**Volatile organic compound signature from co-culture of lung epithelial cell line with *Pseudomonas aeruginosa***

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**Abstract**

Bacteria are found ubiquitously within and on nearly every site within humans, including the airways. Microbes interact with airway epithelial cells in lung infections such as ventilator-associated pneumonia (VAP). Development of infection results in the production of oxidants such as hydrogen peroxide that may further damage the epithelium. VAP is difficult to diagnose and associated with significant mortality. Current methods are invasive and time consuming impacting on appropriate therapy, antimicrobial resistance and financial costs. Volatile organic compound (VOC) analysis in exhaled breath is proposed as a tool for early detection due to its non-invasive property and potential to facilitate timely diagnosis. To investigate potential early VOC markers, A549 epithelial cells that were originally isolated from human alveoli were cultured with and without *Pseudomonas aeruginosa,* and the headspace of the culture vessel analysed using sorbent-based capture of VOCs followed by thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) in order to identify potential discriminatory VOCs. A549 cells were also cultured with hydrogen peroxide to induce oxidative stress in order to investigate potential biomarkers of epithelial cell damage. Previously reported VOCs including acetone and ethanol were observed from the infection experiment along with novel bacterial markers, which we identified as mostly ether based compounds. Alkanes such as decane and octane were also found to be elevated after hydrogen peroxide treatment of A549 cells, likely as a result of peroxidation of oleic acids.

**Introduction**

The airway epithelium serves as a physical barrier against environmental particles and microorganisms 1. Mechanical defenses such as the cough reflex and mucociliary clearance provide additional support in maintaining airway health 2. The insertion of endotracheal tubes when critically ill patients require mechanical ventilation compromises the aforementioned mechanical defenses and may cause injury to the epithelium 3 , and is associated with colonisation of the airways by opportunistic pathogens such as *Pseudomonas aeruginosa* and subsequent development of serious iatrogenic infections such as ventilator-associated pneumonia (VAP).

Diagnosis of VAP is invasive, time consuming, and lacks adequate sensitivity and specificity 4, 5. Exhaled breath volatile organic compound (VOC) analysis is proposed as an alternative for non-invasive diagnosis 6, 7. Establishing links between *in vitro* and *in vivo* results will be important to aid biomarker discovery and validation, as previously demonstrated 8, 9. We also share this view and have performed several *in vitro* bacterial cultures to investigate potential VOC biomarkers. The goal is to unify results from this study and others 10 with results from an ongoing clinical study 11.

To add to the body of knowledge of possible biomarkers, we hypothesise that the interaction between microbes and the epithelium may yield diagnostic VOCs that could facilitate timely administration of appropriate antimicrobials. Genomic studies have shown differential expression of certain genes in A549 epithelial cells when cultured with *P. aeruginosa 12*. These genomic changes may therefore result in downstream modification of released metabolites. We have therefore cultured *P. aeruginosa* with A549 lung epithelial cell line and the headspace VOC profile recovered and analysed by thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS).

Furthermore, we investigated volatile markers of oxidative stress and subsequent epithelial cell damage, as occurs with progression of infection. Oxidative stress is typically due to production of reactive oxygen species (ROS) such as superoxide (O2-) and hydrogen peroxide (H2O2) by activated neutrophils responding to foreign agents such as bacteria 13. These ROS are known to cause damage to macromolecules within cells. In this study, A549 cells were treated with H2O2 to induce oxidative stress and the headspace subsequently analysed for potentially discriminatory VOCs in comparison to untreated cells.

Adherent cells such as the A549 epithelial cells used in this study are typically cultured in plastic tissue flasks. Unfortunately the plastic emits unwanted volatile contaminants 14 and thus these culture vessels might compromise the integrity of the sampled headspace. To circumvent this problem, we have performed experiments using a glass bottle to culture these cells which may be more ideal for VOCs measurement.

