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REVIEW

New Breath Diagnostics for Fungal Disease

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Abstract

Purpose of Review Diagnosis of fungal disease etiology is often difficult, compounded by inaccurate or delayed diagnostic methods. Breath-based biomarkers are being investigated as a novel target for clinical diagnostics. This review aims to summarize recent advancements, identify gaps, and discuss future research directions for breath-based fungal diagnostics.

Recent Findings Studies conducted in vitro, in animal models, and in human breath show fungi produce a large and diverse volatile metabolome. Recent studies on *Aspergillus*, *Candida*, *Rhizopus*, *Coccidioides*, *Trichoderma*, *Fusarium*, and *Alternaria* demonstrate the feasibility of identifying infectious etiology using fungal volatile profiles. However, the majority of data on fungal volatiles come from in vitro analyses, which have limited translatability to in vivo infections; thus, future studies should focus on in vivo volatile profiles to develop breath tests for diagnosing infections and monitoring antifungal therapy.

Summary This review describes recent studies that examine volatile organic compounds (VOCs) as biomarkers to detect and differentiate pathogenic fungi, highlighting the feasibility of breath-based diagnostics for fungal disease.

Keywords Volatile organic compounds · Biomarkers · Fungal disease · Breath · Diagnostics · Mycoses

Introduction

Background on Fungal Disease

Fungal infections are a serious public health problem, resulting in over 1.5 million global deaths per year [1]. Most fungal infections occur in immunosuppressed individuals, caused by opportunistic fungi such as *Aspergillus*, *Candida*, or *Cryptococcus* [2]. Those who are at greatest risk are persons infected with human immunodeficiency virus (HIV); persons receiving immunosuppressive therapy to prevent organ transplant rejection, biological immunomodulatory agents to treat autoimmune diseases, or bone marrow suppression cancer chemotherapies; premature neonates; and the elderly [3]. Though endemic dimorphic fungi including *Blastomyces* spp., *Coccidioides* spp., *Histoplasma* spp., *Paracoccidioides* spp., *Sporothrix* spp., and *Talaromyces marneffei* are capable of infecting immunocompetent individuals, *Coccidioides* spp. is the only dimorphic fungus that routinely causes systemic disease in healthy hosts [2, 4].

In response to the rising threat of fungal infections, the World Health Organization (WHO) developed its first fungal priority pathogen list (WHO FPPL) in 2022 [5]. The list has been broken into three priority groups (Critical, High, and Medium) and includes 19 fungal pathogens associated with serious risk for mortality and/or morbidity and is mainly focused on systemic invasive infections (Table 1). It is important to note that some pathogens are confined to certain geographical areas and therefore not considered a priority on a global scale; however, in areas of endemicity, they are associated with significant disease burden.

There is no current estimate of the overall global economic burden associated with fungal disease; however, in the USA, the economic burden due to fungal disease is conservatively estimated at $11.5 billion annually, which includes direct medical costs ($7.2–$7.5 billion), productivity loss due to missed workdays ($870 million), and premature deaths ($3.2 billion) [7, 8]. This cost could be estimated as high as $48 billion when taking a value of statistical life approach [7, 8]. Still, these estimates do not include costs for excessive testing and inappropriate treatment prior to

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an accurate fungal diagnosis being established, nor do they include undiagnosed infections. Fungal diagnostics are often limited due to the growing diversity of pathogenic species, time-consuming diagnostic methods, a lack of sensitive and specific testing, and a decreasing number of clinical mycologists [9, 10]. In addition, the global public health surveillance for common fungal infections is poor [3].

Relatively little research funding has been applied to the study of fungal diseases or the development of better diagnostics despite the significant global economic burden, proposed geographic range expansion of endemic mycoses due to climate change, growing human migration on a global scale bringing naïve hosts into new areas, and increased use of immune suppressive medications [1, 11]. In 2018, the National Institute of Allergy and Infectious Diseases (NIAID) received $5.3 billion in appropriated funds; however, only $98,193 (less than 0.002%) was used for fungal diseases [12]. In 2022, while the funding portfolio has improved, reflecting the increased burden of mycoses in the USA and worldwide over the past 4 years, there are only 48 projects totaling $30 million (0.4% of the budget) focused on the development of diagnostics for fungal infections based on data from NIH RePORTER.

