Chapter 3

Volatile Organic Compound and Metabolite Signatures as Pathogen Identifiers and Biomarkers of Infectious Disease

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Additional information is available at the end of the chapter

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Abstract

Volatile organic compound (VOC)-based diagnostics have great potential to be the next generation of screening tools for pathogen identification and infectious disease management. VOCs are low molecular weight metabolic compounds that have high vapor pressures and low boiling points, both of which facilitate evaporation at ambient temperatures. There is increasing evidence that particular VOCs, or profiles of VOCs, are unique to various disease states. Different pathogenic species have been found to produce characteristic profiles of VOCs by virtue of their distinct metabolisms. The detection of these metabolite profiles from patient samples could provide an effective means of rapid, non-invasive pathogen identification, thus enabling early diagnosis and treatment. In this review, we will discuss the potential of VOC profiles to be utilized as biomarkers of pathogenic infection, with a focus on bacterial pathogens. Herein we describe the common methods for clinical VOC sample collection, provide an overview of the various instruments and techniques used for VOC detection and analysis, and summarize the key findings of recent studies that have investigated VOC biomarkers in various infectious diseases. We will also discuss the challenges associated with translating VOC analysis into a clinical diagnostic tool.

Keywords: volatile organic compounds, VOCs, metabolites, signatures, biomarkers, profiles, pathogen identification, infectious diseases, clinical diagnostics, non-invasive tools, antibiotic resistance

1. Introduction

Although VOC identification has only been enabled by the development of sophisticated analytical techniques in the last two decades, the premise that VOC profiles can be used as biomarkers for disease can be traced back to ancient times, when physicians diagnosed diseases

based on their senses. The ancient Greek physician Hippocrates (460–370 BC) is said to have poured human sputum over hot coals to liberate the distinct odors indicative of tuberculosis infection. In the early 20th century, it was postulated that bad breath in many mammalian species may be sexually unattractive precisely because it is indicative of disease [1]. Currently, there is a great public health need to develop rapid, non-invasive methods of identifying pathogens and determining their antibiotic resistance or susceptibility status in order to effectively treat infectious diseases. Conventional diagnostic methods offer limited sensitivity and specificity, and can be expensive, invasive for patients, and time-consuming, often requiring several days for cell culture and low-throughput microscopy assays. Delays and limitations in diagnostic results often lead to the initiation of untargeted therapies, such as treatment with broad-spectrum antibiotics, which contribute to the evolution of antibiotic-resistant pathogens.

VOCs represent a diverse group of carbon-based molecules, including alcohols, ketones, aldehydes, hydrocarbons, isocyanates, amines, terpenes and sulfides [2]. VOCs are generally short-lived and become rapidly diluted in microenvironments. Altering growth conditions can modulate VOC profiles, reflecting the unique metabolic state of an organism in specific environments. Many animals, plants, and microbes have evolved chemical sensing mechanisms that can detect minute quantities of VOCs released during growth to protect against antagonists and to act as signaling molecules for intercellular communication. For example, plants use volatiles to communicate with pollinators and to coordinate growth with their own kind to out-compete foreign species.

As a result of normal metabolic functions, the healthy human body produces a vast number of VOCs that are liberated in exhaled breath, skin secretions, saliva, blood, urine and feces. Many of these VOCs likely derive from commensal microbes in the body and are often detectable by odor [3]. Pathogenic infection in humans alters both the quantity and composition of VOCs produced. As a result of their distinct metabolisms, different pathogens produce characteristic VOC profiles, which can often be detected in the headspace of cultures grown *in vitro* [4, 5]. Upon pathogen infection, VOCs released by both the pathogen and infected host can potentially be used as a diagnostic signature of the infection state. Analysis of the VOC profiles released from clinical samples have yielded VOC biomarkers indicative of specific diseases and infections [6–8]. Exhaled breath tests in particular have already proven useful in the diagnosis of a broad range of pathologies, including lung disorders, diabetes, gastrointestinal and liver disease, cancer, and pathogen infection [9–11].

