Breath analysis for noninvasively differentiating *Acinetobacter baumannii* ventilator-associated pneumonia from its respiratory tract colonization of ventilated patients

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**Abstract**

A number of multiresistant pathogens including *Acinetobacter baumannii* (*A. baumannii*) place a heavy burden on ventilator-associated pneumonia (VAP) patients in intensive care units (ICU). It is critically important to differentiate between bacterial infection and colonization to avoid prescribing unnecessary antibiotics. Quantitative culture of lower respiratory tract (LRT) specimens, however, requires invasive procedures. Nowadays, volatile organic compounds (VOCs) have been studied *in vitro* and *in vivo* to identify pathogen-derived biomarkers. Therefore, an exploratory pilot study was conceived for a proof of concept that the appearance and level of *A. baumannii*-derived metabolites might be correlated with the presence of the pathogen and its ecological niche (i.e. the infection and colonization states) in ICU ventilated patients.

Twenty patients with *A. baumannii* VAP (infection group), 20 ventilated patients with LRT *A. baumannii* colonization (colonization group) and 20 ventilated patients with neurological disorders, but without pneumonia or *A. baumannii* colonization (control group) were enrolled in the *in vivo* pilot study. A clinical isolate of *A. baumannii* strains was used for the *in vitro* culture experiment. The adsorptive preconcentration (solid-phase microextraction fiber and Tenax® TA) and analysis technique of gas chromatography–mass spectrometry were applied in the studies.

Breath profiles could be visually differentiated between *A. baumannii* cultivation *in vitro* and culture medium, and among *in vivo* groups. In the *in vitro* experiment, nine compounds of interest (2,5-dimethyl-pyrazine, 1-undecene, isopentyl 3-methylbutanoate, decanal, 1,3-naphthalenediol, longifolene, tetradecane, iminodibenzyl and 3-methyl-indene) in the headspace were found to be possible *A. baumannii* derivations. While there were eight target VOCs (1-undecene, nonanal, decanal, 2,6,10-trimethyl-dodecane, 5-methyl-5-propyl-nonane, longifolene, tetradecane and 2-buty1-1-octanol) exhibiting characteristics of *A. baumannii* VAP derivations. The selected VOC profile *in vivo* could be adopted to efficiently differentiate the presence of LRT *A. baumannii* from its absence, and LRT *A. baumannii* infection from its colonization (AUC = 0.89 and 0.88, respectively).

It is not feasible to simply transfer the metabolic biomarkers from the *in vitro* condition to *in vivo*. The direct detection of exhaled *A. baumannii*-derived VOCs may be adopted for an early alert of the LRT bacterial presence in ventilated ICU patients, and even in different parasitic states of *A. baumannii* (i.e. infection and colonization). However, further refinement and validation are required before its clinical use.
1. Introduction

Ventilator-associated pneumonia (VAP), a subset of hospital-acquired pneumonia (HAP) or nosocomial infection, represents a significant cause of morbidity, mortality and resource utilization within intensive care unit (ICU) patients. The mortality attributed to VAP is estimated to be 6–10% [1, 2]. Late-onset VAP is most critical due to infection with multidrug-resistant pathogens. In fact, multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) pathogens currently pose a serious threat to clinical practice, especially the prevalence of carbapenemase-producing Gram-negative bacilli (GNB), including Acinetobacter baumannii (A. baumannii) [3, 4]. A. baumannii infections in critically ill patients were therefore associated with increased mortality [5, 6]. CHINET 2014 surveillance of bacterial resistance in China illustrated the serious resistance problem of clinical bacterial isolates, including A. baumannii. Approximately 65% of Acinetobacter spp. (A. baumannii accounts for 93.0%) strains were resistant to carbapenem antibiotics [7]. VAP was the predominant infection that the pathogenic bacterium A. baumannii was responsible for, and the mortality rate of A. baumannii VAP ranged from 35–60% [8, 9].

Acinetobacter spp. strains are non-fermentative, strictly aerobic and opportunistic bacterial pathogens, of which A. baumannii has been the most frequently discovered from clinical isolates. It can contaminate inanimate hospital surfaces and devices, and colonize patients’ normal skin, wounds or oropharynx. Respiratory tract colonization is common in intubated patients, which has been regarded as a high risk factor for VAP. It is a vital challenge to differentiate colonization from infection. Failure to do this often results in the unnecessary prescription of antibiotics, and the subsequent aggravation of antimicrobial resistance.

Breath volatile organic compound (VOC) analysis has increasingly attracted clinicians’ interest, due to its noninvasiveness and rapidity. Various respiratory diseases including pneumonia, chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis, cancer and tuberculosis, have been studied for their respective breath VOC profiles [10–14]. Several typical breath tests have been approved by the United States Food and Drug Administration (FDA) for diagnostic and screening tools in humans [15]. Their clinical uses include the detection of breath ethanol and acetaldehyde concentrations after alcohol consumption, 13C-urea or ammonia breath tests for the diagnosis of Helicobacter pylori infection, breath carbon dioxide concentration monitoring (i.e. capnography) for use in anesthesia and intensive care, and nitric oxide test for asthma screening.

