Diagnosis of pulmonary injury and infection by exhaled breath analysis
Bos, L.D.J.

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Chapter 7.

Volatile metabolites of pathogens – a Systematic review

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Lieuwe DJ Bos, Peter J Sterk & Marcus J Schultz
Abstract

Ideally, invading bacteria are detected as early as possible in critically ill patients: the strain of morbific pathogens is identified rapidly, and antimicrobial sensitivity is known well before start of new antimicrobial therapy. Bacteria have a distinct metabolism, part of which results in the production of bacteria–specific volatile organic compounds (VOCs), which might be used for diagnostic purposes. Volatile metabolites can be investigated directly in exhaled air allowing for non–invasive monitoring. The aim of this review is to provide an overview of VOCs produced by the six most abundant and pathogenic bacteria in sepsis, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*. Such VOCs could be used as biological markers in the diagnostic approach of critically ill patients.

A systematic review of existing literature revealed 31 articles. All 6 bacteria of interest produce isopentanol, formaldehyde, methyl–mercaptan and trimethyl–amine. Since humans do not produce these VOCs they could serve as biological markers for presence of these pathogens. The following volatile biomarkers were found for identification of specific strains: isovaleric acid and 2–methyl–butanal for *Staphylococcus aureus*, 1–undecene, 2,4–dimethyl–1–heptane, 2–butanone, 4–methyl–quinazoline, hydrogen–cyanide and methyl–thiocyanide for *Pseudomonas aeruginosa* and methanol, pentanol, ethyl–acetate and indole for *Escherichia coli*. Notably, several factors that may effect VOC production were not controlled for, including used culture media, bacterial growth phase and genomic variation within bacterial strains.

In conclusion, VOCs produced by bacteria may serve as biological markers for their presence. Goal–targeted studies should be performed to identify potential sets of volatile biological markers and evaluate the diagnostic accuracy of these markers in critically ill patients.
**Introduction**

Sepsis is increasingly prevalent in the developed world comprising 240 per 100,000 persons per year [1]. Early start of targeted antibiotics lowers mortality [2]. However, in the majority of cases empirical antibiotic treatment is untargeted due to inadequate diagnostics, resulting in a three-fold increase in mortality when compared to targeted antibiotic treatment [3].

Ideally, invasion of morbific pathogens is detected as early as possible; the strain of the causative pathogens is identified swiftly, and antimicrobial sensitivity is rapidly known, preferably before start of antimicrobial therapy. However, cultures may take days to become positive and have limited sensitivity, especially in patients already receiving antibiotics because of a previous infection [4]. In addition, contamination could lead to false-positive results and therefore may increase prescription of unnecessary antibiotics [5]. Gram–stain results and direct cellular examination (e.g. of broncho-alveolar lavage fluid) are rapidly available but have limited sensitivity and specificity, and do neither tell the exact strain of pathogen nor its antimicrobial sensitivity [6, 7]. These disadvantages also apply to several biomarkers (c-reactive protein, pro-calcitonine, pro-adrenomedullin and endotoxin) [8-10]. PCR–based diagnostics are currently under investigation and although the results are promising, PCR takes hours before results are available and is laborious and costly [11].

In ancient times, physicians relied heavily on their senses before sophisticated analytical techniques became available. Color, taste, and smell were used to detect biological markers [12]. Bacteria are known to have a characteristic smells. Bacterial strains have a distinct metabolism, part of which results in the production of bacteria–specific volatile organic compounds (VOCs) [13-15]. The metabolic pathways have been described for bacteria in several excellent review articles [14, 15]. However, reviews not yet focused on pathogens and clinical problems.