Breath-Based Diagnostics

Breath-based diagnostics, utilizing volatile organic compounds (VOCs), are a promising novel approach for diagnosing respiratory and other systemic infections in a non-invasive manner. An infection alters both the host and pathogen's metabolism, affecting the presence and/or quantity of VOCs in the breath, which can be leveraged as biomarkers to determine the identity of the infectious agent in a non-invasive and culture-independent manner [13]. Due to the chemical complexity of breath, various sampling, preconcentration, and analysis techniques have been investigated. For offline analyses of breath VOCs [14, 15], the most common approach is to use thermal desorption tubes (TDTs) to preconcentrate and trap the VOCs for storage and transport, followed by VOC analysis using various combinations of gas chromatography (GC) and mass spectrometry (MS) techniques (e.g., gas chromatography–time-of-flight mass spectrometry) [16]. Online analyses of breath VOCs are most commonly performed using direct-injection mass spectrometry techniques such as proton transfer mass spectrometry (PTR-MS), secondary electrospray ionization mass spectrometry (SESI-MS), and selected ion flow tube mass spectrometry (SIFT-MS); details of these methods are thoroughly described in the second edition of Breathborne Biomarkers and the Human Volatilome [17••]. The most common instrumentation for VOC biomarker discovery and breath analysis has also recently been reviewed [16, 18].

The majority of breath-based diagnostic research has been directed at respiratory diseases, including bacterial, fungal, and viral infections [19]. In vitro studies have demonstrated that microbial pathogens produce large and unique volatile metabolome (volatilome) profiles that can be used to differentiate and identify organisms to the genus, species, and strain level [13]. Animal model respiratory infections have shown that the combination of pathogen and host volatile metabolites has high diagnostic accuracy for detecting and identifying lung infection etiology [20–29]. Clinical studies of community-acquired pneumonia, ventilator-associated pneumonia, and chronic lung infections have shown that volatile biomarkers can sensitively detect and identify the etiologies through the analysis of respiratory samples, such as sputum, bronchoalveolar lavage fluid (BALF), and breath [18, 30–32]. To date, relatively few studies have specifically focused on identifying volatile biomarkers for fungal lung disease; the work published over the last decade (since 2013) is summarized in Table 2 and presented herein.

Recent Advances in Breath Biomarkers for Specific Fungal Diseases

Aspergillus

Aspergillosis encompasses a variety of infections caused by *Aspergillus*, a genus of opportunistic fungal pathogens

Table 1 2022 World Health Organization fungal priority pathogen groups [5]

Critical priority	High priority	Medium priority
Cryptococcus neoformans	*Candida glabrata* (*Nakaseomyces glabrata*[a])	*Scedosporium* spp.
Candida auris	*Histoplasma* spp.	*Lomentospora prolificans*
Aspergillus fumigatus	Eumycetoma causative agents	*Coccidioides* spp.
Candida albicans	Mucorales	*Candida krusei* (*Pichia kudriavzevii*[a])
	Fusarium spp.	*Cryptococcus gattii*
	Candida tropicalis	*Talaromyces marneffei*
	Candida parapsilosis	*Pneumocystis jirovecii*
		Paracoccidioides spp.

[a]Reclassified in 2023 [6]. The new classification is in parentheses

Table 2 Research investigating volatile compounds associated with human fungal disease

Genus	Species	Disease	Sample type			
			In vitro	Murine BALF	Murine breath	Human breath
Alternaria	*A. alternata*		[33]			
Aspergillus	*A. calidoustus*		[34]			
	A. flavus		[34]			
	A. fumigatus	Invasive aspergillosis	[34–41]		[42]	[34, 37, 43]
	A. niger	Invasive aspergillosis	[34, 37]			[34, 37]
	A. terreus		[34]			
		Chronic pulmonary aspergillosis				[44•]
		Pulmonary invasive aspergillosis				[45]
Candida	*C. albicans*	Oral candidiasis	[46–48, 49•, 50–53]			[54]
	C. glabrata (Nakaseomyces glabrata[a]*)*	Oral candidiasis	[46–48]			[54]
	C. krusei (Pichia kudriavzevii[a]*)*		[46, 50]			
	C. parapsilosis	Oral candidiasis	[48, 49•, 50, 52]			[54]
	C. tropicalis	Oral candidiasis	[46–48]			[54]
		Ventilation-associated pneumonia				[55]
Coccidioides	*C. immitis*	Coccidioidomycosis	[56•]	[57•]		
	C. posadasii	Coccidioidomycosis	[56•]	[57•]		
Fusarium	*F. oxysporum*		[33, 58]			
	F. proliferatum		[58]			
	F. solani		[58]			
	F. verticillioides		[58]			
Rhizopus	*R. arrhizus var. arrhizus*	Mucormycosis			[42]	
	R. arrhizus var. delemar	Mucormycosis			[42]	
	R. microsporus	Mucormycosis			[42]	[42]
Trichoderma	*T. asperellum*		[59]			
	T. afroharzianum		[59]			
	T. atroviride		[59]			
	T. longibrachiatum		[59]			