It is of great value to seek specific pathogen-derived volatile metabolites in breath for the underlying microbial etiology identification of pneumonia, though certain endogenously produced biomarkers in breath have been reported to be derived from the host, not the pathogen. These host-derived volatiles include saturated hydrocarbons, such as pentane, propane, malondialdehyde and methylated hydrocarbons involved in lipid peroxidation, and unsaturated hydrocarbons, such as isoprene involved in the mevalon pathway of cholesterol synthesis [16]. To identify the pathogen-derived or pathogen-specific VOCs, many viruses and bacteria have been cultured in vitro and analyzed, including the influenza virus, human rhinovirus, Enterococcus faecalis, Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus aureus (S. aureus) and Klebsiella pneumonia (K. pneumonia) [17–20]. These microorganisms constantly induce VAP. Of the above bacteria, some VOCs were discovered to be emitted by more than one strain, yet several were found to be potentially exclusive, which was properly reviewed by Bos et al [21]. Additionally, the aim should be shifted from finding one single VOC representative of the presence or absence of a specific pathogen, to exploring entire VOC fingerprints, i.e. the volatome. The concentration profile of volatile compounds is likely correlated with the course of bacterial metabolism and host-pathogen interactions. To date, the prevalent bacterium A. baumannii has not been critically involved, which was frequently separated in lower respiratory tract (LRT) specimens of VAP. Several recently published reports associated with VAP and its breath VOC profile have implicated multiple different types of pathogens, and the limited number and heterogeneity of subjects, thus hindering the identification of specific strain-derived VOCs in vivo [22–24].

We conducted this prospective pilot study to test the primary hypothesis that exhaled A. baumannii-derived VOCs could be used for an early alert of the presence of LRT bacteria in ventilated ICU patients. We further hypothesized that the metabolite concentration profiles might be associated with the different states of A. baumannii infection and colonization.

2. Materials and methods

The study was performed at the 52-bed adult mixed-ICU of the tertiary teaching hospital (Sir Run Run Shaw Hospital, SRRSH), Zhejiang University, China, over a period of 24 months, from February 2014 to February 2016. The study was approved by the SRRSH Ethics Committee (protocol number 20130703-4) and adhered to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki [25]. Informed consent was required from the patient, family/relative, guardian, or proxy according to Chinese law. The study taken here was explorative and the findings obtained had no impact on patient care.

2.1. Definitions of VAP and colonization

Because most definitions of VAP lack perfect sensitivity and specificity, we chose a combination of the two
widely used VAP criteria, the clinical pulmonary infection score (CPIS) and the definition from the Centers for Disease Control and Prevention (CDC) of America (tables 1 and 2). The CPIS was initially developed by Pugin et al [26], and later modified by Luna et al [27]. This diagnostic tool for VAP was recommended by the American Thoracic Society and the Infectious Diseases Society of America (ATS/IDSA) [28]. The cut-off value of six was adopted for pulmonary infection diagnosis. Ventilator setting parameters were added to the streamlined surveillance definition of CDC, to promote the sensitivity and specificity of the VAP diagnosis [29]. Chest radiographs were judged by one radiological specialist and one clinical attending doctor, who were both blinded to the study procedure. Quantitative cultures from LRT specimens, including endotracheal aspirate (ETA), bronchoalveolar lavage (BAL) and protected specimen brush (PSB) samples, were taken to guide the choice of antibiotic therapy.

Respiratory tract A. baumannii colonization was defined by the bacterium A. baumannii cultured and isolated from LRT specimens, but the VAP standard and bacterial threshold of quantitative culture, as described above, could not be reached. The diagnosis of VAP due to A. baumannii infection or just its respiratory tract colonization was decided by two clinical attending doctors independent of the study, based on the above two VAP criteria.

### 2.2. Admission criteria and clinical practice

Twenty critically ill patients with identified A. baumannii VAP (infection group) and 20 critical care patients requiring ventilation with defined respiratory tract A. baumannii colonization (colonization group) were enrolled. Of these, seven patients overlapped, which meant that A. baumannii VAP and its respiratory tract colonization occurred to them during different stages of the ICU stay. These two groups could be combined as a group with the presence of A. baumannii (bacterium group). Another 20 ventilated patients with neurological disorders, but without pneumonia or respiratory tract A. baumannii colonization were included as a negative control (control group). Patients with confirmed structural lung diseases, such as COPD, respiratory tract neoplasms and asthma were excluded.

In our present study, the LRT microbiological spectrum varied, particularly with antibiotic usage, during the ICU stay of the enrolled patients. These LRT pathogens were also cultured and isolated before or after this A. baumannii VAP or colonization episode (i.e. the time point of breath sampling). Actually, we chose the time point for breath sampling under the condition that the only LRT microorganism isolated was A. baumannii, and the duration of positive culture was at least 3 d. The condition of LRT co-existing microorganisms (co-infection or co-colonization or both crossed) was deliberately excluded for breath sampling in our present study.

The LRT specimens taken with a fiberoptic bronchoscope (BAL and PSB) or without (ETA) were stored in a sterile sealed container. Then, the samples were transferred quickly to the microbiology laboratory (<1 h) or temporarily preserved under the conditions of 2°C–8°C. The standardized procedure for specimen inoculation, smear, Gram staining, and quantitative or semi-quantitative cultivation was performed according to the guidelines from the American Clinical and Laboratory Standards Institute (CLSI) and Chinese Standard for Bacterial Culture Procedures of Lower Respiratory Tract Infections. The blood biomarkers for liver, renal and inflammation intensity were tested daily or every two days routinely. The results of the parameters detected at a time closest to breath sampling were recorded for the following analysis.

Characteristics of all eligible patients were recorded, such as age, gender, diagnosis, smoking history, serum markers for liver and renal function (e.g. bilirubin, alanine transaminase (ALT); aspartate transaminase (AST), gamma glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), urea and creatinine), blood biomarkers for infection besides core temperature (e.g. white blood cell (WBC), C-reactive protein (CRP) and procalcitonin (PCT)), sources of the LRT specimen (e.g. ETA, BAL and PSB), positive end-expiratory pressure (PEEP)/oxygen concentration in inspired air (FiO₂) and antibiotic usage.

The prescription of antimicrobial agents was performed in accordance with the guideline from a Chinese consensus statement on clinical management and infection control of the infections induced by XDR GNB [30]. Other routine therapies, such as a head elevation, prophylaxis of peptic ulcer and deep vein thrombosis, sedative and analgesic supervision, ventilation adjustment and weaning were executed according to the corresponding standardized clinical protocols by two independent attending doctors.

