was in electron ionisation mode set at 70 eV. The source temperature was set to 200 °C, at a trap current of 200 μA, and spectra were acquired in dynamic range extension mode at 5 scans s⁻¹ over a range of 40–500 m/z. For E. coli minimal media experiments, and cyclopentanone authentic standard analysis, the same parameters were used albeit using a different analytical platform: a thermal desorption-gas chromatography triple quadrupole mass spectrometer (Markes TD-100 and 7010 series triple quadrupole GC/MS, Agilent technologies, Manchester, UK) as described in (van Oort et al 2017).

Data processing and analysis

Data pre-processing
GC-TOF-MS data were acquired and analysed using Masslynx (Waters Corp, Manchester, UK). Chromatographic peaks and mass spectra were cross-referenced with National Institute of Standards and Technology (NIST) library 14 for putative identification purposes, and followed the metabolomics standards initiative (MSI) guidelines for metabolite identification (Sumner et al 2007). Quanlynx (Waters Corp, Manchester, UK) was used as a quantitative tool for obtaining the response of peaks of interest with high NIST matching factor (>750 match). Masshunter quantitative analysis software (Agilent, Manchester, UK) was used to quantify the response of indole-d₈ for the minimal medium experiments.

Raw data in the manufacturer’s data format were converted into a netCDF format utilising the DataBridge software (Waters Corp, Manchester, UK). All statistical analyses were performed using R software (version 3.3.2; R Core Team 2016). The xts package was used to pre-process the netCDF files in R following the approach as outlined by Tautenhahn and colleagues (Tautenhahn et al 2008). Isotopologue parameter optimisation (IPO) was used to obtain optimised parameters for pre-processing raw data (Libiseller et al 2015). IPO is an R package which reiteratively pre-processes a selected number of netCDF files using a specified range of parameters and outputs the recommended parameters. The product of raw data pre-processing is a data file containing ion fragments, their corresponding m/z, retention times, and integrated areas. Normalisation using the IS, 4-bromofluorobenzene, was based on the 174 m/z parent ion.

Univariate and multivariate analyses
Where applicable, for univariate analysis the non-parametric Kruskal–Wallis test was performed. The Games–Howell post hoc test was subsequently used to investigate statistically significant bacterial groups. A critical α = 0.05 value was used in tests.

For multivariate analysis, normalised data were log₁₀ transformed and autoscaled. Principal component-discriminant function analysis (PC-DFA) was then applied to find differences between bacterial groups and the variables that contribute to this property. Briefly, the concept of PC-DFA is to maximise the variation between groups and minimise the variation within groups (Goodacre et al 1998, Jombart et al 2010). Principal components (PCs) are used as input variables so PCA is performed initially on the X block (VOC profiles) and this is the variable that is tuned for optimisation. The R package adegenet was used for this analysis (Jombart 2008). In order to determine the number of PCs to retain, the dataset was split into 70% training set and 30% test set by stratified random sampling. PC-DFA was then performed on the training set using varying numbers of PCs, and the ability of each PC-DFA model to accurately predict new membership (test set) was then evaluated to select the optimal number of PCs. At each level of selected PCs, the procedure was repeated 1000 times. Thirty PCs were included for DFA as it achieved the lowest root mean square error (RMSE). This proportion accounted for 86.9% of conserved variance extracted from the dataset. For validation purposes, again the dataset was split into 70% training and 30% test sets. A PC-DFA model was built using the training set and the test set was then projected into the subspace created by the training set to visualise the prediction of the test data on the basis of proximity to the training samples originating from the same bacterial groups.
Results

Growth curves

Figure 1 depicts the growth response of distinct bacterial species when cultured in ASM and NB. For *E. coli*, it can be observed that growth in both media reached a similar maximum OD, but a prolonged lag phase is observed for bacteria cultured in ASM. *P. aeruginosa* grown in ASM, has an extended log phase and thus almost twice the biomass in comparison to the NB culture. For *S. aureus*, growth patterns in both media appear similar while also obtaining comparable ODs. Growth was only observed for *K. pneumoniae* in NB and not in ASM.

VOC profiling in ASM and NB cultures

The VOCs from the headspace of the bacterial cultures putatively identified using the NIST library are listed in the table 1. Some of these markers such as indole (figure 2(a)) have been previously reported in literature and others have not been associated with the pathogens in this study (figure 3). The mass spectra of one of the novel VOCs, cyclopentanone, has been compared to an authentic standard and matches in terms of mass spectra and retention time. Thus this compound is considered as MSI level 1. The putatively annotated compounds are considered MSI level 2.