VOC signatures uniquely associated with specific pathogens can be clinically relevant for diagnosing various infectious diseases, elucidating antibiotic resistance versus susceptibility, designing treatment regimens, and monitoring disease progression. The ability to reliably distinguish between different pathogenic species, based on their VOC signatures, will facilitate the development of rapid, highly-sensitive, and non-invasive diagnostic methods and tools, ultimately leading to improved patient outcomes.

2. Overview of clinical sample collection for VOC analysis

There are potential advantages to employing VOC signatures for disease diagnostics, such as ease of collection from all patients, including the critically ill, children, and the elderly.

Furthermore, longitudinal samples from patients could be more easily obtained to track disease progression and monitor therapeutic interventions during follow-up studies. The following section describes VOC collection and detection from various types of clinical samples.

2.1. Breath

The vast majority of studies on VOC biomarkers have been conducted using exhaled breath samples, as they are the most easily obtained [12]. VOC analysis from breath samples has proven useful for diagnosing a wide range of diseases and various infections [11, 13]. Exhaled breath contains hundreds of VOCs that can be attributed to either exogenous or endogenous sources. Exogenous volatiles include compounds inhaled from the external environment, such as the ingestion of food or smoking cigarettes. Endogenous volatiles consist of compounds derived from the body. These may include compounds produced by the human body's assortment of commensal bacteria, or in the case of infectious disease, compounds released by pathogenic microbes. Endogenous volatiles are transported from different organs via the bloodstream to the lungs, excreted via diffusion across the pulmonary alveolar membrane, and subsequently exhaled via breath. Distinguishing exogenously derived VOCs from the endogenous compounds in a breath sample is a significant challenge in elucidating VOC signatures related to disease. The detection of exogenous VOCs in a breath sample may suggest exposure to a drug or environmental toxin, which can confound the search for disease biomarkers [14].

VOCs contained in clinical breath samples and bacterial culture headspace samples are present at very low levels. To concentrate and analyze breath VOCs, several methods have been developed, such as chemical trapping, sorbent trapping, cold trapping, or condensate trapping, followed by thermal desorption to analyze the VOC content [15]. Pre-concentration of breath VOCs can further be achieved by solid-phase micro-extraction (SPME), in which different VOCs in a sample are adsorbed by a coated microfiber. These VOCs are then delivered directly into the mass spectrometer or other instrument for analysis. A more recently developed method is membrane extraction with a sorbent interface, which combines sampling and pre-concentration in a single step [16]. Exhaled breath condensate (EBC) is another new technique in which aerosolized micro-droplets from the lower respiratory tract are captured by directing the exhaled air through a cooling device, resulting in the accumulation of EBC in the collection chamber. In general, EBC collection is an inefficient VOC capture method, due to the abundance of non-volatile components in the micro-droplets [11].

2.2. Saliva

Human saliva from healthy subjects is a complex secretion containing peptides, proteins and metabolites. Saliva is not a homogenous fluid, but a mixture of different fluids made from three distinct salivary glands (the parotid, the submandibular, and the sublingual glands). The protein composition of these fluids varies significantly depending on the gland sampled. A small amount of saliva is also secreted through hundreds of minor glands located within the mouth. As such, when sampling saliva, it is essential to characterize the sample in terms of its location. Most studies utilizing saliva samples collect the whole saliva mixture comprised of all the various glandular saliva types produced in the mouth [17]. While the protein composition arising from each salivary gland is well characterized, the metabolite composition is not well understood. However, it has been determined that saliva contains numerous VOCs

including alcohols, aldehydes, ketones, carboxylic acids, esters, amines, amides, lactones and hydrocarbons [18]. The various bacterial species found in the oral cavity also contribute to the chemical composition of saliva through secretion of their metabolic by-products. Analysis of sulfur-containing volatile compounds in exhaled breath has linked malodorous breath to anaerobic bacterial activity in the oral cavity [19]. Furthermore, exogenous VOCs inhaled through the lungs or absorbed through the skin can be excreted into saliva. Other considerations for saliva sampling include time since brushing teeth, time since ingesting sugary or acidic foods, evidence of oral injury, and the presence of contaminating fluids such as blood [20]. The endogenous VOC profile of human saliva is of particular interest in medical forensics, where oral fluids are routinely analyzed for the presence of drugs or toxins.

