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Bacterial Volatiles and Diagnosis of Respiratory Infections

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Abstract

Breath testing has enormous potential in the medical diagnostic field. The underlying complexity and perceived availability of adequate specimens, combined with a lack of knowledge of the metabolic pathways that give rise to compounds that are sources of analytes detectable in breath, has greatly slowed development. These real obstacles have recently been largely overcome in the use of breath testing to identify patients with cystic fibrosis associated *Pseudomonas aeruginosa* infection and tuberculosis. This review summarizes progress made in the characterization of microbial volatiles produced by major lower respiratory tract bacterial pathogens, and their potential use as diagnostic markers in patient breath testing.

1. INTRODUCTION

Respiratory infectious diseases are among only a few common causes of patient visits to a doctor. The most prevalent, the common cold, is likely the most frequent human symptomatic infection (Eccles, 2005). Laboratory culture, a central aspect of the science of microbiology, remains the definitive tool in diagnosis of common bacterial respiratory infections in otherwise healthy patients. Nevertheless, culture and biochemical characterization are no longer frequently used in routine medical practice simply because of the modest expense and minimal time associated with growth of bacteria that can be cultivated overnight in the laboratory media (Bartlett, 2011). Due to the typical low morbidity associated with the most common upper respiratory infections in otherwise healthy people, more often antibiotics are instead prescribed based on a patient history, doctor patient relationship, or self-report of sputum characteristics and length of symptoms (Tonkin-Crine, Yardley, & Little, 2011). Broad spectrum drugs are preferred, as most of the time the suspect bacterial agent is never confirmed. This contributes to acquisition of resistance in normal colonizers that can then be transferred to less benign residents and invaders by a variety of known mechanisms. Levels of use of antibiotics are directly related to levels of antibiotic resistance (Costelloe, Metcalfe, Lovering, Mant, & Hay, 2010), and an obvious goal is to reduce the inappropriate professional application of antibiotics in the treatment of respiratory infections.

A way to reliably identify a subset of agents causing respiratory infection in less than an hour at the site of patient contact would make a substantial contribution in this area

(Ustianowski, 2012). Perhaps the most intuitively appealing approach to rapid point of care diagnosis of specific bacterial infections is analysis of patient breath, or “breath testing”. It is now possible to identify infecting microorganisms based on volatiles they are known to produce. (In this summary of the current state of the field, breath testing will refer to detection of volatile metabolites in the breath, as opposed to “breath condensate” analysis, which should be considered as a distinct approach) (Hunt, 2011; Montuschi, 2007). While the proximity and accessibility of live microbes with distinct and diverse metabolic activities in or near airways provides a fundamentally important opportunity for efficient diagnosis, a series of challenges have greatly slowed use of the strategy. These include typically higher levels of both relevant environmental volatiles and potentially useful host-derived volatile metabolites in the same breath samples. Other obstacles include the need to concentrate and then efficiently recover volatiles present at very low levels for even the most sensitive of analyses, and that identical and very similar volatile compounds from different sources can contribute identical products to the analytical assays now used to identify them.

2. IDENTIFICATION OF VOLATILES AND VOCs

Volatile compounds are those whose vapor pressures at ambient temperatures are sufficient to escape from a liquid or solid surface and enter the air. For organic volatiles, these most often contain fewer than 20 carbons, and are under 500 Da (Skogerson, Wohlgemuth, Barupal, & Fiehn, 2011). Many are major secondary metabolites. All living things produce volatiles, but only a few have known roles in mediating ecological interactions. Volatile organic compounds or VOCs produced by plants are those best characterized, and include thousands of terpenoids, aromatic ring compounds, and amino and fatty acid derivatives (Baldwin, 2010). Several have known roles in pollination, defense, and fruit maturation (Kesselmeier & Staudt, 1999).

Gas chromatography (GC) together with electron impact (EI) mass spectrometry (MS) is the most established, reliable, and widely used approach to identify and characterize gasses and volatile compounds. Wider availability of this approach and the resolving power for different isomers in GC, and the existence of large reference databases and efforts at standardization (e.g. Kovats Index of *n*-alkane standards) (Phillips et al., 2012; Zhang et al., 2011) will increase the possibility for similar results to be obtained in different laboratories. Various methods of concentrating volatiles from reasonable volumes of air or breath (e.g. 1 L), including adsorbents (Woelfenden, 2010), solid-phase microextraction or SPME (Laaks, Jochmann, & Schmidt, 2011), and reactive coatings (Fig. 2.1) (Li, Biswas, Nantz, Higashi, & Fu, 2011; Strand, Bhushan, Schivo, Kenyon, & Davis, 2010), allow GCMS to be a sufficiently sensitive breath volatile detection method. Conventional high-temperature (>200° C) desorption on injection and “hard” ionization can complicate identification and reporting of novel volatiles and the definition of new putative diagnostic markers (Spanel & Smith, 2011). Original compounds may be altered prior to ionization, and ions generated are not readily interpreted for microbial volatiles not in databases and lacking reference standards. Complex samples are thereby made more complex, and analyses lengthy. This approach will nonetheless likely continue to remain essential in defining volatiles, and indicating the target diagnostic markers for dedicated sensors or other more direct MS analyses more likely to be used in the point of care testing. Current successful methods

(Chambers, Bhandari, Scott-Thomas, & Syhre, 2010) and the major analytical and statistical approaches used in breath testing have been recently reviewed (Boots et al., 2012; Buszewski, Keszy, Ligor, & Amann, 2007).