**Materials and methods**

*Cell line and bacteria culturing*

The human lung carcinoma A549 epithelial cell line (ATCC CCL 185) was used in this study. The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM 1X, Gibco®, Paisley, UK) supplemented with foetal bovine serum (FBS, Gibco®, EU Approved Origin: South America), pen-strep (5 mL containing 10,000 Units/mL Penicillin, 10,000 µg/mL Streptomycin, Gibco®), and GlutaMAXTM-l (5 mL, 100X, Gibco®) were incubated at 37 °C in 5 % CO2 (Forma, Steri-Cycle i160 CO2 incubator, Thermo Scientific). Passaging of cells occurred every 3-4 days and maintained in T75 flasks (Thermo Scientific, NuncTM, EasYFlaskTM, Kamstrupvej, Roskilde, Denmark). Briefly, the medium was removed using a combination of a suction pump and aspiration pipette. The cells were then washed twice with Dulbecco’s Phosphate Buffered Saline (DPBS 1X, Gibco®) and subsequently detached from cell culture vessels using 0.25 % Trypsin-EDTA (1X, Gibco®). Cell count was performed using a Vi-CELLTM XR Cell Viability Analyser (Beckman Coulter, Fullerton, CA, USA) which uses a Trypan blue exclusion technique to distinguish between viable and dead cells 15.

Freeze-dried stock of *P. aeruginosa* ATCC 10145 was obtained from American Type Culture Collection. This strain was cultured on tryptic soy agar (TSA; Oxoid, Basingstone, UK) to obtain axenic colonies. Liquid cultures were obtained by inoculating single isolated colonies into tryptic soy broth (TSB; Oxoid).

*Infection and treatment optimisation*

A multiplicity of infection (MOI) of 50 and 100 were tested for the *P. aeruginosa* infection experiment as they are commonly utilised in literature 12, 16. MOI is the ratio of infecting agent (bacteria) to target cell (epithelial cell). A549 cells were initially grown in T25 flasks (Thermo Scientific, NuncTM, EasYFlaskTM, Kamstrupvej, Roskilde, Denmark) for 4 days. After washing with DPBS, the cells were infected with *P. aeruginosa* and incubated for 4 h at 37 °C in 5 % CO2. The incubation time was selected as described in literature 12, 16.Cell viability was determined after incubation by using an automatic counter as described above.

The cell viability assay alamarBlueTM (invitrogen, Paisley, UK) was used to assess the viability of A549 cells after treatment with hydrogen peroxide-urea adduct (Sigma-Aldrich, Germany). Hydrogen-peroxide-urea adduct is a source of free hydrogen peroxide (H2O2) when dissolved in aqueous solution. The assay is based on the conversion of non-fluorescent blue dye to a fluorescent pink colour by the reduction power of viable cells. This fluorescence can then be measured to determine the number of viable cells; i.e., the more viable cells present the greater the measured fluorescence. Briefly A549 cells (*ca*. 3 x 105 cells/well) were seeded into 96 well plates (NunclonTM Delta surface, Kamstrupvej, Roskilde, Denmark) and were treated with different concentrations of H2O2 and incubated for 24 h at 37 °C in 5 % CO2. The assay was subsequently prepared as described by the manufacturer. A fluorescence excitation wavelength of 544 nm was used and fluorescence emission was read at 590 nm.

*Infection and treatment experiments*

After reaching ~80-85% confluency, A549 cells (~ 4 x 106 cells) were sub-cultured in a Schott Duran glass bottle 24 h prior to the experiment. On the day of the experiment, the medium (DMEM supplemented with FBS, pen-strep, and glutamax) was removed and washed with DPBS twice. The cells were then replenished with 25 mL serum- and antibiotics-free medium (DMEM supplemented with glutamax) and infected at a MOI of ~100 and incubated for 4 h following the protocol of Hawdon *et al. 16* (these we refer to as ‘A549-Pa’) (Fig S1). For the treatment experiment, 100 mM of H2O2 was added to the cells for 24 h (‘A549-H2O2’) (Figs S2 and S3). Environmental incubation conditions for both experiments were at a temperature of 37 °C in 5 % CO2.

*Headspace collection*

After incubation, the headspace of sample bottles was flushed with dry nitrogen at a flow rate of 60 mL min-1 whilst simultaneously trapping on dual-bed sorbent tubes containing Tenax GR-Carbograph 5TD using a pump at a flow rate of 200 mL min-1. Prior to capture on the sorbent tube, the headspace sample was mixed with dry nitrogen supplied at a flow rate of 140 mL min-1 to minimise condensation effects. A volume of 1200 mL was collected and a schematic representation of the headspace sampling method is shown in Fig 1.



**Fig 1.** A schematic representation of headspace sampling of A549 epithelial cells (blue) and *P. aeruginosa* (red) co-culture. The metabolites are represented as red dots above the sample.