2.3. Skin

VOCs emitted from the skin surface are mainly derived from a combination of sweat secreted by the sweat glands and sebum, an oily substance secreted by the sebaceous glands. Although some of these VOCs result from internal hormonal or metabolic changes, many VOCs appear to be derived from commensal skin bacteria that metabolize compounds secreted in sweat and sebum. Samples are easily obtained by wiping the patient's skin with an organic solvent (e.g. acetone), or by collecting the VOCs directly from the affected skin onto an absorbent SPME fiber. However, care must be taken to avoid contaminating the sample with cosmetics, perfumes, or compounds present in the ambient air. This may be especially difficult given that even trace VOCs associated with the preservatives found in skin creams and gels are detectable in skin swab samples [21].

2.4. Blood

Blood directly reflects the internal environment of the body, including nutritional, metabolic, and immune status. Given that most endogenous VOCs are secreted from cells directly into the bloodstream, as the main conduit of communication between different parts of the body, the analysis of plasma-derived VOCs has garnered much interest from researchers and clinicians alike. Recent work has focused on building up a compendium of blood-borne VOCs in healthy human subjects to compare these profiles with patient samples, and thereby identify VOC biomarkers unique to disease states [22]. However, acquiring blood samples is more invasive than either breath or skin, and the requisite pre-treatment of blood to remove red and white blood cells has the disadvantage of being very time-consuming. The SPME method has been shown to detect a range of volatiles at very low concentrations in human blood, including hydrocarbons (pentane and isoprene), ketones (acetone), halogenated compounds (isoflurane), and thioethers (dimethyl sulfide). Since blood-borne VOCs can also be liberated in exhaled breath, a greater understanding of blood VOCs will also contribute to the diagnostic potential of breath analysis [23].

2.5. Urine

Given that urine samples are routinely used for diagnosis of disease, urine components and urine profiles have been well characterized [24]. The compounds predominantly found in urine are intermediate products or end-products of many metabolic pathways. These substances contain a variety of chemical motifs, such as ketone, alcohol, furan, pyrrole and sulfide, which

often generate specific odors. Since there are many components present in urine samples, VOC patterns will only be evident after statistical analysis of many patient samples. In addition, considerable variation among individuals has been found in profiles of urine-derived VOCs [7]. Importantly, urine components are affected not only by the metabolic status of the body, but also significantly by ingested foods and drinks. Therefore, caution must be taken when determining whether or not any particular VOC biomarker is the result of disease-related changes in metabolism or an exogenous cause.

2.6. Feces

Fecal samples contain dietary end-products resulting from intestinal bacterial metabolism and digestive and excretory processes. The composition of a patient's gut microbiota is reflected in their fecal sample [25]. As such, fecal VOCs may provide the best non-invasive means of diagnosing gastrointestinal and liver diseases. Although many volatile compounds might be easily detected in fecal samples, they are still influenced by a range of confounding factors, such as diet, gender, age, smoking and certain medications. Also, a large number of VOCs in healthy patients is derived from the breakdown of food by intestinal normal flora, and is not indicative of any disease. Nevertheless, distinct patterns of VOCs have been discovered in the fecal samples of patients with certain bacterial infections, including *V. cholerae*, *C. jejuni* and *C. difficile* infections [8, 26].

3. Overview of VOC detection methods and analytical instruments

Over the last two decades, significant advances in analytical chemical techniques and instruments have facilitated the identification of VOCs with improved sensitivity and accuracy. Here we summarize the various methods used for VOC detection. The advantages and limitations associated with these techniques and instruments are summarized in **Table 1**, and reviewed in detail elsewhere (see [17, 21, 27, 28]).