3. BACTERIAL VOLATILES

Our knowledge of VOCs produced by bacteria is quite limited compared to those released by plants. Although volatiles from different types of bacteria have been characterized, bacterial VOCs for the most part do not appear in the major databases (i.e. Wiley, NIST, Massfinder) used for initial presumptive identification of compounds by comparison of mass spectra (Kai et al., 2009; Schulz & Dickschat, 2007). Fully defining more microbial volatiles will require novel methods to obtain sufficient quantities necessary for structural studies by isotope labeling and NMR. In our laboratories, we are currently using a covalent reactive capture approach with Fourier transform ion cyclotron mass spectrometry (FTICR-MS), together with GCMS, to define small volatile compounds (<500 Da) whose specific molecular formulae can be reliably defined in this way (Feng & Siegel, 2007).

Our knowledge of what bacterial volatiles and VOCs actually are and do is limited (Kai et al., 2009), and the corresponding metabolic pathways they derive from are mostly unknown. Volatiles are indeed abundantly produced, and characteristic of different types of bacteria. For example, *Escherichia coli* growing on the conventional yeast extract-tryptone agar produces large amounts of the volatile indole. Actinomycete bacteria in the genus *Streptomyces* are abundant in soil, and produce geosmin and related volatiles. Cultures of *Pseudomonas aeruginosa*, discussed below, often have a distinctive “grape-like” odor due to the production of 2-amino-acetophenone (Scott-Thomas et al., 2010), which has demonstrated good potential as a volatile breath marker of respiratory infection.

Identification of bacterial volatiles typically involves growth in laboratory cultures where production of different volatiles is dependent on type of media, time of incubation, amount of aeration, and other environmental variables (Carey et al., 2011; Chippendale, Spanel, & Smith, 2011; Nawrath, Mgode, Weetjens, Kaufmann, & Schulz, 2012; Preti et al., 2009). These parameters can also influence our ability to detect different volatile chemicals produced by the bacteria, which although largely stable can be reactive (e.g. aldehydes and ketones) and of different solubilities and likelihood of being adsorbed onto surfaces. For the most reproducible mid-logarithmic phase laboratory broth cultures, only volatiles capable of escaping broth culture media with reasonable Henry partition constants ($K_{\text{air/water}}$) can reach detectable headspace levels (in the nanomolar or 0.1 ppm range). The different media typically used in the culture of bacteria are most often derived from biological sources, and produce their own characteristic volatiles, which may be increased or altered by autoclaving, or made more volatile by bacterial metabolic activity or physical alteration of agar surfaces (Schulz & Dickschat, 2007). Experiments designed to identify bacterial volatiles then must identify those released from media and subtract levels for what may appear to (and may) be identical volatiles by available analytical methods. Headspace air above the cultures can be collected by a variety of means, and for laboratory cultures a custom “closed loop stripping apparatus” is often constructed to allow volumetric determination from a hermetically closed culture system. Alternately, headspace air may be sampled directly by MS approaches that

do not rely on prior separation of compounds by chromatography (Boots et al., 2012). Bacterial culture volatiles may also be adsorbed onto different surfaces or “trapped” in inherently less quantitative but still highly useful approaches, as described in the numerous primary studies cited below.

It is unlikely that breath volatile makers originating from bacteria important in respiratory and other infections will become mainstream and reliable indicators of disease until their metabolic origins and chemical structures are fully defined using isotopes and laboratory cultures. We also may not find those of actual utility unless we also look simultaneously at microbes growing in their normal ecological niches. For the bacteria of high relevance to respiratory infection, these are of course the host cells and tissues in which they normally reside.

4. VOLATILES FROM MAJOR BACTERIAL RESPIRATORY PATHOGENS

Bacteria cause roughly a third to a half of adult community-acquired respiratory infections (Fine et al., 1996; Johansson, Kalin, Tiveljung-Lindell, Giske, & Hedlund, 2009; Lim et al., 2001). These are often secondary to viral infections, warranting consideration of the use of antibiotics in only about half of cases. Current Medicaid guidelines in the USA call for administration of antibiotics within 6 h for community-acquired pneumonias (File, Solomkin, & Cosgrove, 2011). A few types of bacteria are of very high specific interest with regard to the common adult community-acquired lower respiratory infections. These include *Streptococcus pneumoniae* and *Haemophilus influenzae*, both of which are often present in the upper respiratory tract of healthy individuals. *Moraxella* (formerly *Branhamella*) *catarrhalis* is only normally prevalent in children, frequently causing ear infections, but is also being increasingly recognized as an as a contributor in adult pneumonia (Ramirez & Anzueto, 2011). *Mycobacterium tuberculosis* is estimated to currently inhabit about a third of the world's population, most often resulting in a life long asymptomatic infection. This becomes of enormous importance for respiratory disease diagnosis when these bacteria instead grow to high levels in the extracellular spaces or cavities in the lung that are created by a delayed-type hypersensitivity (DTH) immune response. While not commonly seen in community-acquired infections, *P. aeruginosa* and *Burkholderia cepacia* are of high interest in terms of colonization of the lung in cystic fibrosis, the most common genetically inherited disease in most countries. For all of these pathogens, there is at least one report of volatile compounds produced during growth in different environments, and Table 2.1 summarizes these data. *M. tuberculosis* and *P. aeruginosa* have been more extensively characterized, and breath testing has already been used in individual trials to identify patients infected by these microbes and is discussed below.