### Table 1. Simplified version of the CPIS.

| Characteristic | 0 | 1 | 2 |
|----------------|---|---|---|
| Temperature (°C) | ≥36.5 and ≤38.4 | ≥38.5 and ≤38.9 | ≥39.0 or ≤36.0 |
| Blood leukocytes (x 10⁹ l⁻¹) | ≥4000 and ≤11000 | <4000 or >11000 | — |
| Tracheal secretions | Few (purulent +1) | Moderate (purulent +1) | Large (purulent +1) |
| Oxygenation P/F (mmHg) | >240 or presence of ARDS | — | ≤240 and absence of ARDS |
| Chest radiograph | No infiltrate | Patchy or diffuse infiltrate | Localized infiltrate |

Abbreviations: ARDS: acute respiratory distress syndrome; P/F, PaO₂/FiO₂, PaO₂: arterial partial pressure of oxygen; FiO₂: fraction of inspired oxygen. The cut-off score of six was employed for the diagnosis of ventilator-associated pneumonia.
Table 2. Streamlined surveillance definitions for VAP according to the Centers for Disease Control and Prevention.

| Category       | Criteria                                                                 |
|----------------|---------------------------------------------------------------------------|
| Radiology      | Two or more serial chest radiographs with at least 1 of the following:   |
|                | 1. New or progressive and persistent infiltrate                          |
|                | 2. Consolidation                                                          |
|                | 3. Cavitation                                                            |
| Systemic signs | (at least 1)                                                              |
|                | 1. Fever (>38 °C) with no other recognized cause                          |
|                | 2. WBC count >12 000 mm\(^{-3}\) or <4000 mm\(^{-3}\)                     |
| Pulmonary signs| 1. >25 neutrophils per low power field on Gram stain of ETA or BAL specimen |
|                | 2. ≥2 d of stable or decreasing daily minimum PEEP followed by a rise in daily minimum PEEP of ≥2.5 cm H\(_2\)O, sustained for ≥2 calendar days; or ≥2 d of stable or decreasing daily minimum FiO\(_2\) followed by a rise in daily minimum FiO\(_2\), of ≥0.15 points, sustained for ≥2 calendar days |

Abbreviations: BAL: bronchoalveolar lavage; ETA: endotracheal aspirate; FiO\(_2\): fraction of inspired oxygen; PEEP: positive and expiratory pressure; VAP: ventilator-associated pneumonia; WBC: white blood cell.

2.3. **In vitro experiment**

In order to determine which substances in the breath gas were potentially A. baumannii-derived, an *in vitro* experiment was simultaneously conducted with microbial cultures at comparable densities to the *in vivo* setting. A clinical isolate of an *A. baumannii* strain was used for the present *in vitro* experiment, which was inoculated at 10\(^{8}\) cfu ml\(^{-1}\) in 1 ml. The strain’s serial dilution within sterile physiological saline for inoculation was determined by turbidometry, and the finishing suspension was confirmed by a quantitative plate count. The culture was incubated at 37.0 °C for 12 h in a standard BacT/ALERT\textsuperscript{®} SA (Aerobic bioMerieux, Inc., Durham, NC, USA) plastic disposable bottle containing 40 ml medium. Uninoculated medium underwent the same procedure as the negative control. Forty bottle cultures and another forty corresponding controls were available for the following treatment.

The methods for strain cultivation, headspace gas extraction, thermal desorption and gas chromatography–mass spectrometry (GC-MS, QP2010 Plus, Shimadzu\textsuperscript{®}) settings have been detailed elsewhere [13, 31], and they were utilized with certain revisions in this experiment. Briefly, a solid-phase microextraction fiber (SPME; divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm; Supelco, Sigma-Aldrich, St. Louis, MO, USA) was used for the preconcentration of the volatile compounds in the headspace, through exposure in a culture bottle for 60 min at 37 °C. Then, the SPME fiber was inserted into the GC inlet and the adsorbed headspace chemicals were desorbed for 2 min at 250 °C. The GC cycle was set as follows: 6 min hold at 55 °C followed by 7 °C min\(^{-1}\) ramp to 97 °C, 2 min hold followed by 2 °C min\(^{-1}\) ramp to 110 °C, 5 °C min\(^{-1}\) ramp to 130 °C, 4 min hold followed by 5 °C min\(^{-1}\) ramp to 160 °C, 4 min hold followed by 4 °C min\(^{-1}\) ramp to 230 °C, 10 °C min\(^{-1}\) ramp to 280 °C and hold 4 min. The constant flow rate of 1.8 ml min\(^{-1}\) was adopted for helium as a carrier gas. The total ion chromatogram (TIC) mode within the range of 35–350 m/z was used for the identification of potential target compounds.

The target compound (i.e. *A. baumannii*-derived compound) was defined as the level of which in the headspace from bacterial cultivation increased by comparing with that from medium control. Only the produced VOC was considered as a target, as the VOC consumed in metabolism (i.e. with a decreased level by comparison) was probably based on different culture medium.

2.4. **Breath sample collection and GC-MS analysis**

The instrumentation as well as procedure for breath sample collection, thermal desorption and subsequent GC-MS analysis were performed according to previous descriptions with some modifications (figure 1) [22, 32, 33]. In brief, a T-piece (polytetrafluoroethylene) was connected to the expiratory tube for breath sampling. The length of the transfer line from the T-piece to the sorption tube was approximately 50 cm. All samples were collected in triplicate. In the present study, the oral tracheal tube (Mallinckrodt\textsuperscript{™}, COVIDIEN\textsuperscript{™}, Mexico, USA) and ventilator (Puritan Bennett 7200 and 840) were available. The preconditioned Tenax\textsuperscript{®} TA steel-tube (PerkinElmer\textsuperscript{®}, a porous polymer 2,6-diphenyl furan resin, 60/80 mesh, 200 mg) was placed in an airtight stainless cylinder (internal diameter 26 mm) with a temperature controller. The cylinder was thermostated at 40 °C during the adsorption process, to avoid excessive water vapor sorption. The gas samples were preconcentrated by adsorption on the stainless tube, with the flow generated by a vacuum pump at a rate of 200 ml min\(^{-1}\) for 20 min. Therefore, approximately 41 of expiratory gas was adsorbed in the Tenax tube.