Detection and identification of VOCs using sophisticated technology may have diagnostic value in medicine [14, 16, 17]. These techniques include gas–chromatography and mass spectrometry (GC–MS), selected–ion flow tube mass–spectrometry (SIFT–MS) [18], ion–molecule reaction mass–spectrometry (IMR–MS) [19, 20] and electronic noses (eNoses)
GC–MS is used as a gold-standard for separation, detection and identification of VOCs. SIFT–MS and IMR–MS allow for real-time measurement of some VOCs. eNoses do not identify VOCs but rely on pattern-recognition [16]. Volatile compounds can be investigated in \textit{in–vitro} (in culture media or directly in patient material) or directly in the exhaled air (\textit{in–vivo}) allowing for non-invasive monitoring.

Three goals could be pursued with VOC-detection: (1) proof absence of bacterial pathogens (i.e., very high sensitivity and negative predictive value, and therefore no start of antibiotic treatment), (2) identify the presence of a specific strain of bacteria (i.e., very high specificity and positive predictive value, and thus start of appropriate antimicrobial therapy), and (3) separation between phenotypes within bacterial species and therefore prevention of start of antibiotics for which the causative pathogens are not sensitive. However, before VOCs can be tested for these goals in clinical trials, possible diagnostic targets per goal should be known. Therefore, the aim of this review is to provide an overview of volatile organic compounds produced by the six most abundant and pathogenic bacteria in sepsis: \textit{Staphylococcus aureus} (SA), \textit{Streptococcus pneumoniae} (SP), \textit{Enterococcus faecalis} (EF), \textit{Pseudomonas aeruginosa} (PA), \textit{Klebsiella pneumoniae} (KP) and \textit{Escherichia coli} (EC) [22].
Methods

A broad systematic search in the EMBASE library was performed on the 1st of August 2012 using the following terms: “(mass and spectrometry) and bacteria and volatile”.

Articles were selected for full-text examination if the title and/or abstract suggested the investigation of bacterial pathogens in a clinically relevant setting and the measurement of volatile organic compounds.

Selected articles were read and included if (a) one or more of the following, most frequently cultured pathogens on the ICU [22], was investigated: Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae or Escherichia coli and (b) a summary of detected volatile organic compounds per pathogen was provided. Furthermore, all references of the selected articles were scanned based on title and selected based on the previous criteria. Double publications of the same data were disregarded.

All volatile organic compounds described in the included articles were summarized in 9 tables (see supplemental information) based on the following molecular structures (adapted from Hakim et al. [64]): hydrocarbons, alcohols, acids, aldehydes, ketones, cyclic compounds, esters, S−containing and N−containing. They were referred to in the text by number (#). If a molecule could be included in more than one table, the most appropriate category was chosen to avoid duplicates.

The production of a VOC by a pathogen in an article was indicated with a “+” and the absence of a molecule with a “−”. The results section focuses on metabolites found in more than one study. The rows of these metabolites also received a coloring in tables S1-S9 based on level of evidence for absence, or presence of a metabolite. Cells were colored based on the pooled results for a VOC per pathogen, for all included studies. A clear cell indicated there is little evidence (zero or one study). When there is convincing evidence a VOC is produced by a pathogen, the cell is colored green (more positive than negative evidence, with more than one study difference). A red cell means that pathogen is not known or rarely found to produce that molecule (more negative than positive evidence, with more than one study difference). Contradicting evidence resulted in an
orange cell.

**Results**

The MEDLINE search resulted in 837 articles of which 778 were excluded based on title and/or abstract (figure 1). Fifty-nine articles were read and tested for inclusion criteria. This resulted in the inclusion of twenty-seven articles (table 1). Ten articles were read based on references, of which 4 were included, bringing the sum of included articles to thirty-one.

The articles originated from 1977 to 2012, with a rapid increase in number of publications from 2006. Fifteen articles reported on data collected with GC–MS, 7 on data collected with SIFT–MS, three with IMR–MS, and six with other techniques. Seven studies used clinical samples; twenty studies used reference strains. Results on 161 metabolites were obtained of which a minority was studied in multiple papers. The findings are reported in tables S1 to S9 and summarized below, per functional group. The most prominent VOCs and their (cross-)association with the six selected gram positive and gram negative bacteria are illustrated in figure 2.