S. pneumoniae is by far the most prevalent and important cause of significant lower respiratory tract infections among otherwise healthy individuals. A recent meta-analysis of patient data indicates that it is most likely that a recent unusual drop in the reported frequency as the causative agent of community-acquired pneumonia (CAP) is due to a dramatic failure in continuing to identify specific bacterial or other agents causing respiratory disease (Bartlett, 2011). While there is a great deal of information on volatile components produced by related Streptococci used in food and beverage fermentation, very

few studies have attempted to characterize those produced by the pneumococcus. It appears none have yet analyzed the potential contributions of this diverse microbe (Hiller et al., 2007) to human breath.

Growth of these bacteria in conventional clinical roller bottle media was shown to produce different levels of acetaldehyde, acetone, and ethanol in culture headspace (Allardyce, Langford, Hill, & Murdoch, 2006), but no unique compounds were identified. In contrast, Ishimaru et al. (Ishimaru, Yamada, Nakagawa, & Sugano, 2008) identified numerous analytes including many potentially unique to cultured *S. pneumoniae* by the soft ionization MS approach APCI-MS (atmospheric pressure chemical ionization). Their sensitive and high resolution approach did not allow identification of the corresponding volatiles. In contrast, a recent carefully calibrated approach to sampling volatiles produced by *S. pneumoniae* growing on sheep's blood agar using multicapillary column mobility spectrometry (MCC-IMS) found only one unidentified *S. pneumoniae* volatile compound, but it was also detected above plates growing 10 of other potential bacterial respiratory pathogens (Junger et al., 2012). A recent important study of volatiles produced by *S. pneumoniae* and other upper respiratory pathogens frequently cultured from infected nasal mucus by Preti et al., (2009) showed that *S. pneumoniae* volatiles produced in culture and in vivo included both common bacterial volatiles and unique compounds. In vitro, *S. pneumoniae* produced benzaldehyde, benzylalcohol, 2-phenylethyl alcohol, acetic acid, and methyl mercaptan, all of which were produced by *H. influenzae* and *M. catarrhalis* with the exception of methyl mercaptan, which has been identified in other studies as produced by *P. aeruginosa* (Table 2.1). *H. influenzae* and *M. catarrhalis* each produced a subset of volatiles produced by *S. pneumoniae* on this agar, and no distinct volatiles for these isolates were detected by the authors' approach. Difficulty in distinguishing volatiles produced by *S. pneumoniae* and *P. aeruginosa* grown on plates and resuspended in buffer was previously noted using a polymer composite sensor array (or electronic nose) that was able to distinguish among other major bacteria in lower respiratory infection (*Staphylococcus aureus*, *S. pneumoniae*, *H. influenzae*, and *P. aeruginosa*), and these pathogens and control samples lacking bacteria (Lai, Deffenderfer, Hanson, Phillips, & Thaler, 2002). Taken together, these few studies suggest that a breath test for known microbial volatiles intended to determine "bacteria positive or negative" is more likely than one able to be able to distinguish among the three most prevalent "typical" bacterial lower respiratory pathogens. Identifying these three will more likely require considering more than a single or small group of defined microbial marker volatiles, as is currently being done with the pattern recognition and sensor arrays, and statistical analysis of data on many analytes from mass spectra (Fend et al., 2006; Phillips et al., 2012; Robroeks et al., 2010; Thorn, Reynolds, & Greenman, 2010).

A great deal of effort to provide a description of the limited number of known bacterial metabolic pathways that generate volatile compounds has been put forth by Schulz and Dickschat (2007). Those described are now included in an MS database known as Superscent (Dunkel et al., 2009). A recent review by Thorn and Greenman (2012) gives an excellent broad overview of the central pathways leading to major classes of known bacterial volatiles currently of interest in developing respiratory infection diagnostics. It will only be through understanding exactly what respiratory bacterial diagnostic marker volatiles are, and

how and when they are generated during infection, that we will be able to use them as targets to develop highly reliable breath tests for the most common bacterial respiratory infections.

