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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 healthcare-acquired infection arising from the invasion of the lower respiratory tract by opportunistic pathogens in ventilated patients. The current method of diagnosis requires the culture of an airway sample such as bronchoalveolar lavage, which is invasive to obtain and may take up to seven days to identify a causal pathogen, or indeed rule out infection. While awaiting results, patients are administered empirical antibiotics; risks of this approach include lack of effect on the causal pathogen, contribution to the development of antibiotic resistance and downstream effects such as increased length of intensive care stay, cost, morbidity and mortality. Specific biomarkers which could identify causal pathogens in a timely manner are needed as they would allow judicious use of the most appropriate antimicrobial therapy. Volatile organic compound (VOC) analysis in exhaled breath is proposed as an alternative due to its non-invasive nature and its potential to provide rapid diagnosis at the patient's bedside. VOCs in exhaled breath originate from exogenous, endogenous, as well as microbial sources. To identify potential markers, VAP-associated pathogens *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were cultured in both artificial sputum medium and nutrient broth, and their headspaces were sampled and analysed for VOCs. Previously reported volatile markers were identified in this study, including indole and 1-undecene, alongside compounds that are novel to this investigation, cyclopentanone and 1-hexanol. We further investigated media components (substrates) to identify those that are essential for indole and cyclopentanone production, with potential implications for understanding microbial metabolism in the lung.

Introduction

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 (Safdar *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 (Safdar *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), diethylenetriaminepentaacetic 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 N_2; 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, cryo-focussed 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, 0.25 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-d_5 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 *xcms* 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 $\alpha = 0.05$ value was used in tests.

For multivariate analysis, normalised data were $\log_{10}$ 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.

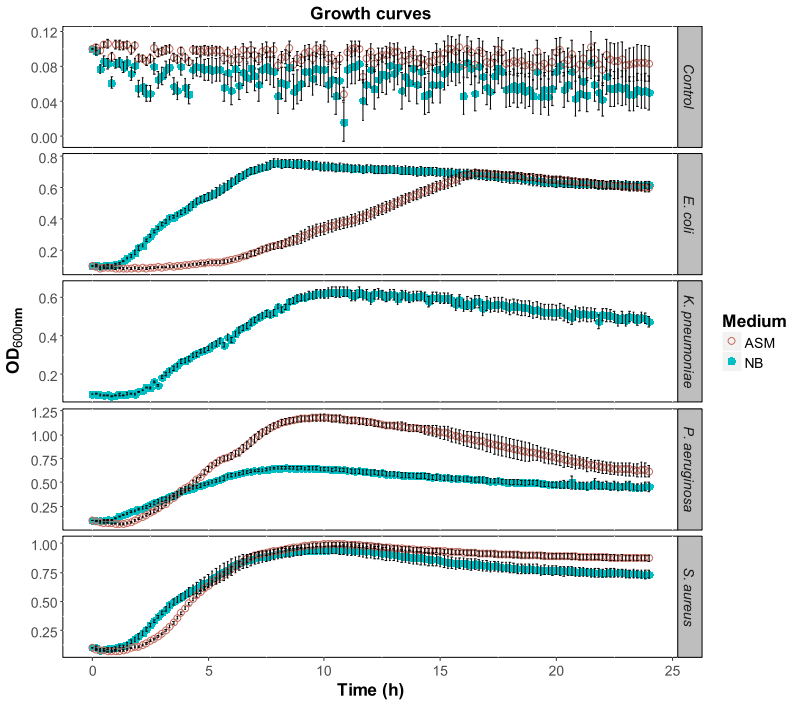

Figure 1. Growth curves of distinct bacterial species grown in nutrient broth (NB) and artificial sputum medium (ASM). Optical density (OD_{600nm}; arbitrary units) versus time (h) is shown. Individual growth curves are averages of 10 replicates, and the error bars show standard deviations.

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-d_5). 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-d_5 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.