Indole production by *E. coli*

Indole was detected in the headspace of *E. coli* cultures with a higher level observed in the NB headspace in comparison to ASM (figure 2(a)). The enzyme tryptophanase (EC 4.1.99.1) (encoded by *tnaA* gene) is stated to catalyse the reaction of converting tryptophan to indole (Goodacre and Kell 1993). To confirm this, a wild type (WT) *E. coli*, along with a *tnaA* knockout (KO), and *trpR* KO strains were grown in minimal medium spiked with deuterated tryptophan (tryptophan-d₅). Figure S1 is available online at stacks.iop.org/JBR/12/026002/mmedia shows the growth curves of the WT *E. coli* and KO strains in minimal medium. *trpR* gene encodes the tryptophan repressor. These KO strains were selected as they exhibited decreased indole levels on performing KOVACs test for indole production (see figure S2). Accordingly, high levels of indole-d₅ were observed in the WT and low concentrations were sampled in the KO strain’s headspace (figure 2(b)) thus confirming the identity and origin of this VOC.

Origin of new markers

To establish the origin of cyclopentanone, *P. aeruginosa* was grown in variants of ASM where ingredients were sequentially omitted. Cyclopentanone was observed in the headspace of all media types except media lacking salmon sperm DNA (figure 4). Similarly, furfural was also absent in the headspace of *P. aeruginosa* cultured in DNA-free media (figure 4). Individual growth curves can be seen in the supplementary information (figure S3).
Table 1. VOCs identified from the headspace of bacterial cultures.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>VOC</th>
<th>Growth medium</th>
<th>MSI level</th>
<th>In vitro studies</th>
<th>Breath studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>indole</td>
<td>Both</td>
<td>2</td>
<td>(Allardyce et al 2006, Zscheppank et al 2014)</td>
<td>—</td>
</tr>
<tr>
<td>E. coli</td>
<td>1-hexanol</td>
<td>ASM</td>
<td>2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>3-methyl-1-butanol</td>
<td>NB</td>
<td>2</td>
<td>(Bees et al 2017)</td>
<td>—</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>1-undecene</td>
<td>Both</td>
<td>2</td>
<td>(Labows et al 1980, Zscheppank et al 2014)</td>
<td>—</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>thioncyanic acid, methyl ester</td>
<td>Both</td>
<td>2</td>
<td>(Shestivska et al 2011)</td>
<td>(Shestivska et al 2011)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>cyclopentanol</td>
<td>ASM</td>
<td>2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E. coli, P. aeruginosa</td>
<td>cyclopentanone</td>
<td>ASM</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>2-furanmethanol</td>
<td>ASM</td>
<td>2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>K. pneumonia, S. aureus</td>
<td>3-methylbutanal</td>
<td>NB</td>
<td>2</td>
<td>(Filipiak et al 2012)</td>
<td>—</td>
</tr>
<tr>
<td>E. coli, P. aeruginosa, S. aureus</td>
<td>2-cyclopenten-1-one</td>
<td>ASM</td>
<td>2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E. coli, P. aeruginosa, S. aureus</td>
<td>dimethyl disulfide</td>
<td>Both</td>
<td>2</td>
<td>(Labows et al 1980, Allardyce et al 2006)</td>
<td>—</td>
</tr>
<tr>
<td>E. coli, P. aeruginosa, S. aureus</td>
<td>dimethyl trisulfide</td>
<td>Both</td>
<td>2</td>
<td>(Labows et al 1980)</td>
<td>—</td>
</tr>
</tbody>
</table>
Multivariate analysis

PC-DFA was utilised to investigate the most discriminant VOCs that can be used to differentiate bacterial species. Included for DFA were 30 PCs which achieved the lowest RMSE and accounted for 86.9% variance in the dataset (see scree plot in figure S5). For validation, a PC-DFA model was built using the training set and the test set was subsequently projected into the PC-DFA space created by the training set to estimate the validity of the model (figure 5). The test data appears congruent with the training data for the various groups and separation of bacterial species along DF1 appears to be based on differences due to media type. From the loadings plot (figure S6), fragments from 2-cyclopenten-1-one and 1-hexanol were found to be contributing factors to separation along DF1.