The sample with Tenax TA was thermally desorbed inside a thermal desorption unit. Firstly, the tube was heated to 250 °C within 2.5 mins, and simultaneously the sample was relocated onto a trap at ~10 °C. The cold trap was then heated to 250 °C at 40 °C s\(^{-1}\), and volatiles were transferred to the chromatographic column for separation. A GC capillary column (Rtx-5MS, film thickness 0.25 μm, inner diameter 0.25 mm, length 30.0 m, dimethylpolysiloxane) was used. The temperature program of the column was set as follows: 40 °C held for 1 min, then ramped 5 °C min\(^{-1}\) up to 250 °C, and an isothermal hold for 1 min. The constant helium flow rate was 1.8 ml min\(^{-1}\) throughout the analysis.
flow rate of 0.47 ml min\(^{-1}\) was used as a carrier gas. The TIC mode within the 20–200 m/z range was applied by MS for the identification of potential target compounds. A high-precision molybdenum quadrupole mass filter/analyzer was available for the detection of product ions.

GC-MSSolution (LabSolutions, Shimadzu\textsuperscript{®}) and Automated Mass Spectral Deconvolution and Identification System (AMDIS, NIST) software were available for raw data preprocessing, including denoising, baseline correction (the drift and slope parameters were preset), resolution of overlapped chromatographic peaks and alignment. The detected compounds corresponding to the peaks were identified by spectra matching with the above software and Mass Spectral Library (NIST 05). The retention time and mass spectrum similarities were used to identify the compounds of interest [23, 34]. The highest similarity matches were presumed to be the most likely candidate compounds. Manual checking was initiated for cautious identification if the similarity matching results were less than 80%. The area under the curve (AUC) of each chromatographic peak was associated with one quantity of compound.

Because we emphasized the different breath profiles between \textit{A. baumannii} VAP and its colonization in ventilated patients, we did not measure the absolute VOCs quantities using pure substances.

The ‘20% rule’, which was introduced by Professor van Schooten \textit{et al}, was applied for data selection [20, 35]. Briefly, a variable was adopted when nonzero data were available for at least 20% of all samples within at least one of the experimental groups. Certain compounds, including siloxanes, phthalate and adipate plasticizers, e.g. bis(2-ethylhexyl) phthalate and its degradation product 2-ethylhexanol, were also excluded initially [36]. After this step, the 349 VOCs detected primarily in the \textit{in vitro} experiment were reduced to 21 VOCs, and the 245 VOCs detected in the \textit{in vivo} study reduced to 19 VOCs. Actually, the selected compounds were present at least 90% of all samples in at least one of the groups.

2.5. Statistical analysis
Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were adopted to visually differentiate between groups using Simca-P software (Umetrics, Sweden). The deduced plots (PCA and PLS-DA) were based on the proximities between the samples of the enrolled groups. Due to the relatively small number of detected VOCs, a nonparametric statistical analysis was employed for each variable (detected VOC) comparison between groups. The Mann–Whitney U-test was used in the \textit{in vitro} experiment, while the Kruskal–Wallis H-test followed by the Nemenyi test were applied for inter-group comparison in the \textit{in vivo} study.

The \textit{in vivo} target VOC (i.e. possibly \textit{A. baumannii} VAP-derived) was defined as the value in which significant differences were all achieved when comparing inter-group (infection group versus control group, infection group versus colonization group, and colonization group versus control group). The whole target compounds were also adopted to compare the bacterium group (i.e. infection group and colonization group combined) with the control group, to assess the presence or absence of \textit{A. baumannii}.

Distinction capabilities of detected metabolites were evaluated by receiver operating characteristic (ROC) curves, from which the AUC, sensitivity and specificity could be obtained. The differential accuracy was characterized as follows: AUC values between 0.5 and 0.7 indicated low accuracy, values between 0.7 and 0.9 indicated moderate accuracy, and values greater than 0.9 indicated high accuracy [37]. Optimum sensitivity and specificity were achieved, when maximum (sensitivity – (1 – specificity)) was reached.
### Table 3. The VOCs from the headspace of A. baumannii cultivation in vitro.