Bacteria	VOC	Growth medium	MSI level	*In vitro* studies	Breath studies
E. coli	indole	Both	2	(Allardyce *et al* 2006, Zscheppank *et al* 2014)	—
E. coli	1-hexanol	ASM	2	—	—
K. pneumonia	3-methyl-1-butanol	NB	2	(Rees *et al* 2017)	—
P. aeruginosa	1-undecene	Both	2	(Labows *et al* 1980, Zscheppank *et al* 2014)	—
P. aeruginosa	thiocyanic acid, methyl ester	Both	2	(Shestivska *et al* 2011)	(Shestivska *et al* 2011)
P. aeruginosa	cyclopentanol	ASM	2	—	—
E. coli, P. aeruginosa	cyclopentanone	ASM	1	—	—
E. coli, S. aureus	2-furanmethanol	ASM	2	—	—
K. pneumoniae, S. aureus	3-methylbutanal	NB	2	(Filipiak *et al* 2012)	—
E. coli, P. aeruginosa, S. aureus	2-cyclopenten-1-one	ASM	2	—	—
E. coli, P. aeruginosa, S. aureus	dimethyl disulfide	Both	2	(Labows *et al* 1980, Allardyce *et al* 2006)	—
E. coli, P. aeruginosa, S. aureus	dimethyl trisulfide	Both	2	(Labows *et al* 1980)	—

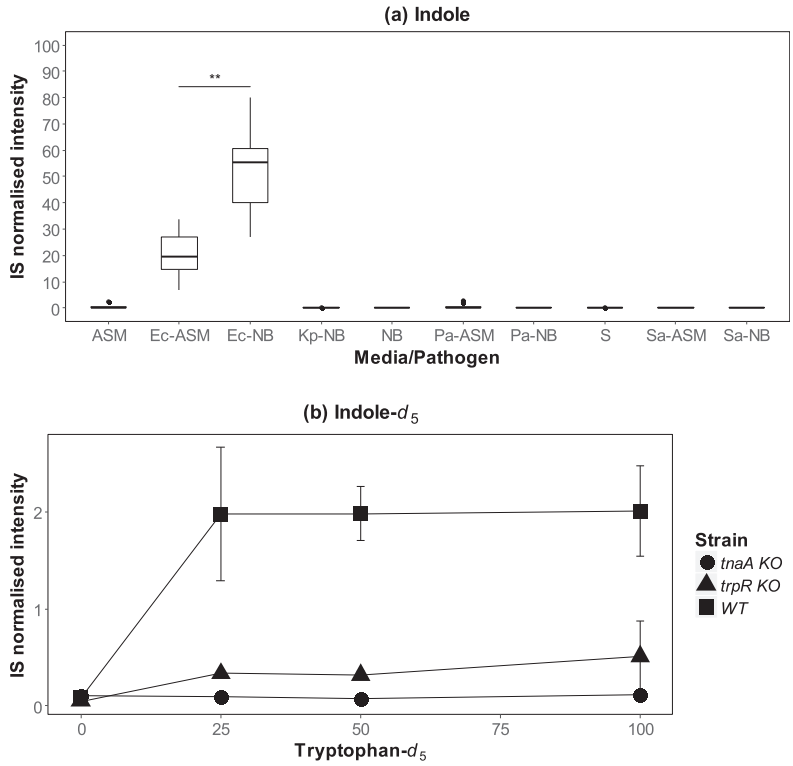

Figure 2. (a) Boxplot showing the internal standard (IS) normalised concentrations of indole in nutrient broth (NB), artificial sputum medium (ASM), bacteria-media conditions (e.g. *E. coli*-ASM (Ec-ASM), and sorbent only blank (S)). (b) Concentrations of indole-d_5 in wildtype *E. coli*, *tnaA* and *trpR* knockouts (KOs). After performing a Kruskal–Wallis test, a Games–Howell test was utilised for post hoc analyses. Statistical significance: NS non-significant; * denotes $0.05 < p \geq 0.01$; and ** $p < 0.01$. The error bars show standard deviations from six and three repeats, respectively.