**Figure 1: Flowchart**

![Flowchart diagram](image)
<table>
<thead>
<tr>
<th>Year</th>
<th>1st Author</th>
<th>Pathogen</th>
<th>Method</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1977</td>
<td>Hayward</td>
<td>SA, PA, EC</td>
<td>GLC</td>
<td>Through references</td>
<td>[65]</td>
</tr>
<tr>
<td>1979</td>
<td>Cox</td>
<td>PA</td>
<td>GC + Colometric</td>
<td>Through references</td>
<td>[66]</td>
</tr>
<tr>
<td>1980</td>
<td>Labows</td>
<td>PA</td>
<td>GC−MS</td>
<td>Pathway description</td>
<td>[39]</td>
</tr>
<tr>
<td>1984</td>
<td>Davies</td>
<td>SA, PA, EC</td>
<td>HS−GLC</td>
<td></td>
<td>[67]</td>
</tr>
<tr>
<td>1986</td>
<td>Zechman</td>
<td>SA, PA, KP</td>
<td>GC−MS</td>
<td></td>
<td>[28]</td>
</tr>
<tr>
<td>1995</td>
<td>Kuzma</td>
<td>PA, EC</td>
<td>GC−MS</td>
<td>Pathway description</td>
<td>[24]</td>
</tr>
<tr>
<td>1997</td>
<td>Scholler</td>
<td>PA</td>
<td>GC−FID</td>
<td></td>
<td>[68]</td>
</tr>
<tr>
<td>2000</td>
<td>Juliák</td>
<td>SA, SP, EF, PA, KP, EC</td>
<td>GC−MS</td>
<td></td>
<td>[69]</td>
</tr>
<tr>
<td>2003</td>
<td>Juliák</td>
<td>SA, SP, EF, PA, KP, EC</td>
<td>GC−FID</td>
<td>Clinical samples</td>
<td>[53]</td>
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<tr>
<td>2005</td>
<td>Carroll</td>
<td>PA</td>
<td>SIFT−MS</td>
<td>Clinical samples</td>
<td>[70]</td>
</tr>
<tr>
<td>2005</td>
<td>Hamilton−Kemp</td>
<td>EC</td>
<td>GC−MS</td>
<td>Through references</td>
<td>[71]</td>
</tr>
<tr>
<td>2006a</td>
<td>Allardyce</td>
<td>SA, SP, PA, EC</td>
<td>SIFT−MS</td>
<td>Antibiotic effects</td>
<td>[56]</td>
</tr>
<tr>
<td>2006b</td>
<td>Allardyce</td>
<td>SA, SP, PA, EC</td>
<td>SIFT−MS</td>
<td>Two different timepoints</td>
<td>[51]</td>
</tr>
<tr>
<td>2006</td>
<td>Julak</td>
<td>PA</td>
<td>SIFT−MS</td>
<td>Clinical samples</td>
<td>[52]</td>
</tr>
<tr>
<td>2006</td>
<td>Scotter</td>
<td>SA, SP, PA, EC</td>
<td>SIFT−MS</td>
<td></td>
<td>[72]</td>
</tr>
<tr>
<td>2008</td>
<td>Bunge</td>
<td>EC</td>
<td>PTR−MS</td>
<td>Different timepoints</td>
<td>[73]</td>
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<td>2008</td>
<td>Syhre</td>
<td>SA, SP, EC</td>
<td>GC−MS</td>
<td>Clinical samples</td>
<td>[35]</td>
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<tr>
<td>2009</td>
<td>Maddula</td>
<td>EC</td>
<td>MCC− IMS + GC−MS</td>
<td></td>
<td>[74]</td>
</tr>
<tr>
<td>2009</td>
<td>Preti</td>
<td>SA, PA</td>
<td>GC−MS</td>
<td>Clinical samples</td>
<td>[54]</td>
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<tr>
<td>2010</td>
<td>Scott−Thomas</td>
<td>PA</td>
<td>GC−MS</td>
<td>Clinical samples</td>
<td>[40]</td>
</tr>
<tr>
<td>2010</td>
<td>Thorn</td>
<td>SA, EF, PA, EC</td>
<td>SIFT−MS</td>
<td>Multi−variate analysis</td>
<td>[13]</td>
</tr>
<tr>
<td>2010</td>
<td>Zhu</td>
<td>SA, PA EC</td>
<td>SESI−MS</td>
<td></td>
<td>[75]</td>
</tr>
<tr>
<td>2010</td>
<td>Chambers</td>
<td>SP</td>
<td>GC−MS</td>
<td></td>
<td>[34]</td>
</tr>
<tr>
<td>2011</td>
<td>Savelev</td>
<td>PA</td>
<td>GC−MS</td>
<td>Clinical samples</td>
<td>[60]</td>
</tr>
<tr>
<td>2011</td>
<td>Shestivska</td>
<td>PA</td>
<td>GC−MS</td>
<td></td>
<td>[76]</td>
</tr>
<tr>
<td>2011</td>
<td>Storer</td>
<td>SA, EF, PA, KP, EC</td>
<td>SIFT−MS</td>
<td>Inoculated urine</td>
<td>[77]</td>
</tr>
<tr>
<td>2012</td>
<td>Bean</td>
<td>PA</td>
<td>GC/GC−TOF−MS</td>
<td></td>
<td>[78]</td>
</tr>
<tr>
<td>2012a</td>
<td>Dolch</td>
<td>PA, EC</td>
<td>IMR−MS</td>
<td>Two different timepoints</td>
<td>[49]</td>
</tr>
<tr>
<td>2012b</td>
<td>Dolch</td>
<td>SA, EF</td>
<td>IMR−MS</td>
<td>Two different timepoints</td>
<td>[50]</td>
</tr>
<tr>
<td>2012</td>
<td>Filipiak</td>
<td>SA, PA</td>
<td>GC−MS</td>
<td>Different timepoints</td>
<td>[48]</td>
</tr>
</tbody>
</table>