| #  | CAS#      | Name                      | Mol Form | RT (min) | Ab versus Mediuma | Possible originb |
|----|-----------|---------------------------|----------|----------|-------------------|-----------------|
| 1  | 95-47-6   | 1,2-dimethyl-benzene      | C_{6}H_{13} | 4.440    | —                 | Medium          |
| 2  | 123-32-0  | 2,5-dimethyl-pyrazine     | C_{6}H_{12}N_{2} | 5.990 ↑  | Bacterium         | Medium          |
| 3  | 6214-51-7 | 3-nitro-benzensulfonic acid, methyl ester | C_{6}H_{4}NO_{3}S | 7.340 ↓   |         | Medium          |
| 4  | 3658-80-8 | Dimethyl trisulfide       | C_{2}H_{5}S_{3} | 8.058 ↓   | Medium          |
| 5  | 104-76-7  | 2-ethyl-1-hexanol         | C_{3}H_{8}O   | 10.911 —   | Medium          |
| 6  | 821-95-4  | 1-undecene                | C_{11}H_{22}  | 11.800 ↑  | Bacterium         | Medium          |
| 7  | 659-70-1  | Isopentyl 3-methylbutanoate | C_{10}H_{22}O_{2} | 12.199 ↑  | Bacterium         | Medium          |
| 8  | 1989-2-1  | 2,4-dinitro-benzensulfonic acid | C_{6}H_{4}NO_{3}S | 13.320 —   | Medium          |
| 9  | 576-26-1  | 2,6-dimethyl-phenol       | C_{6}H_{12}O  | 13.542 —   | Medium          |
| 10 | 91-20-3   | Naphthalene               | C_{10}H_{8}   | 14.608 —   | Medium          |
| 11 | 112-31-2  | Decanal                   | C_{10}H_{20}O | 16.014 ↑  | Bacterium         | Medium          |
| 12 | 132-86-5  | 1,3-naphthalenediol       | C_{10}H_{12}O | 17.233 ↑  | Bacterium         | Medium          |
| 13 | 475-20-7  | Longifolene               | C_{10}H_{18}  | 21.501 ↑  | Bacterium         | Medium          |
| 14 | 629-59-4  | Tetradecane               | C_{14}H_{30}  | 27.853 ↑  | Bacterium         | Medium          |
| 15 | 5451-98-9 | Chloroacetic acid, octyl ester | C_{4}H_{10}Cl_{2} | 30.038 —   | Medium          |
| 16 | 494-19-9  | Iminodibenzyl             | C_{4}H_{12}N  | 34.674 ↑  | Bacterium         | Medium          |
| 17 | 767-60-2  | 3-methyl-indene           | C_{10}H_{16}  | 34.799 ↑  | Bacterium         | Medium          |
| 18 | 4891-44-5 | 2-(2-ethylhexyl)-thiophene | C_{14}H_{14}S | 42.534 ↓   | Medium          |
| 19 | 24468-13-1| 2-ethylhexyl chloroformate | C_{9}H_{17}ClO_{2} | 47.387 —   | Medium          |
| 20 | 123-00-2  | 3-morpholino-1-propylamine | C_{13}H_{26}N_{2}O | 53.733 —   | Medium          |
| 21 | 7278-65-1 | 3,7,11-trimethyl-3-dodecanol | C_{15}H_{32}O | 59.009 —   | Medium          |

*a The mark ‘↑’ indicated that the level of headspace compounds from A. baumannii cultivation increased compared with that from the medium control using the Mann-Whitney U-test, and ‘↓’ meant that the level decreased correspondingly, while ‘—’ denoted that the compound level did not change after comparing the two groups.

*b Only the headspace compound produced (↑) during bacterial metabolism was defined as possible bacterial derivation. While the compound consumed (↓) or unchanged in level (–) during in vitro cultivation was considered as a possible medium origin.

Abbreviations: Ab: A. baumannii, Acinetobacter baumannii; CAS#: Chemical Abstracts Service Number; Mol Form: Molecular Formula; RT (min): average retention time (minute); VOCs: volatile organic compounds.

SPSS for Windows (Statistical Package for the Social Sciences, IBM SPSS Statistics 19) was available for all statistical analysis. A p value of <0.05 was considered to be statistically significant.

### 3. Results

#### 3.1. Bacterial cultivation in vitro

The VOCs detected in the headspace of A. baumannii cultivation are listed in detail in table 3. Twenty-one compounds were included for the following analysis, of which nine were presumed to be derived from the bacterial metabolism in vitro.

#### 3.2. Profile differentiation in vitro

The headspace profiles consisting of the above detected 21 VOCs from A. baumannii cultivation in vitro and culture medium were visually distinguished based on PCA (figure 2).

#### 3.3. Included patients characteristics

Patients’ baseline characteristics are presented in table 4. There were statistically significant differences in the levels of liver and renal function, systemic inflammatory intensity and respiratory support, when comparing those from A. baumannii VAP patients to corresponding ones from ventilated patients of A. baumannii respiratory tract colonization, or to ones from ventilated patients without pneumonia or A. baumannii colonization.

The individual value of each related parameter, the bacterial quantity related to sources of LRT species (ETA, BAL and PSB), disease diagnosis and antibiotic usage during breath sampling, and ever isolated microorganisms pertaining to individuals before or after the time point of breath sampling (≥3 d) are presented in detail in the supplementary data (stacks.iop.org/JBR/10/027102/mmedia).

#### 3.4. Breath profile from A. baumannii VAP

The expiratory VOCs profile from A. baumannii VAP patients is presented in detail in table 5. There were 19 compounds of interest included in the following analysis. Of these, eight target ones were considered to be possibly derived from A. baumannii VAP after comparing inter-groups.

#### 3.5. Profile differentiation in vivo

The exhaled breath profiles consisting of the above selected 19 VOCs could be fairly discriminated among A. baumannii VAP patients, ventilated patients with A. baumannii colonization in the respiratory
tract, and ventilated patients without pneumonia or *A. baumannii* colonization, by using the PLS-DA method (figure 3).

Based on ROC analysis, eight target compounds could be adopted to efficiently distinguish ventilated patients with the presence of *A. baumannii* in the respiratory tract from those without, though the former group exhibited heterogeneity (i.e. patients with *A. baumannii* infection and colonization). Furthermore, the *A. baumannii* VAP patients could be differentiated from ventilated patients with *A. baumannii* colonization in the respiratory tract due to ROC analysis for these eight breath compounds. A moderate accuracy was reached for both (AUC = 0.89 and 0.88, respectively). The ROC curves are displayed in figure 4. The relative abundances of the eight target VOCs in infection, colonization and control groups are presented in the supplementary data.

4. Discussion

This prospective, controlled and non-blinded clinical pilot study provides a proof of concept that the appearance and concentration profile of *A. baumannii*-derived metabolites might be correlated with the pathogen presence and its ecological niche (i.e. the infection and colonization states) in ventilated patients. The direct detection of breath VOCs could be potentially employed as a novel, noninvasive and rapid means to identify *A. baumannii* VAP patients, especially in the precise targeting of antibiotic treatment and the reduction of invasive diagnostic manipulations.