Gas chromatography–mass spectrometry (GC–MS) is currently considered the gold standard for separation, detection, identification and quantification of VOCs. Samples for GC–MS must be in the gaseous phase such that a pure inert carrier gas can transport the sample through the chromatographic column. Depending on the VOC concentration, sample pre-concentration may be required [21]. Compound resolution improves as the length of the chromatographic column increases. Each unique compound is eluted from the column at a different time (termed the retention time) and detected by the mass spectrometer via compound ionization and measurement of the mass to charge ratio of each ion, thereby generating a unique mass spectrum for each compound. The class of volatile that can be detected by GC depends on the type of detector used. Examples include time of flight (TOF), plasma ionization, photoionization and electron capture detectors [17].

There are also several analytical methods that can be coupled with GC to achieve different outputs. As mentioned earlier, SPME followed by GC–MS can provide a solvent-free and easily automated system for quantifying trace amounts of VOCs [29]. However, it is important

Technique	Description	Advantages	Limitations
GC–MS	GC–MS combines separation, GC and MS. Separation is typically performed by a capillary column, with compounds being separated by their boiling point and polarity. As compounds are eluted, they are detected by the mass spectrometer as a function of their mass to charge ratio. Different MS detectors are available, with Time Of Flight (TOF) and tandem quadrupoles (MS–MS) being the most common.	• Good sensitivity (ppm-ppb) • Separates, identifies and quantifies VOCs all in one • High chromatographic resolution achievable • Highly reproducible results • Can analyze VOCs from complex mixtures • Can tentatively identify unknown compounds based on comparison to known mass spectra	• Often requires sample pre-concentration • Lengthy processing and analysis times • Unsuitable for screening unknown compounds • Requires a supply of pure inert carrier gas
GC-IMS	GC-IMS combines separation, GC and IMS. Separation may be performed using standard GC capillary columns or multi-capillary columns. Dual separation occurs first through the column and then in the detector according to the compound's gas-phase ion mobility. Ionized molecules are accelerated by an electric field towards a Faraday plate, where the impact of single ions is detected.	• High sensitivity (ppb-ppt) • Rapid results • Best for identifying differences between non-identical samples • Simple to use on site • Can use ambient air as the carrier gas	• Detection is compound-specific and depends on the ion's mass and charge • Limited dynamic range for quantitation • Unsuitable for screening unknown compounds • Confusing mass spectra can arise when high levels of solvents are present
Direct detection	These methods include SIFT-MS, IMR-MS, PTR-MS. They are popular for their sensitivity, rapid analysis times, and ability to extract target compounds from samples with little or no pre-separation.	• High sensitivity (sub-ppb) • Rapid results • Absolute quantification • Can detect trace compounds in mixtures	• Very expensive • Unsuitable for screening unknown compounds • PTR-MS only suitable for compounds with higher proton affinity than water
E-nose	A variety of E-nose detectors exist today. They generally consist of a micro-array of sensors which differ from each other in polarity. The sample passes through the array, and compounds adsorb to varying degrees on the different sensors depending on their composition. Compound adsorption on sensors changes the mass or resistance of each sensor, and this change is detected to provide different outputs.	• Best for identifying the differences between non-identical samples • Rapid results • Does not require sample separation or pre-concentration. • Relatively small, portable, and simple to use on site	• Cannot quantify VOCs • Can only identify known patterns of VOCs stored in its database • Unsuitable for screening unknown compounds • Sensitive to high ambient temperature and humidity

Table 1. Summary of most common VOC analytical techniques and their advantages and limitations.

to note that SPMEs are coated with different materials for selective compound adsorption. Therefore, individual SPMEs may not trap all VOCs present in a sample. GC ion mobility spectrometry (GC-IMS), based on separation of ions relative to their gas phase mobility, is highly sensitive and enables rapid quantification of separated VOCs, but is not suitable for

identifying unknown compounds. The advantage of GC-IMS over GC–MS stems from its ability to use ambient air as the carrier gas, which negates the need for a pure inert gas supply and allows GC-IMS devices to be portable and particularly useful for breath sample analysis [30]. GC flame ionization detection (GC-FID), which is also widely used for breath analysis, detects VOCs with high sensitivity and low background noise.