SA = Staphylococcus areus, SP = Streptococcus pneumoniae, EF = Enterococcus faecalis, PA = Pseudomonas aeruginosa, KP = Klebsiella pneumoniae, EC = Escherichia coli.
Hydrocarbons (table S1)

In table S1, the hydrocarbons investigated in pre-specified pathogens are listed. One of the most investigated hydrocarbons is isoprene [13], which seems to be produced in both Gram-positive and Gram-negative bacteria, although studies show contradicting results on their presence. The production is most likely through the methylerythritol phosphate pathway and is growth dependent (high during the log-phase and low during the stationary phase) and occurs primarily in a nutrient rich environment [23, 24]. Isoprene is also one of the main volatiles in the breath of mammals and is less applicable as focus for in-vivo studies [25]. 1-Undecene [6] and other less well-studied alkenes [3-5] are suggested to be produced mainly by PA, and are most likely the product of degradation of fatty acids through the B-oxidation pathway, a pathway that is suggested for most volatile hydrocarbons [15, 26]. 1,3-Butadiene [2] is reported to be produced by Gram-positive bacteria, but not by Gram-negative bacteria.

Alcohols (table S2)

1-Alcohols are produced through b- or a-oxidation of fatty acid derivates through acetyl-CoA. Ethanol [30] is one of the most studied volatiles [15]. It can be produced by all investigated bacteria, but some (SA, SP, KP and EC) produce it almost always, while others (EF and PA) have been found to lack ethanol in the headspace. Methanol [32], propanol [34], butanol [27], pentanol [36] and some longer chain 1-alcohols [28, 29, 35] are most prominently produced by EC, however not exclusively. EC might use these alcohols to inhibit the growth of other bacteria [27]. The branched alcohol isopentanol [25] is found less frequently in EC, compared to the other pathogens. This metabolite is produced through another pathway, possibly via isovaleryl-CoA, since concentrations increase when leucine is added to the growth medium [28].