It is still under study for the precise diagnosis of VAP and accurate prescription of antibiotics. Serum PCT has recently been regarded as an important marker in the diagnosis and treatment of severe sepsis, including LRT infection. It can be used to assist clinicians in initiating or discontinuing antibiotic therapy [38, 39]. However, the test results should be interpreted carefully, and the clinical value requires further validation [40, 41]. In fact, the biomarker has a relatively low sensitivity in the diagnosis of pneumonia. Other important biomarkers are extensively used for bacterial infection screening, such as WBC and CRP, and also have a limited value for specificity. Thus, it is worth searching alternative breath markers taken noninvasively from *A. baumannii* VAP in ICU patients.

Quantitative cultures from LRT specimens, such as ETA, BAL and PSB samples, could be adopted for the rational use of antibiotics. A diagnostic threshold (ETA \( \geq 10^5 \) cfu ml\(^{-1}\), BAL \( \geq 10^4 \) cfu ml\(^{-1}\), PSB \( \geq 10^3 \) cfu ml\(^{-1}\), respectively) was recommended by ATS/IDSA in the decision to continue or discontinue antibiotics [28]. However, its application is restricted because it is an invasive procedure with imperfect sensitivity and specificity [42]. Moreover, the culture results are not immediately available. The time-consuming microbiological culture of LRT specimens often presents false negative results, particularly already with antibiotic therapy. Herein, we propose this novel noninvasive approach for the early detection of LRT *A. baumannii* presence by monitoring breath volatile metabolites of ventilated patients.

Breath analysis using e-nose and GC-MS techniques have been previously studied in ICU VAP patients. Initially, the e-nose method was applied in breath-print research for VAP patients [43–45]. The diagnostic accuracy of exhaled breath profiling in discriminating between VAP (+) and VAP (−) patients was fairly well, when compared to that from chest computed...
Table 4. Patient baseline characteristics.

| Characteristic                  | Infection (n = 20) | Colonization (n = 20) | Control (n = 20) |
|--------------------------------|--------------------|-----------------------|-----------------|
| Age (year)                     | 55 (35–85)         | 50 (34–78)            | 61 (44–77)      |
| Male, n (%)                    | 13 (65)            | 14 (70)               | 12 (60)         |
| Smoking history, n (%)         | 8 (40)             | 10 (50)               | 9 (45)          |
| Diseases, n (%)                |                    |                       |                 |
| Hypertension                   | 6 (30)             | 9 (45)                | 7 (35)          |
| Diabetes mellitus              | 8 (40)             | 7 (35)                | 8 (45)          |
| Stroke                         | 6 (30)             | 5 (25)                | 8 (45)          |
| Trauma                         | 3 (15)             | 4 (20)                | 6 (30)          |
| Valvular heart disease         | 4 (20)             | 5 (25)                | 2 (10)          |
| Liver function (normal range)  |                    |                       |                 |
| Bil (0–20.5 μmol l⁻¹)          | 96 (34–146)        | 33 (17–55)            | 35 (22–41)      |
| ALT (7–40 U l⁻¹)               | 103 (56–243)       | 56 (32–84)            | 28 (16–35)      |
| AST (13–35 U l⁻¹)              | 96 (48–167)        | 48 (34–95)            | 32 (20–43)      |
| γ-GT (7–45 U l⁻¹)              | 46 (21–68)         | 32 (21–59)            | 24 (14–34)      |
| ALP (35–135 U l⁻¹)             | 176 (55–268)       | 75 (51–158)           | 35 (46–111)     |
| LDH (120–250 IU l⁻¹)           | 359 (165–581)      | 188 (98–305)          | 189 (131–221)   |
| Renal function (normal range)  |                    |                       |                 |
| Cr (40–90 μmol l⁻¹)            | 165 (78–223)       | 96 (56–188)           | 77 (60–106)     |
| Bun (2.5–7.1 mmol l⁻¹)         | 5.6 (3.1–7.9)      | 3.0 (2.4–4.5)         | 4.5 (3.1–6.0)   |
| Infection biomarkers (normal range) |                |                       |                 |
| Tmax. core (36.5–37.5 °C)      | 38.7 (37.2–39.8)   | 37.6 (37.1–38.0)      | 37.4 (36.9–37.4) |
| WBC (3.5–9.5 × 10⁹ l⁻¹)        | 14.2 (8.6–16.9)    | 7.6 (5.6–9.4)         | 6.3 (4.1–7.9)   |
| CRP (0–5 mg l⁻¹)               | 156 (76–189)       | 13 (4–17)             | 6 (3–12)        |
| PCT (0–0.05 mg ml⁻¹)           | 2.6 (0.5–4.2)      | 0.15 (0.09–0.37)      | —               |
| LRT specimen sources, n (%)    |                    |                       |                 |
| ETA                            | 9 (45)             | 7 (35)                | 11 (55)         |
| BAL                            | 6 (30)             | 7 (35)                | 5 (25)          |
| PSB                            | 5 (25)             | 6 (30)                | 4 (20)          |
| FiO₂ (%)                       | 58 (40–80)         | 38 (30–45)            | 33 (29–40)      |
| PEEP (mmHg)                    | 12 (8–15)          | 7 (4–8)               | 6 (5–7)         |

* The infection group consisted of *A. baumannii* VAP patients, and the colonization group consisted of *A. baumannii* respiratory tract colonization of ventilated patients, while the control group consisted of ventilated patients without pneumonia or *A. baumannii* colonization. Data were presented as median (25th percentile–75th percentile) or n (%) of patients.

* The blood biomarkers for liver, renal and inflammation intensity were tested daily or every two days routinely. The results of parameters detected at a time closest to breath sampling were recorded for following analysis.

* Statistically significant differences were both reached when comparing inter-groups (infection group versus control group and infection group versus colonization group) using the Kruskal–Wallis H-test followed by the Nemenyi test.