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-d_5) was observed in the headspace of the WT *E. coli* when it was grown with tryptophan-d_5 (figure 2(b)). Moreover indole levels diminished in the *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 *trpR* KO strain in comparison to the *tnaA* KO. The *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-d_5 and the potential production of

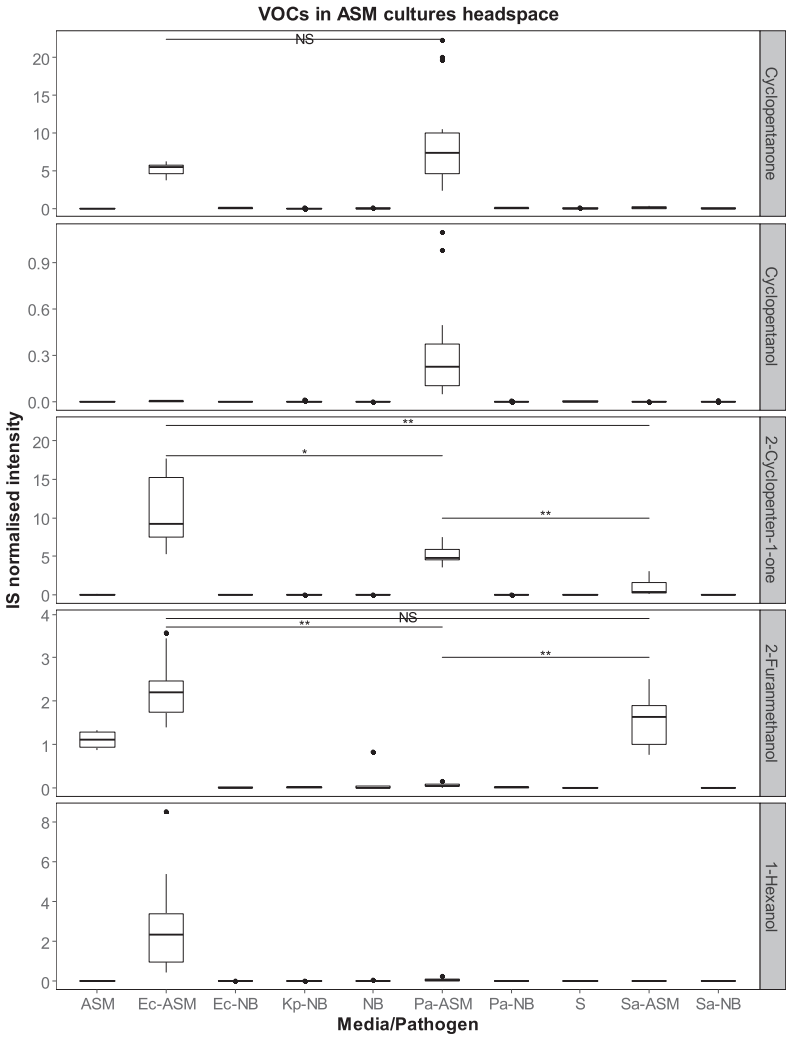

Figure 3. Boxplots showing the internal standard (IS) normalised concentrations of cyclopentanone, cyclopentanol, 2-cyclopenten-1-one, 2-furanmethanol and 1-hexanol in nutrient broth (NB), artificial sputum medium (ASM), bacteria-media conditions (e.g. *E. coli*-ASM (Ec-ASM) and sorbent only blank (S)). After performing a Kruskal–Wallis test, a Games–Howell test was used for post hoc analyses. Statistical significance: NS non-significant; * denotes $0.05 < p \geq 0.01$; and ** $p < 0.01$. The boxplots were generated from six repeats.

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

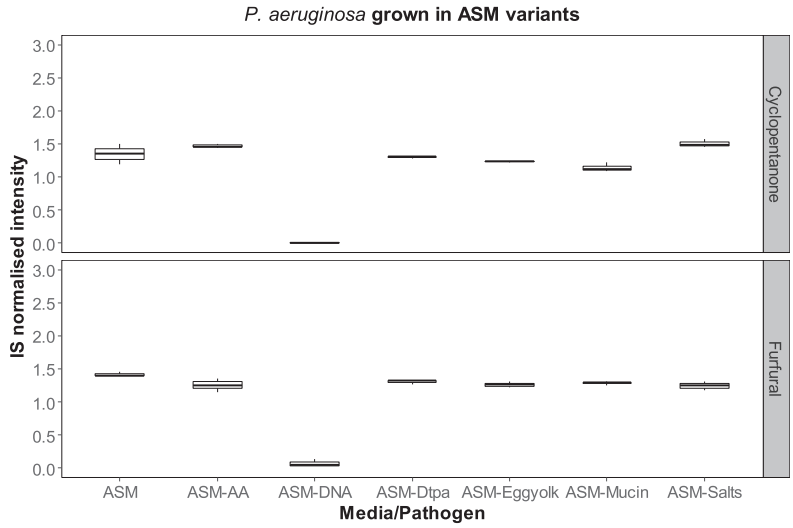

Figure 4. Boxplots showing the internal standard (IS) normalised concentrations of cyclopentanone and furfural when in full ASM, and modified ASM. The error bars show standard deviations based on three measurements.