5. DEVELOPMENT OF BREATH TESTING

In contrast, volatiles found in human breath are rather well characterized. Hippocrates is said to have used breath odor as a means of diagnosing patient disease (Geist, 1957). The possibility of obtaining and analyzing human exhaled air for its volatile chemical components by GC was first demonstrated 50 years ago by Linus Pauling, who trapped breath in 5 ft-long coiled stainless steel tubes or “traps” (Pauling, Robinson, Teranishi, & Cary, 1971; Teranishi, Mon, Robinson, Cary, & Pauling, 1972). These were cooled to freeze and remove excess water and all “condensate” (see below) prior to analysis. A heat gun was then used to immediately “flash” air in the metal tube into a GC column for analysis by an ion flame detector. The initial experiments showed that normal breath gases and volatiles produced about 250 analytes. We now know these are primarily from nitrogen, oxygen, carbon dioxide, water vapor, and much lower amounts of VOCs including acetone, isoprene, ethane, and pentane (Kwak & Preti, 2011; Miekisch, Schubert, & Noeldge-Schomburg, 2004; Whittle, Fakhrazadeh, Eades, & Preti, 2007). Even abundant VOCs are present at only parts per billion (ppb, approximately nMol) levels, and others likely of utility in diagnosis at parts per trillion (ppt, approximately pMol) concentrations. Detection at such low levels has been achieved by different MS approaches, where sensitivity (Kwak & Preti, 2011) and the use of widely different and quickly evolving analytic methods (Thorn & Greenman, 2012) have resulted in very little overlap in reports of potentially useful breath test biomarkers by different research groups. As demonstrated by Boshier, Marczin, Hanna (2010) with quantitative selective ion flow tube or SIFT-MS directly on exhaled breath, relative levels of normal breath VOCs can be reliably assessed in individual laboratories. Reasonably sensitive diagnosis of *P. aeruginosa* (Savelev et al., 2011; Scott-Thomas, Pearson, & Chambers, 2011; Scott-Thomas et al., 2010), *M. tuberculosis* (Kolk et al., 2012; Phillips et al., 2012, 2010, 2007; Syhre, Manning, Phuanukoonnon, Harino, & Chambers, 2009), and *Aspergillus fumigatus* (Chambers, Syhre, Murdoch, McCartin, & Epton, 2009) lung infections by the patient breath testing has also recently been demonstrated, as described below.

A second major challenge that has slowed the development of diagnostic breath testing is that most human breath VOCs and many proposed disease biomarkers are present in the environment. Breath is composed of a mixture of endogenously produced and environmental compounds. Early studies by Michael Phillips, a leader in this area, proposed the calculation of an “alveolar gradient” (Phillips, Greenberg, & Awad, 1994), or identification and quantification of local ambient air volatile levels to “subtract” from levels found in the exhaled breath. While clearly a required consideration, others have since pointed out (Bajtarevic et al., 2009; Kwak Preti, 2011) that inspired volatile compounds can be concentrated in the body, and therefore higher levels in the breath relative to the environment do not necessarily indicate an endogenous origin of an identical compound. With volatiles of potential diagnostic interest present in both the environment and produced by healthy patients, breath volatile quantification becomes one of paramount importance.

Unfortunately, this is not a strength of the most widely used sampling and analysis methods. Nonetheless, numerous studies are now available that clearly establish that individual specific volatiles or combinations of detected MS analytes in breath analyses can be used with good accuracy to identify diseases, including diabetes, asthma, breast and lung cancer, cystic fibrosis, COPD, and liver disease (reviewed by (Buszewski et al., 2007; Whittle et al., 2007)). Standardizing approaches across different laboratories (as described below) and then bringing breath testing to respiratory infection patients for point of care testing will likely require further reducing the cost of tests by a second level of development (Smith, 2011). There is considerable potential for contributions in the form of dedicated sensor technology (e.g. “electronic noses”) capable of monitoring individual previously identified or unknown volatiles in complex mixtures (Lai et al., 2002). Sensor approaches, like soft ionization analyses with SIFT-MS and proton transfer reaction spectrometry (PTR-MS), are also capable of defining temporal patterns of evolution of identified markers (Bunge et al., 2008; O’Hara & Mayhew, 2009) of diagnostic value directly in breath.

Defining changes in endogenous volatile compounds in breath that result from infection rather than microbial volatiles is also likely to contribute to our ability to diagnose bacterial respiratory infections by breath testing. Although endogenous markers are not ideal in terms of contributing specificity across patient populations, their inherently higher levels may make their use in breath testing necessary. Alterations of better characterized human volatile metabolites in the breath do occur with respiratory disease, including infections. These changes likely result from the different and specific types of inflammation and temporal recruitment of different populations of immune cells producing different factors and cell signaling molecules. All pathogens entering the alveoli first encounter resident alveolar macrophages capable of secretion of proinflammatory cytokines including interleukin (IL)-8, IL-1 β , and tumor necrosis factor (TNF)- α . Inflammation associated with pneumococcal infection, for example, is characterized by the central role of the innate immune response and influx of neutrophils (Delclaux & Azoulay, 2003). The potential for a characteristic pattern of breath volatiles to reflect this kind of immediate and innate inflammatory response has been demonstrated recently in a rare animal model of breath testing with Sprague–Dawley rats (Guaman et al., 2012). In contrast, *M. tuberculosis* is able to limit proinflammatory signaling when encountering alveolar macrophages not previously activated by other stimuli. If able to survive phagocytosis, local inflammation is then initially limited for a time until a DTH develops, characterized by infiltration of cells with central roles in acquired immunity (T lymphocytes, including regulator T-cells, and B-cells). This results in a very different granulomatous inflammation and remodeling of lung tissues and may eventually result in active disease and large scale tissue destruction (including cavity formation, with high levels of extracellular bacteria directly in the airway). Differences in metabolism among relevant cells and different populations of cells are then expected to lead to changes in endogenous volatile production in the breath (Mashir et al., 2011), as now well described for other noninfectious diseases (Whittle et al., 2007).