*Gas chromatography-mass spectrometry (GC-MS) analysis*

After sample collection, internal calibration standards were loaded onto the sorbent tubes by dilution of gaseous calibration standards (10 ppmv acetone-*d*6, hexane-*d*14, toluene-*d*8, xylene-*d*10 in nitrogen, standards supplied by Air Products, Amsterdam, The Netherlands) using a custom-made dilution system. As a quality assurance procedure, empty and sorbent-only tubes are also analysed. Sorbent tubes were at a temperature of 225 °C (TDSA, Gerstel, Mülheim an der Ruhr, Germany) into the GC column. Solvent venting mode was used to transfer the sample to the packed liner (filled with Tenax TA) held at -55 °C and subsequently heated to 280 °C. A cold trap (CTS2, Gerstel, Mülheim an der Ruhr, Germany) was used to minimise band broadening (initial temperature was -150 °C, and heated to 220 °C after 1.6 min). A capillary gas chromatograph (7890N GC, Agilent, Santa Clara, CA, USA) using a VF1-MS column (30 m × 0.25 mm, film thickness 1 μm, 100% dimethyl-polysiloxane, Varian Chrompack, Middelburg, The Netherlands) was used with the following temperature program: 30 °C, hold 3.5 min, ramp 5 °C/min to 50 °C, hold 0 min, ramp 10 °C/min to 90 °C, hold 0 min, ramp 15 °C/min to 130 °C, hold 0 min, ramp 30 °C/min to 180 °C, hold 0 min, ramp 40 °C/min to 280 °C, hold 1 min. A Time of Flight mass spectrometer (LECO Pegasus 4D system, LECO, St. Joseph, MI, USA) was used in electron ionization (EI) mode at 70 eV, with a scan range of *m/z* 29–400 Da, scanning rate 20 scans s–1.

*Data processing and analysis*

*Data pre-processing*

LECO Chromatof software (LECO, St. Joseph, MI, USA) was used for acquisition and curation of GC-MS raw data. The acquired chromatograms can be investigated using this software and (EI fragment) mass spectra from individual peaks cross-referenced with National Institute of Standards and Technology library 14 (NIST, Gaithersburg, MD, USA) for putative identification purposes (MSI Level 2), and followed the metabolomics standards initiative (MSI) guidelines for metabolite identification (Sumner et al., 2007). Metabolites with a matching factor of ≥800 within the NIST software were deemed to be identified and investigated further.

Prior to analysis, raw data in the instrument manufacturer’s data format were converted into a netCDF format utilising the LECO Chromatof software. All statistical analyses were performed using the R software (version 3.4.2; R Core Team (2017)). The *xcms* package following the approach as outlined by Smith *et al.* (Smith et al., 2006) was used to pre-process the netCDF files in R. The product of raw data pre-processing is a data file containing ion-fragments, their corresponding *m*/*z*, retention times, and also integrated areas. Normalisation using the internal standard (IS), toluene-*d8*, was based on the 100 *m*/*z* parent ion.

*Univariate and Multivariate Analyses*

For univariate analysis, the non-parametric Mann-Whitney U test was performed on individual ion-fragment for pairwise comparisons; i.e., A549 *vs.* A549-Pa, and also A549 *vs.* A549-H2O2. A critical *α*=0.05 value was used in tests. False discovery rate method was used to adjust the obtained *p*-values from the tests as described in 17. Statistically significant fragments that appear to originate from the same VOCs were identified. Fragments grouping into unique compounds were identified during pre-processing using the R package *CAMERA 18*. Representative VOC fragments were then re-tested.

For multivariate analysis, principal component-discriminant function analysis (PC-DFA) was also used to detect similarities and differences visually between the experimental groups and to discern the associated VOCs. The R package *adegenet* was used for this analysis 19. The input variables for DFA are PCs and are optimised, as previously described 10. Briefly, the data are split into 70% training set and 30% test set by stratified random sampling. The training set was then used to build several PC-DFA models (using different number of PCs) and the ability for each model to correctly classify the test set aided in selecting the optimal model (number of PCs). This optimisation was repeated 1000 times at each PC level and an optimal model was selected based on having the lowest root mean square error (RMSE). For validation, again the data was split into 70% training and 30% test sets and the former was then used to build a PC-DFA model using the optimal number of PCs. After this tuning process the test set was projected into the subspace created by the training set to visualise the prediction of test data of distinct experimental groups.