[a]Reclassified in 2023 [6]. The new classification is in parentheses

primarily infecting immunosuppressed hosts and individuals with underlying pulmonary disease. Infections include invasive forms of disease (i.e., invasive pulmonary aspergillosis, rhinosinusitis), chronic disease (i.e., aspergilloma, chronic pulmonary aspergillosis), asthma with fungal sensitization, and cutaneous aspergillosis, an infection of the skin [60, 61]. At least 24 species of *Aspergillus* are capable of causing disease in humans; however, *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus flavus*, and *Aspergillus niger* are the species primarily responsible for human infections [61]. Currently, histopathology and culture are the gold standards for diagnosing *Aspergillus* infection despite their low sensitivities; in patients with an active inflection, *Aspergillus* is grown from sputum and BALF in only 35% and 63% of samples, respectively [62]. Additionally, diagnosing *Aspergillus* is often complicated by naturally colonizing *Aspergillus* species and morphological similarities to other filamentous fungi [60, 62]. In the USA, the approximate economic burden of aspergillosis is estimated at $1.8 billion per year, including direct medical costs and productivity loss due to hospitalizations, outpatient visits, and premature deaths [8]. Global cases of invasive aspergillosis and chronic pulmonary aspergillosis are estimated at over 350,000 and 3 million per year, respectively [63].

Most studies into the in vitro volatilome of *Aspergillus* species have focused on *A. fumigatus*. The volatile profile of *A. fumigatus* has been analyzed with altered growth conditions [34, 35, 38], at different incubation times [36, 38, 39, 41], and in comparison to metabolic mutants [35, 39].

Heddergott et al. found the most consistent volatiles across growth conditions to be the monoterpenes α-pinene, camphene, and limonene and the sesquiterpenes α-bergamotene and β-trans-bergamotene, while 1-octen-3-ol, 3-octanone, and pyrazines were dependent on nutrient conditions and growth environment [35]. Oxygen concentration strongly influences the *A. fumigatus* volatilome [38]; Rees, et al. identified a panel of 19 volatiles capable of discriminating between four growth conditions (early hypoxia, late hypoxia, early normoxia, and late normoxia). The effect of growth phase on the volatilome of *A. fumigatus* has also been investigated, with several studies observing time-dependent changes in VOC abundances [36, 38, 41].

The volatilome of *A. fumigatus* changes in co-culture versus monoculture, whether in the presence of another pathogen or the host [34, 36, 41]. Chippendale et al. investigated the presence of species-specific biomarkers in co-cultures of *A. fumigatus* with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Streptococcus pneumoniae*, three common respiratory bacteria [41]. Hydrogen cyanide and methyl thiocyanate, biomarkers of *P. aeruginosa* [64, 65], were still detectable in co-culture with *A. fumigatus*; however, there was a lack of propanol in co-culture, which was detected in *A. fumigatus* monoculture. Organosulfur compounds associated with *A. fumigatus* were not compromised by the presence of *S. aureus* or *S. pneumoniae*, although characteristic *Staphylococcus* aldehydes were missing in co-culture. Gerritsen et al. compared the headspace volatilomes of three clinical *Aspergillus* isolates and the exhaled breath profiles of the same patients [37]. The authors noted differences between the volatile profiles of the strains when cultured in vitro; however, the in vitro volatile patterns were not replicated in vivo.