Optical spectroscopic methods, such as laser absorption spectrometry, are also useful for the detection and quantification of specific VOCs in a mixture. These methods are highly selective and sensitive, and can be connected to different types of spectroscopic sensors, such as conductive polymer sensors and acoustic wave sensors, to detect the specific VOCs of interest [31]. Non-optical direct-injection methods for VOC measurement include Ion Molecule Reaction mass spectrometry (IMR-MS), Selected Ion Flow Tube mass spectrometry (SIFT-MS) and Proton Transfer Reaction mass spectrometry (PTR-MS). These methods do not require pre-concentration and little or no pre-separation. However, unknown compound identification is not possible. SIFT-MS provides real-time absolute quantification of several VOCs simultaneously, and therefore is well suited for analyzing clinical samples. A small SIFT-MS-based analytical instrument has been developed for routine use in a clinical setting [32]. Both GC and PTR ionization technology can be coupled to a Time Of Flight mass spectrometer (GC-TOF-MS, PTR-TOF-MS), thereby making real-time VOC analysis possible [33, 34].

Finally, devices that electronically mimic the human olfactory system, termed electronic noses or 'e-noses', have been developed and improved upon since the 1980s [28]. E-noses employ several gas sensors combined with pattern recognition software to detect overall odor fingerprints rather than specific compounds. This may be considered a limitation of the technology, as it cannot identify individual biomarkers. On the other hand, unlike GC–MS, e-noses have the advantage of being able to differentiate between non-identical samples without the need to separate the mixture into its individual components, a process which can be highly variable based on the technique(s) used. E-noses provide rapid results, but are limited by the VOC patterns they are programmed to detect, and thus cannot be used for screening unknown compounds. Before e-noses can be used routinely for practical diagnosis, it will be necessary to improve their accuracy and sensitivity to enable reliable recognition of a large number of VOC profiles.

While the informatics approaches used in conjunction with the aforementioned instruments can vary, three main methods are typically used, alone or concurrently, to confirm VOC identification: (1) comparing mass spectra data obtained to those in reference libraries and databases, (2) comparing mass spectra and peak retention times to those obtained from pure standard compounds, and (3) comparing mass spectra data obtained to those characterized in the literature.

4. VOC analysis for detecting infectious diseases

A growing number of studies clearly demonstrate the efficacy of VOC analysis in identifying a wide range of non-infectious diseases, including inflammatory disease [35], diabetes [36], lung cancer [37], and even Alzheimer's disease [38]. In the context of infectious diseases, VOC detection has clinical value in three aspects of diagnostics: (1) identifying the absence of pathogens (i.e. no antibiotic treatment), (2) identifying the presence of a specific pathogen

(i.e. start appropriate antibiotic treatment), and (3) distinguishing between pathogenic species (i.e. determine antibiotic resistance versus sensitivity for the pathogen to guide treatment regimens). Examples of candidate VOCs identified as being associated with specific pathogens are summarized in **Table 2**. It should be noted here that many researchers emphasize the importance of considering the entire VOC profile of a pathogen and how it differs from another pathogen, rather than relying on any single VOC biomarker to reveal an association.