The fact that lung tissue inflammation is characteristic of tuberculosis is reflected in the evolution of *M. tuberculosis* to use signals encountered in environments rich in reactive oxygen and nitrogen to regulate expression of factors important for adaptation (Trivedi, Singh, Bhat, Gupta, & Kumar, 2012). Volatile carbonyl compounds and saturated

hydrocarbons that are abundant in breath (e.g. acetone, ethane, and pentane) are thought to arise from membrane lipid and fatty acid peroxidation (Miekisch et al., 2004). Interestingly, this resembles the release of green leaf volatiles from mechanically injured plants (Baldwin, 2010). An important early study by Phillips (Phillips et al., 2007) showed a previously established index of normal breath alkanes thought to be generated by lipid peroxidation did not increase in *M. tuberculosis* culture positive respiratory patients compared to patients hospitalized with other respiratory diseases. A group of 12 other breath markers were significantly associated with culture positive status, and potentially reflect differences in lung inflammation characteristic of infection by *M. tuberculosis*, in addition to a smaller number of volatiles that may have originated directly from the bacteria. Robroeks et al. (2010) also used a combination of 14 volatile markers likely to have included those originating from the patient's response to infection to achieve 100% correct classification of 23 *P. aeruginosa* culture positive among 48 pediatric CF respiratory patients.

6. DIAGNOSIS OF RESPIRATORY INFECTION BY ANALYSIS OF VOLATILES

Breath testing is probably the least developed of any recognized area of medical diagnostic testing. It is also perhaps both the least invasive and most readily accepted by the patient. In terms of identifying bacterial respiratory diseases, breath testing is clearly an area of extremely high potential. The only frequently used breath test for a microbial infection involves *H. pylori*, which specifically colonizes the gastric mucosa rather than the respiratory tract. This application is also not entirely noninvasive, as detection of *H. pylori* urease activity, a metabolic capability shared by many microbes (Jassal et al., 2010) but absent from human hosts, currently involves administration of radiolabeled urea and detection of labeled carbon dioxide. *H. pylori* colonization is also reported to elevate levels of both breath carbon dioxide (Pathak, Bhasin, & Khanduja, 2004) and nitrate and cyanide (Lechner et al., 2005). In contrast to most areas of the life science research, the use of animal models of infection (Guaman et al., 2012; Purkhart et al., 2011) has made only very limited contributions to progress in this area, likely because of the key role of specific environment in microbial volatile production previously described. Successful experimental human breath testing for *P. aeruginosa* and *M. tuberculosis* and the fungal respiratory pathogen *Aspergillus* has recently been demonstrated. *A. fumigatus* breath testing has been reviewed recently by Chambers and is not summarized here (Chambers et al., 2010).

M. tuberculosis and *P. aeruginosa* are the two lower respiratory pathogens that now appear to be the most amenable to detection by breath testing. Interestingly, they are both known for their metabolic capacities and flexibility, having large (~4,000 open reading frame or ORF) genomes and very large (6,000 ORF) genomes, respectively. While *Pseudomonas* normally lives in a variety of environmental reservoirs and is an opportunistic pathogen, *M. tuberculosis* normally inhabits only human cells and tissues. A large genome for an obligate intracellular pathogen provides the reserve capacity needed to live in a variety of different human cells and tissues at different stages of pulmonary infection (Bishai, 1998). Neither of these bacteria is ever considered part of normal flora colonization or "microbiota" of healthy

individuals. This increases our ability to detect their presence from the volatiles they produce and evoke from the host during respiratory infection.

Infection by *P. aeruginosa* is only infrequently associated with CAP (Fine et al., 1996) but is instead one of the most important causes of hospital acquired lower respiratory infection. Children with cystic fibrosis and patients with immunocompromise or chronic lung conditions including chronic obstructive pulmonary disease or COPD (Fujitani, Sun, Yu, & Weingarten, 2011) are most frequently infected. *P. aeruginosa* infection rates are much higher in the hospital setting where they impact patients with chronic lung disease and HIV. In the U.S. hospital intensive care units, *P. aeruginosa* was the most frequently (31.6%) isolated bacterium from respiratory culture (Neuhauser et al., 2003). These infections are often associated with use of ventilators and bronchoscopes.

Enderby, Smith, Carroll, and Lenney (2009) reported an early effort to use SIFT-MS detection of previously reported *P. aeruginosa* volatile hydrogen cyanide to identify infected CF patients. HCN levels were found to be significantly elevated in 16 culture positive patients among 37 pediatric respiratory patients, but the authors found results were not consistent over 6 months in retesting. Hydrogen cyanide and methyl thiocyanate were found to be reliably produced by strains of *P. aeruginosa* in culture but did not prove useful as breath markers as more recently reported by Shestivska et al. (2011).