Investigations into pulmonary aspergillosis have demonstrated that developing breath biomarkers for fungal pneumonias are feasible. Koo et al. characterized the breath VOCs of 34 patients with invasive aspergillosis (IA) and 30 with other pneumonia [34]. They identified a unique secondary metabolite signature—α-trans-bergamotene, β-trans-bergamotene, a β-vatirenene–like sesquiterpene, and trans-geranylacetone—that discriminated IA from other pneumonia with a sensitivity of 94% and a specificity of 93%. A preliminary in vivo study by de Heer et al. analyzed the exhaled breath profiles of prolonged chemotherapy-induced neutropenia (PCIN) patients with pulmonary invasive aspergillosis using an electronic nose (eNose) [45]. Using discriminant analysis, the authors classified invasive aspergillosis cases and PCIN controls with a sensitivity of 100% and a specificity of 83.3%. A subsequent in vivo study by de Heer et al. examined the feasibility of eNose technology to identify *A. fumigatus* colonization in patients with cystic fibrosis (CF) [43]. Comparing *A. fumigatus* colonized CF ($n = 9$) and uninfected CF patients ($n = 18$), they were able to classify the colonized subjects with a sensitivity of 78% and a specificity of 94%. Li et al. conducted a clinical study using breath samples collected from patients with chronic pulmonary aspergillosis (CPA), non-fungal community-acquired pneumonia (CAP), and healthy individuals [44•]. The authors found a sensitivity of 95.8% and a specificity of 96.9% for differentiating the CPA group from the CAP group, with five potential biomarkers: phenol, neopentyl alcohol, toluene, limonene, and ethylbenzene. Thus, these findings support further investigation into a breath-based biomarker panel as a potential diagnostic for *Aspergillus* infection, supported by in vivo and in vitro studies. In vitro studies, such as those previously discussed, offer insight into the diverse composition of the *Aspergillus* volatilome in response to various environmental and metabolic changes.

Candida

Candida species are the primary fungal pathogen isolated from immunosuppressed individuals [66]. Candidiasis, an infection caused by *Candida* species, is responsible for 8–10% of nosocomial infections each year [67]. In recent years, the majority of infections have been attributed to five species: *Candida albicans*, *Candida glabrata*,[1] *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei*[2] [68]. However, the rising prevalence of multidrug-resistant species, including *Candida auris*, has increased hospital outbreaks, morbidity, and mortality [69]. In the USA, the direct medical costs and the related productivity loss due to invasive and non-invasive candidiasis are estimated at $4.3 billion annually [8]. Global cases of invasive candidiasis are estimated at between 934,000 and 2.3 million per year [70]. Recent diagnostic advances for invasive candidiasis include the development of the T2Candida Panel, a non-culture-based method that combines nucleic acid amplification and T2 magnetic resonance detection with an estimated 91% sensitivity and 94% specificity [71, 72]. While the T2Candida Panel is an improvement from the previous diagnostic standard of blood cultures (which are ~ 50% sensitive) [73], the test is currently limited to the identification of five major *Candida* species, with results grouped by suspected antifungal susceptibility and reported as *C. albicans/C. tropicalis*, *C. parapsilosis*, and *C. krusei*[3]/*C. glabrata*[4] [72].

Investigations into the in vitro *Candida* volatilome demonstrate that *Candida* species have unique VOC profiles that are altered by their growth environment [46–48, 49•, 51, 53]. Similar to the findings from other fungal and bacterial volatilome studies, the primary differences between species are not due to the production of unique VOCs by each

[1] Reclassified in 2023 to *Nakaseomyces glabrata* [6]
[2] Reclassified in 2023 to *Pichia kudriavzevii* [6]
[3] Reclassified in 2023 to *Pichia kudriavzevii* [6]
[4] Reclassified in 2023 to *Nakaseomyces glabrata* [6]

Candida spp., but rather differences in the abundances of common VOCs [46–48, 49•]. For example, while the majority (> 98%) of VOCs identified from in vitro cultures were shared among *C. albicans*, *C. glabrata*[5], and *C. tropicalis*, the patterns of VOC abundances differed, facilitating the hierarchical clustering of *Candida* species by their volatile profiles, independent of culture duration (12, 24, or 48 h) [47]. Comparisons of the *Candida* volatilomes between growth media have shown differences in the number of volatiles captured and the abundances of VOCs [48, 49•]. The volatilome composition is also highly dependent on growth phase [46, 47, 51, 53]. Distinct changes in the relative abundance of different chemical classes have been observed from the end of lag phase to stationary phase [47]. Similarly, the mode of growth—planktonic or biofilm—altered the volatilome, with noted volatile changes characteristic of biofilm maturation [49•].