Discussion

Three time points was selected to collect the headspace of bacterial cultures; two at different stages of the growth phase and one in the stationary phase. Several of the VOCs observed were found to be specific to individual pathogens, such as indole (E. coli), 1-undecene and methyl ester thiocyanate (P. aeruginosa), and 3-methyl-1-butanol (K. pneumoniae), whilst others were identified in several species, such as 3-methylbutanal (K. pneumoniae, S. aureus), dimethyl disulfide (E. coli, P. aeruginosa, S. aureus), and dimethyl trisulfide (E. coli, P. aeruginosa, S. aureus).

Indole was detected in the headspace of E. coli cultures throughout the growth period, consistent with recently reported literature (Zhu et al 2010, Tait et al 2014, Zscheppank et al 2014). Indole is a well characterised metabolite stated to originate from the metabolism of tryptophan by the enzyme tryptophanase (EC 4.1.99.1) which is present in E. coli (Goodacre and Kell 1993). In this study, verification of its bacterial origin was demonstrated: deuterated indole (indole-\textit{d}5) was observed in the headspace of the WT E. coli when it was grown with tryptophan-\textit{d}5 (figure 2(b)). Moreover indole levels diminished in the \textit{tnaA} KO, which lacks the gene that codes for the enzyme that catalyses the conversion of tryptophan and produces indole (Li and Young 2013). A slightly higher concentration was observed in the \textit{trpR} KO strain in comparison to the \textit{tnaA} KO. The \textit{trpR} gene encodes the tryptophan repressor which functions to bind tryptophan in the cell thereby acting as a transcription regulator of the tryptophan biosynthetic pathway in E. coli (Arvidson et al 1994). Since the gene has been knocked out, tryptophan synthesis should continue; however, the high abundance of tryptophan-\textit{d}5 and the potential production of...
endogenous tryptophan did not translate into a high indole concentration in the headspace. Thus, it could be that the loss of trpR function has compromised other clearance mechanisms of tryptophan such as conversion to indole and may explain the reduced amount of indole observed.

The growth patterns of *P. aeruginosa* in both media were distinct, with the ASM culture achieving a higher growth density in comparison to the NB culture. This phenomenon may be a factor that contributes to an individual’s susceptibility to infection. The VOCs 1-undecene and methyl ester thiocyanate were found to be produced specifically by *P. aeruginosa*. Both metabolites increased in concentration during growth. Other studies have also reported 1-undecene as a marker for *P. aeruginosa* (Labows *et al* 1980, Goeminne *et al* 2012, Boots *et al* 2014, Zscheppank *et al* 2014). Zscheppank and colleagues (Zscheppank *et al* 2014) reported that when *P. aeruginosa* was grown in three different media, the time of detection and also the response in terms of observed integrated area was different. Methyl ester thiocyanate has also been reported in *in vitro* studies and has been found in the breath of CF patients (Shestivska *et al* 2011). The fact that these markers have been identified in this study alongside reports elsewhere provides considerable encouragement that these VOCs are bacteria-specific, detected following bacterial growth under different environmental conditions and by different analytical techniques.

Culture environment may influence the emitted VOC profile, as is apparent for pathogens grown in ASM. Initially, two markers were observed in the ASM cultures which to our knowledge have not yet being associated with the bacterial species investigated in this study. One of these markers, cyclopentanone, was emitted in both *E. coli* and *P. aeruginosa* cultures (figure 3). The phenotypes of *P. aeruginosa* were

![Figure 3. Boxplots showing the internal standard (IS) normalised concentrations of cyclopentanone, cyclopentanol, 2-cyclopenten-1-one, 2-furanmethanol and 1-hexanol in nutrient broth (NB), artificial sputum medium (ASM), bacteria-media conditions (e.g. *E. coli*-ASM (Ec-ASM) and sorbent only blank (S)). After performing a Kruskal–Wallis test, a Games–Howell test was used for post hoc analyses. Statistical significance: NS non-significant; * denotes 0.05 < p < 0.01; and ** p < 0.01. The boxplots were generated from six repeats.](image-url)
observed to be different as we visually noticed microcolonies in the ASM culture and not in the NB culture. *P. aeruginosa* has been stated to exist in this biofilm-like state in the CF lung (Sriramulu et al 2005). This compound has been reported to be emitted by soil microbes especially the actinomycetes (Scholler et al 2002). The other marker, 1-hexanol, was observed mainly in *E. coli*-ASM culture.