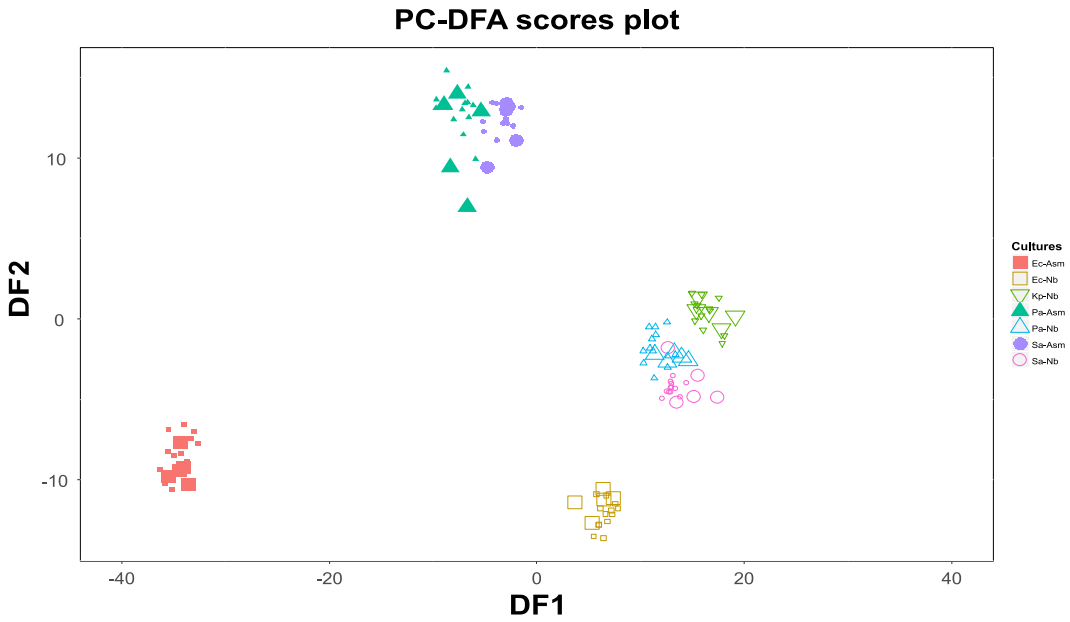

Figure 5. PC-DFA scores validation biplot. For DFA this used 30 PCs (accounting for 86.9% of total explained variance) along with the information on the seven classes used as the *a priori* information. Training samples from the distinct bacterial cultures are indicated as small shapes and the corresponding larger shapes are the projected test samples.

observed to be different as we visually noticed micro-colonies 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

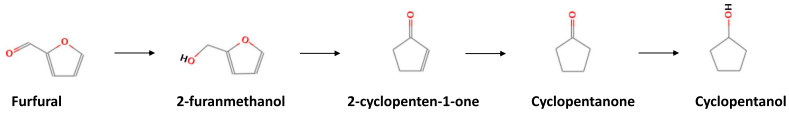

Figure 6. Hypothesised catabolic breakdown of furfural for the generation of novel VOCs. Adapted from (Yang *et al* 2013, Zhu *et al* 2014). Molecular structures were obtained from NIST webbook (webbook.nist.gov/chemistry/).