Robroeks et al. (2010) were the first to describe a comprehensive evaluation of potential breath marker volatiles and their successful use in diagnosis *P. aeruginosa* infections among CF patients. They used what is now a classic approach of collecting breath into 5 L polycarbonate (e.g. Teldar) bags, followed by drawing the contents through carbon adsorbant containing tube. GC Time of flight (TOF) MS was then used to identify ions and a group of 14 marker analytes by statistical discriminant analyses. This combination of 14 markers was able to discriminate 100% of the 23 *P. aeruginosa* culture positive patients among 39 CF patients.

The aromatic ketone 2-acetoaminophenone, which imparts a grape-like odor to *P. aeruginosa* cultures, has been described (Scott-Thomas et al., 2010) as a sensitive and specific (93.8% and 69.2%, respectively) indicator of *P. aeruginosa* culture positive status. Although also previously identified in headspace analyses of *S. pneumonia* growing in BacTec roller bottles (Allardyce et al., 2006; Scotter, Allardyce, Langford, Hill, & Murdoch, 2006), the authors did not identify the volatile as produced by cultures of this or other bacteria (several strains of *H. influenzae*, *Legionella pneumophila*, *M. catarrhalis*, *Pseudomonas fluorescens*, and *Burkholderia multivorans*) in their assay. 2-acetoaminophenone demonstrated high utility as a biomarker in identifying 15 of 16 *P. aeruginosa* infected CF patients among 46 total participants, including other CF patients, many with *S. aureus* infections. The authors used breath collection into 1 L glass bulbs (after finding 2-acetaminophene unstable in Teldar polycarbonate bags), into which were inserted adsorbant SPME needles, and the compound was detected by targeted CG-MS-MS.

The largest studies reporting successful patient breath testing for bacterial respiratory infections to date come from efforts to diagnosis tuberculosis. Diagnosis of *M. tuberculosis*

by breath testing is of great specific interest as it is only in active disease when bacteria grow in extracellular spaces within the lung (Grosset, 2003) that tuberculosis patients are infectious. If these cases can be efficiently recognized in this way, new cases could be dramatically reduced in the remaining high prevalence regions. That *M. tuberculosis* produces recognizable volatiles when growing in the lung is clearly demonstrated by the ability of both honeybees (Suckling & Sagar, 2011), and trained African giant pouch rats (Mgode, Weetjens, Cox, et al., 2011; Weetjens et al., 2009) to identify positive sputum specimens. Recently specific volatiles including three diterpenes have been described (Prach, Kirby, Keasling, & Alber, 2010). Iso-tuberculosinol (originally called Edaxadiene) has been shown to block phagosome maturation in infected macrophages and is only produced in standard media when magnesium levels are reduced (Mann et al., 2009). *M. tuberculosis* also has an extraordinary metabolic capability to synthesize and degrade lipids, and constituent fatty acids are precursors to many different known volatiles including alkanes and carbonyls. *M. tuberculosis* volatile metabolite production changes with culture on different media, and has now been characterized by Nawrath et al. (2012).

In the first reported effort at testing patient breath to diagnose tuberculosis (Phillips et al., 2007), researchers obtained filtered patient-breath specimens from 42 hospitalized potential tuberculosis patients in New York with a portable multistage breath collection device capable of sampling a subset of expired air (Phillips, 1997; Phillips & Greenberg, 1992). The device continuously collects breath samples from which volatiles are adsorbed onto a conventional carbon matrix. Volatiles adsorbed from 1 L of breath (2 min) were thermally desorbed by heating in a stream of helium and the volume of collected gas then reduced by two sequential cryofocusing traps, prior to analysis of the entire sample by conventional GCMS. Room air values were also obtained and subtracted for each spectra. Results from 59 local age matched healthy volunteers were compared to those from 23 culture positive tuberculosis patients. Statistical multivariate analyses were then used to identify a subset of 134 analyte ions identified for each sample. Principal component analysis indicated that a group of 10–12 identified analytes could be used as a test with a sensitivity of 82.6% for culture positive patients, and 100% specificity. (Current widely used sputum smear microscopy was estimated to detect only 63% of cases as of 2007) (WHO, 2010). Two different types of statistical analyses on these data indicated two different groups of putative breath marker volatiles, with both including multiple volatiles related to heptane and benzene (Phillips et al., 2007). Comparison of 1 ml of headspace from *M. tuberculosis* cultures grown in commercial VersaTREK bottles by the same breath collection and analysis approach indicated just 1-methyl-naphthalene and 1,4-dimethyl-cyclohexane as candidate bacterial derived volatiles that could be identified from both laboratory cultures and in culture positive patient breath.

A larger multisite (the U.S., U.K., and Philippines) application of the same approach by these investigators (Phillips et al., 2010) analyzed breath volatiles in 226 symptomatic high risk patients. Markers of good predictive value were identified including both proposed host oxidative stress products (alkanes and alkane derivatives) and the previously described volatile *M. tuberculosis* metabolites cyclohexane and 1,3,5-trimethyl-benzene. Researchers were able to identify active pulmonary tuberculosis with 84% sensitivity and 64.7% specificity when sputum culture, microscopy, and chest radiography were either all positive

or all negative, and 70% specificity when using positive sputum culture as the only criterion for infection.