4.1. Respiratory infections

Although pathogens are capable of producing a large variety of VOCs, very few metabolites are produced exclusively by only one bacterial species. Particularly in cases of polymicrobial pulmonary infections, such as cystic fibrosis (CF), identification of the specific bacterial species responsible for the pathology is critical for correct diagnosis and treatment. Since patient prognoses can decline rapidly following these types of opportunistic infections, particularly in children, early detection is vital for the timely initiation of appropriate therapies [48]. GC-TOF-MS analysis of breath samples from CF patients has demonstrated that a distinctive VOC profile consisting of 22 compounds can discriminate CF patients from healthy controls with 100% accuracy. Furthermore, within the CF patients analyzed, a profile of 14 VOCs was able to correctly discriminate between patients with *Pseudomonas aeruginosa* positive cultures compared to those with negative cultures [49]. Interestingly, genotypically diverse strains of *P. aeruginosa* under the same culture conditions have been shown to exhibit a high degree of variability in detectable VOCs [50], indicating that additional CF patients need to be studied to determine which VOCs are truly discriminatory. In another study, distinct VOCs were characterized in the culture headspaces of four different opportunistic pathogens (*P. aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and the *Burkholderia cepacia* complex) that cause lung and airway infection in CF patients, providing additional VOC signatures to test in infected host systems [51].

Pathogen	Infectious disease(s)	VOC candidates for disease biomarkers	Reference(s)
M. tuberculosis	Active pulmonary tuberculosis	1-methyl-naphthalene, methyl nicotinate, 1,4-dimethyl-cyclohexane	[13, 39, 40]
C. jejuni	Ulcerative colitis, diarrhea	Butanoic acid, 1-octen-3-ol, 1-butoxy-2-propanol	[8]
C. difficile	Ulcerative colitis, diarrhea	Ethanol, Butanol, Isopropanol	[8]
V. cholera	Cholera	Dimethyl disulfide, p-menth-1-en-8-ol	[9, 26]
H. pylori	Peptic ulcers	Hydrogen cyanide	[41]
S. aureus	Sinusitis, pneumonia	acetoin, hydroxyacetone, acetic acid, isovaleric acid, acetaldehyde, 2-propanol, 3-methyl-1-butanol	[42, 43]
P. aeruginosa	Sinusitis, pneumonia	2-aminoacetophenone, pyrrole, 1-vinylaziridine, 3-methylpyrrole, 1-undecene, 2-nonanone, methyl thiocyanate	[43–46]
K. pneumoniae	Bronchitis, pneumonia	butyraldehyde, octyl acetate, tridecanol, dodecenal, butanoic acid	[47]

Table 2. Examples of VOCs associated with specific pathogens and infectious diseases.

Mycobacterium tuberculosis infection is another respiratory disease that has been the focus of much VOC research. GC–MS analysis of urine sample headspaces was used to identify and distinguish VOC profiles from tuberculosis (TB) patients and healthy controls. Five biomarker compounds were able to discriminate between these two groups with 98.8% accuracy: alpha-xylene, isopropyl acetate, 3-pentanol, dimethylstyrene, and cymol. These compounds also served to discriminate TB patients from patients with lung cancer and COPD [52]. In another study using GC–MS analysis of exhaled breath, active pulmonary TB could be distinguished from non-active TB with 85% accuracy. 1,3,5-Trimethylbenzene was identified in active pulmonary TB, whereas 1,2,3,4-tetramethylbenzene was a biomarker for the non-active state. Exhaled breath samples from all the TB patients contained the *M. tuberculosis*-associated biomarkers 1-methyl-naphthalene and 1,4-dimethyl-cyclohexane, which were also observed in *in vitro* cultures [39]. Other studies focusing on headspace VOCs from *in vitro* cultured *Mycobacterium* species have revealed several metabolites of nicotinic acid, four of which are considered specific for *M. tuberculosis* and *M. bovis* strains: methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole [53]. Methyl nicotinate has also been detected at high levels in the exhaled breath of smear-positive TB patients [40]. VOCs derived from *in vitro M. tuberculosis* cultures are distinct from those VOCs produced by an infected host, as a result of oxidative stress. Volatiles related to oxidative stress include alkanes and methylated alkane derivatives, whereas *in vitro*-defined VOCs of *M. tuberculosis* origin include cyclohexane, benzene, decane, and heptane derivatives [13].