Acids (table S3)

Fatty acids could be a marker for anaerobic metabolism, but are not strain specific [29, 30]. However, anaerobic dependent production does not apply to very short, volatile fatty acids. Acetic acid [37] is most frequently produced by SA, but also by the other pathogens. Isovaleric acid [42]
is more exclusively produced by SA. Propionic acid [#44] has only been reported in the headspace of KP. Other acids [#38–41, 45] have not been identified in the headspace of the pathogens studied in that review.

**Aldehydes (table S4)**

Formaldehyde [#56] is produced by many bacteria [31], including the six species on which we focus in this review. Acetaldehyde [#54] is also produced by most pathogens, but PA and KP are less likely to produce this metabolite. Notably, acetaldehyde and benzaldehyde [#55] are known to have antimicrobial activity [32]. Methylpropanal [#49], 3−methyl−butanal [#53] and 2−methyl−butanal [#48] are modifications of amino−acids and intermediates for the formation of many ester and branched ketones [15]. Methyl−propanal and 2−methyl−butanal are mostly produced by SA while methylpropanal is produced by all investigated pathogens.

**Ketones (table S5)**

Methyl−ketones are produced during decarboxylation of fatty acid derivates. The smallest, acetone [#81], is produced by most bacteria, but not under all circumstances. Furthermore, acetone is also present in high concentrations in breath, limiting the in−vivo applicability as a biomarker for bacterial presence. The longer 2−ketones [#62–70] are classically biomarkers for PA [28], but the pooled results provide evidence only for 2−nonanone, 2−dodecanone, 2−pentanone and 2−heptanone. 2−Nonanone is also produced by SA. Acetoin or 3−hydroxybutanone [#79] is used to differentiate between lactose fermenting and nonfermenting Enterobacteriaceae. Surprisingly, in one study acetoin was found in the headspace of the non−fermenting EC. This suggests the involvement of other pathways for the production of acetoin [33]. In SA, acetoin generation has been linked to murein hydrolase activity, stationary−phase survival and antibiotic resistance [33].

**Cyclic compounds (table S6)**

2−Phenylethanol [#87] is one of the most widespread microbial VOCs [15], but not in the hereby investigated pathogens. 2−Pentylfuran [#86] has been proposed as a biomarker for Aspergillus [34, 35] but was also found in the headspace of SP. Two pathways of production via linoleic
acid have been proposed: enzymatically controlled oxidation and direct interaction with reactive oxygen species [34]. Limonene [#89], phenol [#92] and toluene [#93] are identified as potential markers for bacterial presence in this study. This might imply that earlier statements that these compounds should be regarded as exogenous when found in the patients’ breath need reconsideration [36]. In a study by Holland, germfree rats were compared to conventional rats [37]. Urinary acetophenone [#88] was 13-fold increased in conventional rats, suggesting bacterial production of this compound. This was indeed reported in one in-vitro study. Several other compounds were found in conventional rats of which 4-heptanone [#76], 2-heptanone [#66] and 5-methyl-2-hexenal were the most significant and are also produced by bacteria in-vitro.

**Esters (table S7)**

Ethyl acetate [#100] and other acetate containing esters [#99, 108] are the product of esterification between acetic acid and a fatty acid. However, the pathogens producing most acetic acid, such as SA are not the same as the ones with the most prominent ethyl acetate production, such as EC. The factors influencing this reaction remain unknown. Ethyl butanoate can be produced by all six pathogens, but is mostly found in *Enterococcus* and EC.

**S-containing (table S8)**

The most important volatile sulfur containing organic compounds are hydrogen-sulfide [#120], methyl-mercaptan [#122], dimethylsulfide [#118], dimethyldisulfide [#117] and dimethyltrisulfide [#119]. All are highly toxic and might be involved in the induction of inflammation [38]. All bacteria are able to produce these compounds but they might provide additional information about the species of pathogen. Hydrogensulfide is produced mainly by EF and EC, while dimethyldisulfide is more frequently found in the gram negative bacteria. Dimethyltrisulfide might be a marker for PA and dimethylsulfide for PA and SP.
The six investigated pathogenic bacteria are plotted. All the metabolites for which convincing evidence on production by at least one of the bacteria was available were included in the figure and connected with a line to all bacteria known to produce a particular metabolite. Four zones of interest are highlighted. The blue zone in the middle indicates metabolites that are (almost) always produced by all pathogens and are therefore candidate markers with a high sensitivity that might thus qualify for the exclusion of infection (high negative predictive value). The three red zones indicate metabolites that are produced by only or mainly one strain of bacteria; these are possibly volatile biomarkers specific for a pathogen with a very high positive predictive value.