**Results**

**A549 growth curve**

The growth of A549 cells was monitored over a period of four days. Twelve T25 flasks were seeded (~3 x 105 cells/mL) on day zero and three flasks were then counted on subsequent days (Fig 2). Viable cell count was performed by an automatic cell counter based on Trypan blue dye exclusion.



**Fig 2.** Growth curve of A549 epithelial cells over a period of four days. The cell count was performed in triplicate and the error bars represent standard errors (SE).

**Plastic *versus* Glass cell culture vessel**

Schott Duran glass bottles were seeded with *ca*. 4 x 106 cells. Cells were seeded at a higher density in glass bottles to sustain growth. The cells were counted after 24 h to compare growth with cells grown for 24 h in T25 plastic flasks; i.e., day zero to day one in Fig 2. Due to the different seeding amount of cells in the culture vessels, the ratio of cell growth between day zero and day one was used for comparison (Fig 3). Although a slower growth rate was observed in the glass bottle in comparison to the plastic flask from day zero to one, the goal was to observe growth prior to infection or treatment to ensure that any cell death observed is due to the introduced stimulus.



**Fig 3.** A549 cell growth pattern from day zero to day one. This was performed in triplicate and the error bars represent SE.

**VOCs of interest**

Approximately 13,000 ion-fragments were generated after data pre-processing. About 1000 fragments were significantly different between uninfected and infected A549 epithelial cells, and 800 fragments for untreated *versus* treated cells by Mann-Whitney U test. Fragments generated from analytical artefacts such as siloxanes and having a high *m/z* value (>250) were removed. Ungrouped and Unidentified fragments were also excluded. Fragments grouped by *CAMERA* and identified by NIST are shown in Figs 4 and 5 respectively and these are identified to MSI level 2.

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**Fig 4.** Identified VOCs observed from the infection experiment. The boxplots were generated from a minimum of six repeats. The *p*-value was obtained after pairwise comparisons between A549 *vs.* A549-Pa by Mann-Whitney U test and adjusted by the false discovery rate method. Abbreviations in the figure are defined as follows: A549=A549 culture, A549-Pa=A549-*P. aeruginosa* co-culture, Medium-Pa=Medium-*P. aeruginosa* culture.



**Fig 5.** Identified VOCs observed from the treatment experiment. The boxplots were generated from a minimum of six repeats. The *p*-value was obtained after pairwise comparisons between A549 *vs.* A549-H by Mann-Whitney U test and adjusted by the false discovery rate method. A549=A549 culture, A549-H=A549-hydrogen peroxide culture, Medium-H=Medium-hydrogen peroxide.

**Multivariate analysis**

The distribution of the distinct sample groups is shown in the PC-DFA scores plot (Fig 6). Ten PCs (accounting for 76.7% total explained variance) were included for DFA as it achieved the lowest RMSE. After validation, it can be observed that the test set is congruent with the training data. The infected cells can be observed to be separated from the treated cells and unperturbed cells along DF1, while along DF2 the treated and infected cells appear to cluster away from epithelial-only cells. From analysing the extremes of the PC-DFA loadings plot (Fig S4), fragments emanating from 3-methyl-1-butanol and ethylidenecyclopropane were observed to contribute to the separation along DF1. Fragments originating from tert-butyl ethyl ether and methyl tert butyl ether contribute to the separation along DF2.



**Fig 6.** PC-DFA scores validation plot. Training samples from the distinct bacterial cultures are indicated as filled shapes while the projected test samples are shown by open symbols. A549 (circle), A549-H202 (triangle), A549-Pa (square). Abbreviations in the figure are defined as follows: A549=A549 culture, A549-Pa=A549-*P. aeruginosa* co-culture A549-H=A549-hydrogen peroxide co-culture.

**Discussion**

We have cultured adherent mammalian A549 epithelial cell lines in a Schott Duran glass bottle, and identified novel and established VOCs of bacterial origin by infecting these cells with *P. aeruginosa*. Through the addition of hydrogen peroxide, we have also detected VOCs that may serve as biomarkers for the epithelial cells undergoing oxidative stress.