4.2. Gastrointestinal infections

Fecal samples taken from patients suffering from various forms of infectious diarrhea have revealed characteristic VOC profiles depending on the causative pathogen. For example, the absence of hydrocarbons and terpenes indicated a *Campylobacter* infection, whereas the absence of furans and indoles indicated a *Clostridium difficile* infection [54]. Another study focusing on ulcerative colitis, a disease marked by inflammation of the colonic mucosa, found that while hundreds of volatiles were detectable in donor fecal samples, distinct VOC patterns could discriminate between healthy controls and patients infected with *C. jejuni* and *C. difficile* [8]. Typhoid fever is caused by *Salmonella typhi* infection and is spread by consuming contaminated water or food. VOC metabolite profiles specific to *S. typhi* can be detected by GC–MS from the blood samples of typhoid patients. Importantly, such metabolite profiles can also differentiate between *Salmonella typhi* and *Salmonella paratyphi A* infections, enabling targeted therapies [55].

4.3. Urinary tract infections

In cases of urinary tract infection (UTI), appropriate and effective therapy is heavily dependent on early diagnosis. UTIs are most frequently caused by *Escherichia coli* and other enteric pathogens such as *Enterococci*, *Klebsiella*, *Staphylococci*, and *Proteus* species, and also fungal pathogens such as *Candida albicans* [56]. Volatile metabolites released by these pathogens are detectable in the headspace of urine samples [56–58]. E-noses have proven particularly useful in recognizing the VOC patterns of healthy versus infected urine samples, though sample pre-concentration is often required. The relative efficacies of the various types of e-noses currently in use were compared in a recent review [57]. Since urine contains a complex mixture of VOCs that is relatively well-defined

[24], significant changes to the VOC profile of patients with UTIs may serve as diagnostic biomarkers of infection. To this end, more sensitive methodologies that do not require sample pre-incubation are needed to enable the efficient routine diagnosis of UTIs using VOC profiling.

4.4. Blood infections

A review of multiple studies revealed that distinct VOC signatures are produced by each of the six most abundant and pathogenic bacteria in sepsis (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*). While all six species produced isopentanol, formaldehyde, methyl mercaptan, and trimethylamine, each species also produced unique key compounds that can be used as specific VOC signatures [59]. Another blood-borne disease, malaria, is transmitted by mosquitoes that introduce the *Plasmodium falciparum* parasite into the blood of the host. Breath-based VOC analysis offers a rapid and non-invasive alternative to the current approach of visualizing *P. falciparum* on stained blood films. A recent study identified nine malaria-associated VOCs: carbon dioxide, isoprene, acetone, benzene, cyclohexanone, and four types of thioethers. The concentrations of these compounds varied significantly as the disease progressed. Following antimalarial drug treatment, parasite clearance correlated strongly with a decline in VOC levels [60]. Notably, another recent study found that blood cultures of *E. coli* and *S. aureus* yielded different VOC profiles before and after exposure to gentamicin or flucloxacillin, demonstrating that antibiotic susceptibility status can also be rapidly evaluated by VOC analysis [61].

5. Challenges in the clinical application of VOC analysis

There remain both logistical and technical challenges to the translation of VOC analysis from the research laboratory to the clinical setting. On the logistical side, the analytical instruments required for VOC detection are very expensive and require a large footprint and specialized training to operate and analyze the data. Furthermore, the methods are time-consuming and not readily scalable for high-throughput sample processing. There remains a lack of standardization for procedures in sample collection, pre-concentration, and storage, which are essential for effective clinical implementation.