**Figure 2: Interaction plot**

The interaction plot shows the relationships between the six investigated pathogenic bacteria and various metabolites. The metabolites are plotted in different zones according to their production patterns by the bacteria. The blue zone indicates metabolites that are consistently produced by all bacteria, suggesting they might be used for the exclusion of infection. The red zones highlight metabolites produced by specific strains, which could serve as specific biomarkers for particular pathogens.
N−containing (table S9)

The simplest N−containing volatile organic compound, ammonia [#142], is most frequently produced by SA and PA. Hydrogencyanide [#147] is only investigated in cultures of Pseudomonas, but was found to be produced in all studies. Trimethyl−amine [#161] might be a marker for PA and EC. 2−Aminoacetophenone (2−AA) [#130] was recently proposed as a breath marker for PA and is responsible for the grape−like odour associated with PA infections [39, 40]. However, review of the literature clearly shows it can be produced by most bacteria and is frequently found in SP and EC. Furthermore, 2−AA can be found in a variety of food products and after consumptions found in exhaled breath, resulting in false−positive results [41]. Indole [#148] is a direct product of deaminating L−tryptophan by tryptophanase. It is mainly produced by EC, but it is sporadically detectable in the headspace of other pathogens. Tryptophanase is essential for biofilm formation, thus indole can be regarded as a biomarker for this bacterial phenotype [42, 43].

Discussion

Pathogenic bacteria are capable of producing a large variety of volatile metabolites. Our systematic review identified thirty−one articles reporting on VOC production by the most important pathogens of sepsis. However, only a very small fraction of the metabolites is produced exclusively by one of the bacterial species of interest. Notably, some studies failed to replicate the results of previous experiments resulting in contradicting overall results. Despite these limitations, several sensitive and some very specific candidate biomarkers were identified by systematically summarizing the available literature (figure 2).

The large number of contradicting results between the studies might be explained by four variables. Firstly, not all studies used the exact same subtype of bacterial species. In one study, phage types of SA influenced headspace volatile organic compounds [44]. Genomic variation between subtypes could result in differences in efficacy of enzymes within a specific metabolic pathway. These variations might be useful to phenotype within species of bacteria, though. However, this could hamper the clinical applicability of volatile biomarkers for strain identification. Secondly, the
growth medium is the source of building blocks for the produced VOCs and therefore a confounding variable [44-47]. Thirdly, measurements were obtained at different time-points in the growth of bacteria. Several studies investigated this phenomenon and found that depletion of metabolites and growth phase (log or stationary) influence headspace metabolites [48-51]. Lastly, the majority of the included studies investigated cultures of reference strains. However some studies focused on clinical samples, in which within-class variation was increased [52-54]. Patient samples are less well defined than laboratory produced cultures of reference strains and are different in the following aspects: CFU’s, growth phase, host response, viscosity [55], confounding co-morbidities and medications (e.g. antibiotics [56]).

Several biomarkers qualify for clinical investigation with regard to the first goal of biomarker research; proof absence of a bacterial pathogen. Isopentanol, formaldehyde, methyl–mercaptan and trimethyl–amine are produced by all bacteria and not by the host (blue area in figure 2). Ethanol and Isoprene are also sensitive candidate markers but are found in large quantities in breath of mammals. If the aim of a study is to exclude bacterial infection from the differential diagnosis, a set of volatile biomarkers with a high a-priori chance of being produced by a lot of pathogens should be investigated. Not finding any of these candidate markers might have a high negative predictive value.