Abbreviations: *A. baumannii*: Acinetobacter baumannii; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; BAL: bronchoalveolar lavage; Bil: Bilirubin; Bun: Blood urea nitrogen; Cr: Creatinine; CRP: C reaction protein; ETA: endotracheal aspirate; FiO₂: oxygen concentration in inspired air; γ-GT: gamma glutamyl transpeptidase; LDH: lactate dehydrogenase; LRT: lower respiratory tract; PCT: procalcitonin; PEEP: positive end-expiratory pressure; PSB: protected specimen brush; Tmax. core: maximum core temperature; WBC: white blood cell count.

Tomography scanning [43] or CPIS [44], and even better when the breath analysis was combined with CPIS [45]. However, the e-nose cannot precisely identify the VOCs underlying pulmonary infection. Lately, the GC-MS technique has been widely accepted as the gold standard for VOC separation and quantification. Using the GC-MS method, some recently published studies involving exhaled VOCs analysis on ICU VAP patients discovered that the breath profile could accurately distinguish between VAP (+) and VAP (−) patients [22–24]. Pathogens including *S. aureus*, *E. coli*, *Candida albicans*, *K. pneumonia* and *Enterobacter aerogenes* were discovered on VAP patients in one study [22], while in another study, *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumonia*, *Haemophilus influenzae* and *A. baumannii* were the most commonly identified as invasive pathogens for pneumonia [23]. However, the heterogeneity in pathogen population and the limited number of subjects per pathogen has hampered the determination of the specific strain-derived VOCs in vivo. Most individual compounds in these studies cannot correspond to those discovered in our pilot study; this lack in overlap might be explained by the underlying diverse pathogens. Tetradecane was reported in a newly published study [23] containing three VAP patients with *A. baumannii* infection, which was also identified in our study. This alkane probably endows *A. baumannii* with its metabolic characteristics.

Nine target VOCs detected *in vitro* (2,5-dimethylpyrazine, 1-undecene, isopentyl 3-methylbutanoate,
Certain breath compounds detected from the infection group (A. baumannii VAP patients) were considered to be possible A. baumannii VAP derivation, when the compound’s level increased by comparing which from the infection group with the corresponding one from the colonization group (ventilated patients with A. baumannii LRT colonization), and with that from the control group (ventilated patients without pneumonia or A. baumannii colonization), and by comparing which from the colonization group with that from the control group. That meant statistically significant differences were all achieved when comparing inter-groups (infection group versus control group, colonization group versus control group and infection group versus colonization group) using the Kruskal–Wallis H-test followed by the Nemenyi test. If not, the detected compounds were regarded as other origin, including respirator, tubes (e.g. respiratory tubes, tracheal tube, connecting tube and extension tube), and host.

Abbreviation. Ab: Acinetobacter baumannii; CAS#: Chemical Abstracts Service Number; LRT: lower respiratory tract; Ab-VAP: ventilator-associated pneumonia; VOCs: volatile organic compounds.

| #  | CAS#       | Name                      | Mol Form   | RT (min) | Possible origin |
|----|------------|---------------------------|------------|----------|-----------------|
| 1  | 64-17-5    | Ethanol                   | C2H6O      | 1.537    | Others          |
| 2  | 78-79-5    | Isoprene                  | C6H10      | 3.148    | Others          |
| 3  | 108-48-3   | Toluene                   | C6H5       | 4.218    | Others          |
| 4  | 108-94-1   | Cyclohexanone             | C6H10O     | 7.084    | Others          |
| 5  | 100-52-7   | Benzaldehyde              | C6H5O      | 8.956    | Others          |
| 6  | 104-76-7   | 2-ethyl-1-hexanol         | C6H10O     | 10.911   | Others          |
| 7  | 821-95-4   | 1-Undecene                | C11H22     | 11.800   | Ab-VAP          |
| 8  | 124-19-6   | Nonanal                   | C6H10O     | 13.108   | Ab-VAP          |
| 9  | 112-31-2   | Decanal                   | C20H2O     | 16.014   | Ab-VAP          |
| 10 | 3891-98-3  | 2,6,10-trimethyl-dodecane | C20H12    | 18.867   | Ab-VAP          |
| 11 | 17312-75-3 | 5-methyl-5-propyl-nonane  | C15H28     | 20.553   | Ab-VAP          |
| 12 | 475-20-7   | Longifolene               | C15H24     | 21.501   | Ab-VAP          |
| 13 | 571-61-9   | 1,5-dimethyl-naphthalene  | C12H12     | 21.887   | Others          |
| 14 | 50894-66-1 | α-fanebrene               | C10H14     | 22.627   | Others          |
| 15 | 128-37-0   | Butylated hydroxytoluene  | C10H14     | 24.027   | Others          |
| 16 | 469-61-4   | α-cedrene                 | C10H14     | 24.086   | Others          |
| 17 | 2883-2-5   | n-nonylcyclohexane        | C13H10     | 26.287   | Others          |
| 18 | 629-59-4   | Tetradecane               | C14H10     | 27.853   | Ab-VAP          |
| 19 | 3913-2-8   | 2-butyl-1-octanol         | C12H20O    | 27.887   | Ab-VAP          |

* Certain breath compounds detected from the infection group (A. baumannii VAP patients) were considered to be possible A. baumannii VAP derivation, when the compound’s level increased by comparing which from the infection group with the different ecological conditions of LRT colonization is under consideration. Currently, the detection of breath VOC profile has emerged as a promising tool due to its noninvasiveness and rapidness features. Metabolites derived from pathogenic organisms or the host could be analyzed by highly sensitive technology, such as mass spectrometry. This technique can potentially be adopted to diagnose and monitor critical illness usefully. This preliminary study demonstrates that the breath VOC profile could be related to the different ecological conditions of LRT A. baumannii infection and its colonization within mechanically ventilated patients, with a relatively high sensitivity and specificity (both reaching 85.0%). The distinction capability of breath-print detection for LRT A. baumannii infection from its colonization may be more powerful when combined with the aforementioned means available in clinical practice, though further affirmation is required.