A smaller pilot study by Syhre et al. (2009) also assayed 10 smear positive patient specimens and 10 healthy controls by collecting breath into 1 L silanized glass bulbs, which were sampled with adsorbant coated SPME needles. Analytes were thermally desorbed directly into a CGMS-MS and scanned specifically to identify methyl nicotinate, one of the four previously identified culture volatiles (methyl para-anisate, ortho-phenylanisole, methyl phenylacetate), now confirmed by Mgone et al. (2011). Detection of methyl nicotinate were significantly higher in subjects with tuberculosis, but still detectable in the breath of healthy controls.

A good example of how quickly this area is now moving is that during the preparation of this review, two new tuberculosis patient breath-testing studies have been reported. Kolk et al. (2012) studied patients in the endemic setting in South Africa, where 28% of culture proven TB patients had Ziehl-Neelsen (ZN) negative sputum smear. Breath was collected by normal breathing into Teldar bags, and analytes collected by drawing the contents through an adsorbant carbon trap. No environmental sampling was used to remove contributions from room air, and no specific breathing maneuvers were performed. An initial set of breath samples from 50 known sputum culture positive and 50 culture negative patients was analyzed using GC TOF-MS. Using six putative volatiles (dodecane, 3-Heptafluorobutyropentadecane, 5-hexenoic acid, 2-ethyl-1-hexanol, tetradecanoic acid, octanal) and one unknown analyte identified as markers, testing on an independent group of 21 TB and 50 non TB patients from the same area gave a sensitivity of 62%, and a specificity of 84%.

Phillips et al. (2012) have refined their approach to include a portable gas chromatograph coupled with surface acoustic wave (SAW) detector, achieving a major goal in allowing the point care testing. Their previously described (above) approach was used to collect breath from 279 patients in the U.K., Philippines, and India (130 active pulmonary TB, 121 controls), and the results collected and analyzed remotely by internet connection. The authors used an important standardization by modified Kovat's Indices to reduce differences at different sites of deployment. Analytes corresponding to the previously described and novel volatiles camphene, camphene, L-beta-pinene, 1,3,5-trimethyl-benzene, 1-methyl naphthalene, tridecane, 2-butyl 1-octanol, 4-methyl dodecane, and 2,2,4,6,6-pentamethyl-heptane were combined into 4 co-resolving regions in their assay system and scored, indicating 71.2% sensitivity and 72% specificity in identifying active tuberculosis cases among the same specimens used to identify markers.

7. STANDARDIZATION

After an initial delay of perhaps 35 years since the original description of breath testing for volatiles, it now seems clear that we can identify at least *M. tuberculosis* and *P. aeruginosa* lower respiratory infections by patient breath testing. More common bacterial agents of community-acquired adult pneumonia are proving more difficult to diagnose, likely because they are both part of and perhaps more similar to other bacteria of the normal upper and

lower respiratory tracts. Our knowledge of the production of volatiles by these bacteria in the laboratory is also more limited. What caused this delay, and what did we learn from it?

As previously described, breath testing for volatiles is not the same as “breath condensate” analysis (Montuschi, 2007). Breath condensates are far more complex human biological specimens that contain an even broader range of compounds, including nonvolatile leukotrienes, peptides, and cytokines. Condensate are also considerably easier to obtain specimens (Hunt, 2011), and so a greater number of different specific approach to collection, storage, and concentration (Rosias, Robroeks, Hendriks, Dompeling, Jobsis, 2004) have added variability to the already diverse application of very different MS techniques. As with the analysis of breath volatile compounds as described in this review, accurate quantification of changes in endogenously produced components is a central aspect. It is more difficult with condensates because they include aerosolized nonvolatiles escaping from the airway epithelial surface, and breath is saturated with water vapor (Effros, 2010).

The same problems of quantification and question of origins are present in breath testing for volatiles. Within a particular patient breath specimen, no volatile compound has been identified that can be used as an internal standard to evaluate levels of other compounds. Standards can be added exogenously to the collected air or adsorbed/desorbed sample, but then the amount and origin of breath represented by the specimen is not standardized. Variability in collection is expected, particularly as there are typically respiratory issues among those needing diagnosis. Many studies have attempted to address the issue of variability in collection of breath. Issues in standardizing breath sampling that occur before analytes are collected are the same for analyses of breath volatiles and condensates, and have been thoroughly described previously in the context of obtaining breath condensates (Horvath et al., 2005; Rosias, 2012). It does appear that many differences in the manner and environment in which breath is collected have the potential to impact the quantification of a subset of volatiles, and those will perhaps not be useful as diagnostic markers. However, there are many potential markers and methods to increase reliability in obtaining samples. These include avoiding a 10–20% dilution from the initially evacuated upper respiratory air in each breath by “end tidal” sampling, which can potentially improve sampling reliability (Thekedar, Oeh, Szymczak, Hoeschen, & Paretzke, 2011). A laboratory technique of pre-breathing pure bottled air (Basanta et al., 2012) can also reduce quantification issues in some settings. There is also the possibility of using only multiple ratios of detected analytes within each specimen as a potentially recognizable matrix of values for statistical consideration.