Adherent mammalian cells are typically grown in plastic cell culture flasks. This vessel may not be the most appropriate for the application of headspace VOC sampling as the analysis of empty culture flasks reveals the presence of background contaminants from plasticizers or other volatile additives in the plastic flasks 14, some of which have mistakenly been proposed as potential biomarkers. An autoclaved glass bottle was therefore investigated as an alternative cell culture vessel since it is usually devoid of such contaminants. Adequate cell growth was observed in this vessel, although this was not at the same growth rate in comparison to the traditionally utilised plastic flasks. It is possible that the decreased surface area might be the source of this limitation, with consequent reduced gas exchange at the liquid-air interface.

Genomic studies have shown differential expression of genes in A549 epithelial cells in the presence of *P. aeruginosa 12* and thus we expect that potential downstream changes in metabolite profiles may also occur. However the identified VOCs likely originate from the bacteria as similar concentrations were observed in the headspace of bacteria-only culture. These compounds (tert-butyl ethyl ether and methyl tert-butyl ether) have not been reported before for *P. aeruginosa* and so we consider these as novel to this study and potential biomarkers for bacterial infection of the epithelia. We also observed elevation in previously reported bacterial markers including 3-methyl-1-butanol, acetone, ethanol, and ethylidenecyclopropane. It has previously been shown how the culture environment influences the observed VOC profile 10, thus highlighting the ability of these organisms to produce diverse VOCs, but also posing a dilemma regarding a consensus panel of biomarkers that will support *in vivo* detection. A robust and reproducible bacterial culturing and headspace analysis methodology involving a variety of environment may aid in discovering a core VOC set that are discriminatory for pathogens. In addition, meta-analysis of existing results from headspace analysis and *in vivo* measurements may also contribute to this harmonisation.

Oxidative stress is implicated in many conditions including ageing, cancer, inflammatory and infectious disease, as well as in heart disease 20. This process occurs as a result of an imbalance in the equilibrium between reactive oxygen species (ROS) such as hydrogen peroxide (H2O2) and the scavengers of this species 21. These oxidants are reported to damage cell components including proteins, lipids and nucleic acid. H2O2-mediated injury of the A549 cells has previously been reported 22. Stimulation with this compound is stated to induce oxidative stress resulting in lipid peroxidation and subsequent generation of alkanes 23. In our study alkanes including decane, hexane, octane, and cyclohexane were observed to be elevated when the A549 epithelial cells were exposed to H2O2. An increased concentration of octane in the breath of patients with acute respiratory distress syndrome (ARDS) was reported by Bos and colleagues 24, perhaps secondary to peroxidation of oleic acid25, 26.

n7-Polyunsaturated fatty acid (PUFA) is a likely precursor of hexane 23 and has been reported as another volatile marker of oxidative stress in patients with unstable angina 27 and also oral malodor 28. In the latter study, cyclohexane was also reported as an oxidative stress marker; we also noted elevated levels of this in our culture experiments H2O2 treatment.

The utility of volatiles such as alkanes as specific markers maybe limited due to the connection of oxidative stress to a variety of disorders. For infections such as pneumonia, this could mean that bacterial-specific markers may be more useful for detection and pathogen identification as these are unique to the infectious agent and not a host-mediated response to infection and/or inflammation. To be useful as markers, alkanes may be best combined with more specific biomarkers (volatile or non-volatile) of different conditions for accurate diagnosis, or could potentially be useful when measured repeatedly relating to a change in clinical state.

A strength of this study can be attributed to the novel culture vessel and headspace sampling methodology developed. Using glass for sample preparation aided in minimising unwanted contaminants, while the headspace collection aided in minimising condensation effects and thus preserves the VOC profile and reduces artefact formation. This supported the discovery of existing and novel VOCs. Further methodological improvements should focus on improved glass cell culture vessels to aid gas transfer into the culture media; further experiments should investigate VOCs originating from epithelial cell co-culture with activated neutrophils, and the culture of epithelial cells with different oxidants.

**Conclusion**

There is an urgent need for early detection of lung infections and disorders such as VAP and ARDS. The measurement and identification of discriminatory VOCs has been suggested as useful for disease diagnosis. These volatile metabolites are particularly attractive as VOC sampling can be done without distress to the patient 11, 29 . *In vitro* cultivation allows specific organisms to be analysed in isolation, and does not require the use of animals for infection models. Validation of the VOC markers that we and others have reported from *in vitro* experiment in human exhaled breath samples will of course be needed in the future for translation into clinical utility. In addition, especially for pathogen identification, screening of these compounds in the headspace of lower respiratory tract specimens is another viable option that would be clinically useful and should be considered.

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