The variable abundance of breath-prints is supposed to be associated with the corresponding intensity of bacterial metabolism, in addition to the different inflammatory responses in the host to the bacterial invasion [22, 31, 47]. However, we did not conduct...
the correlation analysis between LRT bacterial load and the level of breath VOCs, because of the diverse sources of LRT species and relatively limited number of corresponding subjects. The metabolic and functional mechanisms of the originated VOCs require further research. The different profiles and corresponding VOCs levels under different parasitic states (infection versus colonization) in our present study were probably associated with the intensity of host immunological responses, such as the changes in the concentrations of interferon-γ (IFN-γ), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), granulocyte colony-stimulating factor (G-CSF) and regulated upon activation normal T-cell expressed and secreted (RANTES) [48, 49].

The blood-borne VOCs derived from organs other than the lung can also be detected in exhaled breath. As reported, acetone is produced in diabetes mellitus, methane and hydrogen from intestinal bacteria activity, sulfur-containing compounds (i.e. dimethylsulfide, methyl mercaptan, ethyl mercaptan) in liver impairment, and nitrogen-containing...
compounds (i.e. ammonia, dimethylamine, trimethylamine) in kidney impairment [50, 51]. Some host-derived volatile metabolites in the breath have also been reported after pathogen infection due to non-specific lipid peroxidation and oxidative stress [16, 34]. Certain target compounds in vivo might be influenced by unmatched liver/renal functions or systemic inflammatory reactions due to infection. As discussed by Professors Haick and Cohen-Kaminsky, breath-prints from lung infection are a balanced mixture of pathogen-derived VOCs, host-derived VOCs, host-derived VOCs in response to pathogens (e.g. immune response), host-derived VOCs in response to pathogen volatiles and pathogen-derived VOCs in response to host VOCs [52]. Certain volatiles derived from microorganisms are considered to possess communication and defense functions [38]. Overall, the specific biosynthetic pathways of the detected substances need to be further explored. Whether these VOCs are end products in the metabolic cycle or only regarded as precursors to secondary metabolites and the role in the pathophysiological process, all require elucidation in the future.

Mixed expiratory gas was sampled in our present study. The alveolar fraction in exhaled breath is potentially ideal for exploring pathogen-derived compounds in vivo, as the dilution effect of dead-space gas from the respiratory tract and endotracheal tube could be minimized. It is impossible to absolutely avoid the dilution effect while alveolar samples are taken, even using a CO₂ capnograph monitor. The breath collection devices in ventilated patients need to be optimized for simplicity and ease of use in gas sampling, such as employing a CO₂-triggered valve [15, 34]. Some unknown quantity of VOCs may be lost or decreased due to their condensation and absorption in condensed water on the inner walls of the tubing. The collecting facility will be further improved, as the transfer line is thermostated in addition to the cylinder.

We did not simultaneously detect inspiratory gas for background correction because the emphasis in the present study was placed on comparing breath profiles from ventilated patients with the presence of A. baumannii in the respiratory tract with those without, and those with A. baumannii infection to those with colonization, not the absolute concentration of the detected compounds. Bias in the same direction could be offset by comparisons so that it would not interfere with the outcome of analyses. A simple 1D subtraction method is not a favorable strategy without knowledge of the concentration of the target compound in blood, its pulmonary adsorption and exhalation ratio, and the lung ventilation/perfusion rate [34, 53].

Our finding of sesquiterpene longifolene in the breath VOCs is interesting; it has been previously discovered in pine resin and Nigella sativa oil [54]. Terpenoids are biosynthesized through the mevalonate pathway or the newly discovered deoxyxylulose phosphate pathway. Multiple bacteria are reported to produce many species-related volatiles, including terpenoids released by cyanobacteria, actinomycetes (e.g. Streptomyces) and myxobacteria, which have been reviewed thoroughly by Schulz and Dickschat [20]. Some monoterpenes and sesquiterpenes have also been detected in the breath of patients with invasive aspergillosis and sputum headspace for the presence of P. aeruginosa [55, 56]. The sesquiterpenoid isolongifolene was found to be released by Marine Artic bacteria [36]. Longifolene was detected in the present in vitro and in vivo studies, which endowed A. baumannii with its metabolic characteristics. Further studies involving its actual metabolic pathway and its potential function need to be carried out.

Our present pilot study has some limitations. The first is the relatively small number of subjects. Larger studies on A. baumannii VAP should be further developed. However, the research period could be extended, particularly with a progressively strict control of nosocomial infection, or a multicenter cohort could be utilized for clinical applications. The second is the diagnostic accuracy of VAP. Several patients might be misdiagnosed without histological examination, known as the gold standard, which could possibly have influenced the findings of the current study. Overall, biopsy or autopsy has not been considered as a routine method for clinical VAP diagnosis. The third refers to the unparalleled baseline regarding liver/renal functions and the extent of the inflammatory reaction between groups. As discussed above, further work should be performed for the biosynthetic and metabolic pathways of these compounds. The fourth indicates that the detected values of the compounds were not converted to the absolute quantities, though this method has been employed in previous studies [55, 57]. The integrated area of each compound’s GC peak was calculated as the relative abundance of the metabolites in breath samples. Overall, we have placed emphasis on the comparison between the variable bacterial states.

5. Conclusion
It is not feasible to simply transfer the metabolic biomarkers from the in vitro condition to in vivo due to their distinct volatile profiles. This pilot study mainly provides a proof of concept that the direct detection of exhaled A. baumannii-derived VOCs might be adopted for an early alert of the LRT bacterial presence in ventilated ICU patients and even different parasitic states (i.e. infection and colonization) of A. baumannii, though further refinement and validation are required before clinical use.

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