In terms of identifying the origins of genuine volatiles in the breath, this is a key question in that only by determining the metabolic sources of volatile markers, microbial or host, are we likely to move the field forward and develop superior and less invasive diagnostic tests. As described above, many low molecular weight volatiles are fairly ubiquitous from biological sources, as are all of those so far identified derived from the host whose levels may change with different immune responses. Microbial volatiles present in the body may be metabolically altered by the host and not recognizable in the breath. In the lung, diffusion of volatiles from the blood to the alveolar air across the alveolar capillary membrane also depends on other volatile chemical properties, including polarity and solubility in fat, and will not be the same as those detected in headspace of blood and urine specimens (King et

al., 2010, 2011). This can perhaps only be addressed with cell culture and animal models using labeled isotopes— work now being carried out in the author's laboratory.

8. THE FUTURE OF DIAGNOSIS BY BREATH TESTING

It now seems likely that we will see clinical diagnostic breath tests first for tuberculosis, and then perhaps nosocomial and community-acquired infections by *P. aeruginosa*. A breath test able to detect any bacterial agent relative to viral etiology appears somewhat further off, but would likely see even wider application. Future development of simplified sensor tests will likely benefit next from the experimental use of a combination of comparative direct analyses of just easily obtained “mixed expiratory” breath, for example, identification of bagged breath volatiles by SPME and GCMS (qualitative), and confirmation and quantification directly in breath by SIFT-MS (quantitative). Once biomarkers have been identified and confirmed, targeted and simplified MS (Phillips et al., 2012) or customized sensors arrays can be designed for the point care testing. These are most likely to be used in combination with other diagnostic tests, to ‘triage’, or rule in or out specific infections. It is likely that the ability to obtain and use of temporal patterns (Bunge et al., 2008; Carey et al., 2011) perhaps in combination with an administered microbial substrate (Jassal et al., 2010), as done with diagnosis of gastrointestinal disorders (Saad Chey, 2007), will help to alter otherwise similar volatiles originating from normal colonizers and those responsible for the bacterial respiratory disease.

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Figure 2.1.

Chemically reactive breath sampling plate used to characterize and monitor carbonyls from patient breath (Fu, Li, Biswas, Nantz, & Higashi, 2011; Li et al., 2011). (For color version of this figure, the reader is referred to the online version of this book.)

Table 2.1

Volatile organic compounds produced by microorganisms in standard culture media

Respiratory bacteria	Identified volatiles	Studies
<i>Streptococcus pneumoniae</i>	Ethanol, benzylalcohol, 2-phenylethanol, aminoacetophenone acetaldehyde, benzaldehyde, acetic acid, indole, methyl mercaptan, dimethyl sulfide, trimethylamine, hexanal,	<i>a, e</i>
<i>Haemophilus influenzae</i>	Benzaldehyde, benzylalcohol acetic acid, indole	<i>a</i>
<i>Moraxellacatarrhalis</i>	Benzaldehyde, benzylalcohol, and 2-phenylethanol	<i>a</i>
<i>Pseudomonas aeruginosa</i>	Ethanol, 1-butanol, 1-pentanol, isopentanol, 2-amino-acetophenone, 2-butanone, 2-nonanone, 3-undecanone, isoprene, 1-undecene, hydrogen sulfide, methyl mercaptan, dimethyldisulfide, dimethyltrisulfide, dimethylpyrazine hydrogen cyanide, methyl thiocyanate, formaldehyde, ammonia, trimethylamine	<i>a, b, c, d, e, i</i>
<i>Mycobacterium tuberculosis</i>	1-hexanol, 2-phenylethanol, 2-hydroxy 3-pentanone, g-methyl butyrolactone, methyl nicotinate *, methyl para-anisate *, ortho-phenylanisole *, methyl phenylacetate *, camphor + also those in refs Phillips et al. (2010), Phillips et al. (2007), Nawrath et al. (2012), Kolk et al. (2012)	<i>g, h</i>
<i>Burkholderia cepacia</i>	Ethanol, 1-pentanol, 2-aminoacetophenone, ethylbutanoate, hydrogen sulfide, methyl mercaptan, isoprene, acetoin, ethyl acetate, acetic acid, butanoic acid, phenyl acetic acid, pyrrol, formaldehyde, ammonia, trimethylamine	<i>f</i>

Volatiles detected above the background levels from the media by various approaches in different laboratories.

* These four potentially *M. tuberculosis*-unique aromatic ring volatiles were original identified by Syhre & Chambers, (2008), and recently independently confirmed by Mgode, Weetjens, Cox, et al. (2011).

^a (Preti et al., 2009)

^b (Labows, McGinley, Webster, & Leyden, 1980)

^c (Zechman, Aldinger, & Labows, 1986)

^d (Carroll et al., 2005)

^e (Allardyce et al., 2006)

^f (Thorn et al., 2010)

^g (Syhre & Chambers, 2008)

^h (Mgode, Weetjens, Nawrath, et al., 2011)

ⁱ (Shestivska et al., 2011).