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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References

Ahmed W M, Lawal O, Nijsen T M, Goodacre R and Fowler S J 2017 Exhaled volatile organic compounds of infection: a systematic review *ACS Infect. Dis.* accepted (https://doi.org/10.1021/acsinfecdis.7b00088)
Allardyce R A, Langford V S, Hill A L and Murdoch D R 2006 Detection of volatile metabolites produced by bacterial growth in blood culture media by selected ion flow tube mass spectrometry (SIFT-MS) *J. Microbiol. Methods* **65** 361–5
Arvidson D N, Arvidson C G, Lawson C L, Miner J, Adams C and Youderian P 1994 The tryptophan repressor sequence is highly conserved among the *Enterobacteriaceae Nucleic Acids Res.* **22** 1821–9
Boopathy R, Bokang H and Daniels L 1993 Biotransformation of furfural and 5-hydroxymethyl furfural by enteric bacteria *J. Ind. Microbiol.* **11** 147–50
Boots A W, Bos L D, van Der Schee M P, van Schooten F J and Sterk P J 2015 Exhaled molecular fingerprinting in diagnosis and monitoring: validating volatile promises *Trends Mol. Med.* **21** 633–44
Boots A W, Smolinska A, van Berkel J J, Fijten R R, Stobberingh E E, Boumans M L, Moonen E J, Wouters E F, Dallinga J W and van Schooten F J 2014 Identification of microorganisms based on headspace analysis of volatile organic compounds by gas chromatography-mass spectrometry *J. Breath Res.* **8** 027106
Bos L D, Sterk P J and Schultz M J 2013 Volatile metabolites of pathogens: a systematic review *PLoS Pathog.* **9** e1003311
Buszewski B, Ulanowska A, Ligor T, Jackowski M, Klodzinska E and Szeliga J 2008 Identification of volatile organic compounds secreted from cancer tissues and bacterial cultures *J. Chromatogr.* B **868** 88–94
Diraviam Dinesh S 2010 Artificial sputum medium
Enderby B, Smith D, Carroll W and Lenney W 2009 Hydrogen cyanide as a biomarker for *Pseudomonas aeruginosa* in the breath of children with cystic fibrosis *Pediatr. Pulmonol.* **44** 142–7
Filipiak W, Sponring A, Baur M M, Filipiak A, Ager C, Wiesenhofer H, Nagl M, Troppmair J and Amann A 2012 Molecular analysis of volatile metabolites released specifically by *Staphylococcus aureus* and *Pseudomonas aeruginosa BMC Microbiol.* **12** 113
Gilchrist F J, Alcock A, Belcher J, Brady M, Jones A, Smith D, Spanel P, Webb K and Lenney W 2011 Variation in hydrogen cyanide production between different strains of *Pseudomonas aeruginosa Eur. Respir. J.* **38** 409–14
Gilchrist F J, Bright-Thomas R J, Jones A M, Smith D, Spanel P, Webb A K and Lenney W 2013 Hydrogen cyanide concentrations in the breath of adult cystic fibrosis patients with and without *Pseudomonas aeruginosa* infection *J. Breath Res.* **7** 026010
Goeminne P C, Vandendriessche T, van Eldere J, Nicolai B M, Hertog M L and Dupont L J 2012 Detection of *Pseudomonas aeruginosa* in sputum headspace through volatile organic compound analysis *Respir. Res.* **13** 87
Goodacre R and Kell D B 1993 Rapid and quantitative-analysis of bioprocesses using pyrolysis mass-spectrometry and neural networks—application to indole production *Anal. Chim. Acta* **279** 17–26
Goodacre R, Timmins E M, Burton R, Kaderbhai N, Woodward A M, Kell D B and Rooney P J 1998 Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks *Microbiology* **144** 1157–70
Guo J H, Xu G Y, Han Z, Zhang Y, Fu Y and Guo Q X 2014 Selective conversion of furfural to cyclopentanone with CuZnAl catalysts *Acs Sustainable Chem. Eng.* **2** 2259–66
Hronec M, Fulajtarova K and Sotak T 2014 Highly selective rearrangement of furfuryl alcohol to cyclopentanone *Appl. Catalysis* B **154** 294–300
Hunter J D 2012 Ventilator associated pneumonia *Brit. Med. J.* **344** e3325
Jombart T 2008 adegenet: a R package for the multivariate analysis of genetic markers *Bioinformatics* **24** 1403–5
Jombart T, Devillard S and Balloux F 2010 Discriminant analysis of principal components: a new method for the analysis of genetically structured populations *BMC Genetics* **11** 94
Kalanuria A A, Ziai W and Mirski M 2014 Ventilator-associated pneumonia in the ICU *Crit. Care* **18** 208
Koenig S M and Truwit J D 2006 Ventilator-associated pneumonia: diagnosis, treatment, and prevention *Clin. Microbiol. Rev.* **19** 637–57
Labows J N, Mcginley K J, Webster G F and Leyden J J 1980 Headspace analysis of volatile metabolites of *Pseudomonas aeruginosa* and related species by gas chromatography-mass spectrometry *J. Clin. Microbiol.* **12** 521–6
Li G and Young K D 2013 Indole production by the tryptophanase TnaA in *Escherichia coli* is determined by the amount of exogenous tryptophan *Microbiology* **159** 402–10
Libiseller G *et al* 2015 IPO: a tool for automated optimization of XCMS parameters *BMC Bioinform.* **16** 118
McNerney R, Mallard K, Okolo P I and Turner C 2012 Production of volatile organic compounds by mycobacteria *Fems Microbiol. Lett.* **328** 150–6
Park D R 2005 The microbiology of ventilator-associated pneumonia *Respir. Care* **50** 742–63
Rees C A, Franchina F A, Nordick K V, Kim P J and Hill J E 2017 Expanding the *Klebsiella pneumoniae* volatile metabolome using advanced analytical instrumentation for the detection of novel metabolites *J. Appl. Microbiol.* **122** 785–95
Safdar N, Crnich C J and Maki D G 2005 The pathogenesis of ventilator-associated pneumonia: its relevance to developing effective strategies for prevention *Respir. Care* **50** 725–39
Scholler C E, Gurtler H, Pedersen R, Molin S and Wilkins K 2002 Volatile metabolites from actinomycetes *J. Agric. Food Chem.* **50** 2615–21
Shestivska V, Nemec A, Drevinek P, Sovova K, Dryahina K and Spanel P 2011 Quantification of methyl thiocyanate in the headspace of *Pseudomonas aeruginosa* cultures and in the breath of cystic fibrosis patients by selected ion flow tube mass spectrometry *Rapid Commun. Mass Spectrom.* **25** 2459–67
Sriramulu D D, Lunsdorf H, Lam J S and Romling U 2005 Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung *J. Med. Microbiol.* **54** 667–76
Sumner L W *et al* 2007 Proposed minimum reporting standards for chemical analysis chemical analysis working group (CAWG) metabolomics standards initiative (MSI) *Metabolomics* **3** 211–21
Tait E, Perry J D, Stanforth S P and Dean J R 2014 Identification of volatile organic compounds produced by bacteria using HS-SPME-GC-MS *J. Chromatogr. Sci.* **52** 363–73
Tautenhahn R, Bottcher C and Neumann S 2008 Highly sensitive feature detection for high resolution LC/MS *BMC Bioinform.* **9** 504
US Environmental Protection Agency 1999 Compendium method TO-17 determination of volatile organic compounds in ambient air using active sampling onto sorbent tubes EPA/