Identification of specific strains might be performed using the following VOCs: SA – isovaleric acid and 2–methyl–butanal; PA – 1–undecene, 2,4–dimethyl–1–heptane, 2–butanone, 4–methyl–quinazoline, hydrogen–cyanide and methyl–thiocyanide; EC – methanol, pentanol, ethyl–acetate and indole (red areas in figure 2). No candidate biomarkers for SP, EF and KP could be identified in the literature yet. For the identification of species of pathogens, a combination of volatile organic compounds is recommended. The advantages of this approach are illustrated in a recent paper by Thorn and in several studies using electronic nose technology [13, 55, 57, 58]. It is imperative that diagnostic accuracy (sensitivity and specificity) is reported following the STARD guidelines [59], as is only done in one of the studies included in this review [60].

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Phenotyping of pathogens should focus on infecting/colonizing bacteria, bacterial growth and bacterial resistance. Interestingly, indole was found to be a biomarker for biofilm formation in EC and might thus be used to separate between clinically relevant phenotypes within the same strain of bacteria. Secondly, small volatile sulfide−containing organic compounds were found to induce inflammation in a rat model and might thus serve as a marker for pathogenicity. Thirdly, the production of several VOCs is decreased after the addition of antibiotics in levels above MIC to the culture medium, suggesting that therapeutic response can be monitored. Antibiotic administration below MIC did lower VOC concentrations, but to a lower extend, suggesting a dose dependency. The influence of bacterial resistance on VOCs was not described in the included papers. However, the first steps in this direction are taken in a recent paper on colometric electronic nose technology discriminating methicillin−resistant SA from methicillin−sensitive SA and vancomycin−resistant EF from vancomycin−sensitive EF [58].

This review has several limitations. First of all, most included studies did not include all pre–selected pathogens and thus provide partial evidence for clinical questions involving all pathogens. Secondly, since most studies did not report quantitative measures and used different sampling techniques no headspace concentrations could be given per compound per study. Thirdly, increased headspace concentrations were reported but decreased concentrations may have been missed in some studies. Indeed, the absence of a normally present metabolite might be just as much proof of the presence of a pathogen as the presence of another VOC. Finally, different technologies were used to detect the volatile organic compounds. GC−MS was mostly used, although not always with the same materials and separation methods. However, while keeping the limitations of the used separation methods in mind, GC−MS remains the gold standard for volatile organic compound discovery. In this review, we also included studies using SIFT−MS, IMR−MS and other techniques that allow for compound identification. These technologies are not as powerful as GC−MS in separating and identifying metabolites but were nevertheless included in this review since they did allow for identification of some compounds.
If volatile organic compounds are used in-vivo for diagnostic purposes in sepsis, several considerations must be taken into account. First of all, the growth medium inside the host might be entirely different from in-vitro growth media, resulting in a different set of produced metabolites. Secondly, the host will interact with the bacteria through an inflammatory response, which might alter metabolism. This inflammatory response can alter human metabolism in itself and future studies will need to address the metabolomic difference between and an infectious and a non-infectious inflammatory response [25, 61]. Thirdly, VOCs can be derived from diet and environment. Finally, the body, including the lung, is host to a unique microbiome, even in healthy conditions [62, 63]. It might very well be that these residential bacteria produce similar metabolites and therefore interfere with a VOC-based diagnostic test. In this scenario, VOCs altered by inflammation might be used to further discriminate between colonizing and pathogenic bacteria.

In conclusion, several volatile biomarkers show to be particularly promising candidates for proof of absence of infection, whereas some others qualify for the detection of bacteria and identification of the six investigated bacterial species. However, only a limited amount of research is available. Therefore, targeted studies should be performed to identify potential sets of volatile biomarkers and evaluate the diagnostic accuracy of these markers in critically ill patients.

**Online Supplement**

For the online supplement, containing supporting information please follow this link: files.figshare.com/1058279/Text_S1.doc or access through the 2D-code below.
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