Cyclopentanone and furfural were absent in the headspace of *P. aeruginosa* that had been cultured in ASM without DNA (figure 5). Furfural has been reported to be toxic to microorganisms and is readily converted into the less toxic 2-furanmethanol (also known as furfuryl alcohol) (Boopathy et al 1993). Furthermore, 2-cyclopenten-1-one and cyclopentanol have been postulated as breakdown products of furfural, albeit using catalysts (Yang et al 2013, Guo et al 2014, Hronec et al 2014, Zhu et al 2014). It is possible that enzymes within the microbes are also able to make these conversions. The extent of furfural metabolism may depend on the specific bacterium: 2-furanmethanol and 2-cyclopenten-1-one were observed in the headspace of *S. aureus* and *E. coli*, cyclopentanone was additionally observed in the

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**Figure 4.** Boxplots showing the internal standard (IS) normalised concentrations of cyclopentanone and furfural when in full ASM, and modified ASM. The error bars show standard deviations based on three measurements.

**Figure 5.** PC-DFA scores validation biplot. For DFA this used 30 PCs (accounting for 86.9% of total explained variance) along with the information on the seven classes used as the *a priori* information. Training samples from the distinct bacterial cultures are indicated as small shapes and the corresponding larger shapes are the projected test samples.
latter, whilst *P. aeruginosa* appeared to produce both cyclopentanone and cyclopentanol, and consume 2-furanmethanol. A proposed catabolic breakdown pathway is shown in figure 6. Future work should be aimed at confirming this hypothesised breakdown of furfural. Furfural spiking during growth in minimal media should yield an increase in the proposed markers, and additional molecular biology studies to knock out gene encoding enzymes may further elucidate the mechanism of furfural breakdown.

The metabolite 3-methyl-1-butanol appears to be specific to the headspace of *K. pneumoniae* (figure S4), consistent with recent findings elsewhere (Rees et al. 2017), however reports of its emission by other bacterial species may ultimately limit its utility as a VAP marker (Filipiak et al. 2012, McNerney et al. 2012, Tait et al. 2014). The abundance and diversity of nutrients in NB in comparison to ASM may explain the lack of growth in the latter medium and may indicate that the availability of certain components is necessary to enable the growth of this bacterium. The PC-DFA scores plot (figure 5) shows separation based on different growth media type, with 2-cyclopenten-1-one and 1-hexanol causing separation along DF1 (see DFA loadings plot in figure S6). ASM cultures of *P. aeruginosa* and *S. aureus* appear to be in close proximity in figure 5 which may reflect similar emission strengths of 2-cyclopenten-1-one, whilst the separation of the *E. coli*-ASM culture can be likely attributed to production of both 2-cyclopenten-1-one and 1-hexanol. Unidentified compounds may also be relevant to this multivariate analysis and require further study.

Several markers were not observed in the present study that have been stated in literature (Bos et al. 2013). This may be attributed to the hydrophobic sorbent selected for this study which is known not to quantitatively trap highly volatile compounds such as hydrogen cyanide, previously reported as a marker for *P. aeruginosa* (Enderby et al. 2009, Gilchrist et al. 2011, 2013). We are currently participating in a multi-centre clinical study in which we are collecting the exhaled breath of patients suspected of VAP (van Oort et al. 2017). The VOCs reported here can be targeted in the data analysis of studies such as this to investigate their diagnostic efficacy. These markers are also potentially useful in rapid headspace analyses of sputum and/or bronchoalveolar lavage specimens which will likely confer an advantage over current, often slow, culture methods.

**Conclusion**

Lower respiratory infections commonly occur in the ICU, of which VAP is an example. As diagnosis of this infection is invasive and can be prolonged, impacting on economic costs as well as affecting mortality and morbidity, an efficient and accurate method of diagnosis would be advantageous. In this study, the headspace from several bacterial species associated with VAP were cultivated and analysed to identify VOCs that could potentially be used for discrimination of these bacteria. To better represent *in vivo* conditions, bacteria were cultured in ASM, culminating in the generation and identification of novel markers which include 2-cyclopenten-1-one, cyclopentanone, cyclopentanol, and 1-hexanol. If the species-specific VOCs measured in this study were identified in the exhaled breath of patients, or indeed the headspace of airway sample cultures, then there is potential to use breathomics as a powerful alternative method for diagnosis of VAP, eliminating the element of invasiveness and potentially facilitating a rapid diagnosis.

There is mounting evidence to support the existence of bacteria-specific VOCs based on reports from studies that used different growth conditions and employed various analytical methodologies. There is great potential for bacteria-specific VOC utilisation as a non-invasive point-of-care diagnostic tool and although exact *in vivo* conditions can be difficult to replicate, studies such as this yield important information relevant to the search for disease biomarkers.

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