625/R-96/101b (www3.epa.gov/ttnamti1/files/ambient/airtox/to-17r.pdf)

van Oort P M *et al* 2017 BreathDx—molecular analysis of exhaled breath as a diagnostic test for ventilator-associated pneumonia: protocol for a European multicentre observational study *BMC Pulm. Med.* **17** 1

Yang Y L, Du Z T, Huang Y Z, Lu F, Wang F, Gao J and Xu J 2013 Conversion of furfural into cyclopentanone over Ni–Cu bimetallic catalysts *Green Chem.* **15** 1932–40

Zhu H Y, Zhou M H, Zeng Z, Xiao G M and Xiao R 2014 Selective hydrogenation of furfural to cyclopentanone over Cu–Ni–Al hydrotalcite-based catalysts *Korean J. Chem. Eng.* **31** 593–7

Zhu J, Bean H D, Kuo Y M and Hill J E 2010 Fast detection of volatile organic compounds from bacterial cultures by secondary electrospray ionization-mass spectrometry *J. Clin. Microbiol.* **48** 4426–31

Zscheppank C, Wiegand H L, Lenzen C, Wingender J and Telgheder U 2014 Investigation of volatile metabolites during growth of *Escherichia coli* and *Pseudomonas aeruginosa* by needle trap-GC-MS *Anal. Bioanal. Chem.* **406** 6617–28