From a technical standpoint, it is important to emphasize that the presence of a unique pattern of VOCs (constituting a complete VOC signature), rather than a single VOC biomarker, will be necessary for bacterial species identification [34]. Diagnostic tests based on a single VOC biomarker do not appear possible, given the fact that all pathogens produce a wide range of overlapping volatile metabolites. It should also be noted that the conspicuous absence of certain volatile compounds from a culture or sample actually forms part of the distinct VOC signature for a particular pathogen [36]. Furthermore, the specific profile of VOCs detectable *in vitro* is largely dependent on the bacterial growth state and density (e.g. logarithmic versus stationary phase), sample storage conditions (e.g. short-term versus long-term), and the type of culture media used [34, 62, 63]. To confound analysis further, patient samples are far less well-defined than laboratory cultures of reference strains, and therefore vary greatly in terms of growth phase, host response, viscosity, confounding co-morbidities, and medications (including antibiotics)

[43, 61, 64]. Therefore, reproducibility of VOC signatures, even in patients infected with the same pathogen, remains a challenge given the variability between individual patient samples.

If VOCs from primary patient samples are to be used effectively for clinical diagnostic purposes, we must recognize the confounding factors associated with VOC analysis. Firstly, the environment of the human body is entirely different from *in vitro* growth media for pathogen and human cell culture, thereby resulting in a completely different set of metabolic by-products [43]. Secondly, genotypic variability between different strains of a pathogen can strongly influence the types and concentrations of volatile metabolites detected [50]. Thirdly, the human body mounts an inflammatory response against pathogen infection, potentially leading to a change in bacterial and host metabolism. Future studies should address the metabolic differences between infectious and non-infectious inflammatory responses [65]. Fourthly, VOCs derived from exogenous sources, such as the host environment and diet, can easily contaminate a sample [66]. Before diagnostic tests based on endogenously produced VOCs can be routinely used on patient samples, it is necessary to definitively separate true biomarkers from contaminating components. Lastly, the human body plays host to an entire microbiome unique to each individual. It may be that these commensal bacteria produce many metabolites that are indistinguishable from those generated by disease-causing pathogens, and therefore may interfere with a VOC-based diagnostic test [67].

6. Conclusions and future perspectives

In the last two decades, diverse studies have used emerging and established technologies to assess the applicability of the VOC profiling approach to the diagnosis and treatment of pathogenic infections. At present, numerous studies have identified VOC profiles and candidate biomarkers for certain infectious diseases, which allow researchers to discriminate between different pathogenic species and between healthy and diseased individuals. VOC analysis continues to be a rapidly expanding field of inquiry. However, as outlined in the previous section, VOC-based diagnostics will require further development and vetting of reproducibility before transition from the laboratory to the clinic.

Existing VOC profiles and candidate biomarkers must still be corroborated across several coordinated studies before there can be sufficient confidence in their diagnostic efficacy. For example, independent *in vitro* studies that investigate the same organism, but subjected to different sampling methods and analytical techniques, have led to identification of different VOC patterns. Similarly, direct comparisons of independent clinical studies are difficult, given that experimental design and parameters differ between studies. In addition, very few studies to date have compared individuals with active disease to individuals at other disease stages (e.g. comparison of active TB and latent TB). Likewise, little data exists on the effect that comorbidities or co-infections (e.g. TB co-infection with HIV) may have on the range and type of detectable VOCs. Targeted studies are still required to fully characterize VOC disease signatures and to further evaluate the diagnostic accuracy of these biomarkers in patient samples. It is clear that before this approach can become integrated into routine clinical practice, it must first be validated by clinical trials using sufficiently large numbers of test subjects across a range of infections.

Despite the challenges, it is foreseeable that continued research in this area may pave the way for the design of unique diagnostic tools, such as disease-specific sensor arrays and targeted metabolite breathalyzers, that could also have potential applications in forensics, pharmacokinetics, and toxicology. Furthermore, the development of portable, sensor-based devices for the personalized monitoring of disease states and therapy progress would represent a clear advancement beyond the current state-of-the-art in clinical practice. In the long term, such tools could enable a more selective approach to antimicrobial drug use, while also opening up the possibility of individually tailored treatments.

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