A couple of recent studies have explored the volatile profile of *Candida* species in human breath [54, 55]. The feasibility of breath analysis was investigated for mechanically ventilated patients by collecting breath samples from 22 patients with ventilator-associated pneumonia [55]. *Candida* species were detected in five patients, three of which were co-infected with *S. aureus*. When Filipiak and colleagues examined the breath for the presence of 29 *Candida* VOCs that were previously detected in in vitro cultures [53], eight of the VOCs were detected in the breath of four out of five candidiasis cases. A preliminary breath study by Hertel et al. explored the volatile profiles of oral candidiasis patients versus healthy controls [54]. The authors did not detect the same signature *Candida* volatiles from their previous in vitro study [46] or significant differences between patients with confirmed candidiasis versus healthy individuals. However, significant differences in the abundance of nine VOCs—2-methyl-2-butanol, hexanal, longifolene, methyl acetate, 1-heptene, acetophenone, decane, 3-methyl-1-butanol, and chlorobenzene—were noted after antifungal therapy. These findings warrant future study into a breath test for candidiasis, potentially utilized to monitor recovering patients and/or susceptible patient groups. While in vitro data has demonstrated the potential to discriminate between *Candida* species based on VOC abundance, in vivo studies utilizing breath samples from cohorts reflective of various *Candida* species etiologies would be necessary to determine if this result can be replicated in vivo.

Rhizopus

Rhizopus is the primary genus responsible for mucormycosis, an opportunistic fungal infection often found in immunosuppressed individuals [74]. The current diagnostic standards for mucormycosis include histopathology, direct microscopy, and cell culture, which are often limiting due to a lack of specificity and an inability to differentiate between pathogens [75, 76]. The economic burden of mucormycosis-related hospitalizations in the USA from 2005 to 2014 was approximately $48 million per year [77]. Global estimates have found over 10,000 cases of mucormycosis per year [63]; however, the true prevalence is likely orders of magnitude higher, as mucormycosis is not a reportable disease in many countries [78]. As just one example, Prakash et al. estimate 900,000 cases in India per year, attributed to uncontrolled diabetes as a primary risk factor [78]. Additionally, mucormycosis has been reported as a secondary infection in current or recovered COVID-19 patients, contributing to a rise in global cases [79]. Diagnosis is often complicated by nonspecific symptoms resembling those of COVID-19 and/or other fungal infections; subsequently, COVID-19-associated mucormycosis has been linked to increased morbidity and mortality [79].

Koshy et al. examined the breath profiles of neutropenic mice infected with three different *Rhizopus* strains (*Rhizopus arrhizus* var. *arrhizus*, *Rhizopus arrhizus* var. *delemar*, and *Rhizopus microsporus*) commonly known to cause invasive human mucormycosis and the breath profiles of five human patients [42]. By analyzing the murine breath profiles obtained via thermal desorption gas chromatography tandem mass spectrometry (GC-MS/MS), unique profiles of sesquiterpene metabolites (*R. arrhizus var. arrhizus*: β-isocomene, epicubebol, and γ-patchoulene, *R. arrhizus var. delemar*: α-guaiene, alloaromadendrene, and *R. microsporus:* cedrene, selina-5,11-diene, 8,14-cedranoxide) were identified. The sesquiterpene profiles were able to differentiate between each *Rhizopus* species and the *Aspergillus fumigatus* control. In comparison to the five human patients diagnosed with *R. microsporus*, some similarities to the murine *R. microsporus* profile were noted; however, additional sesquiterpene metabolites were found in human breath. Additionally, in an individual with breath samples collected after antifungal treatment, there was a significant decrease in the abundance and an eventual disappearance of the sesquiterpene metabolite features. These results indicate the possibility of a human breath test capable of distinguishing invasive mucormycosis, while also highlighting the non-invasive nature and potential utility in clinical monitoring.

Coccidioides

Coccidioides immitis and *Coccidioides posadasii* are the two species responsible for coccidioidomycosis, or Valley fever, a fungal pneumonia endemic to the arid and semi-arid regions of North and South America. *Coccidioides* spp. are one of the few fungi that routinely infect immunocompetent hosts and it is estimated there are 350,000

[5] Reclassified in 2023 to *Nakaseomyces glabrata* [6]

new US cases each year, though underdiagnosis leads to 10- to 20-fold fewer confirmed cases [80]. Valley fever is estimated to cost the US $385 M annually [8], which is likely conservative; in Arizona and California, where over 95% of all reported US cases arise, the estimated economic burden of the disease is significantly higher than this national estimate [81, 82]. Coccidioidomycosis, like other fungal infections, is difficult to diagnose, leading to a median 23-day time-to-diagnosis from the time that a patient seeks healthcare [83]. The only truly definitive diagnosis for coccidioidomycosis requires a positive fungal culture or histopathology within lung tissue or bodily fluids [84]; however, obtaining a suitable sample is difficult and invasive. In practice, most patients are diagnosed through serological tests: enzyme immunoassay, immunodiffusion, and complement fixation; however, these tests have poor sensitivity, especially in the early stages of infection [85] and in persons who are immunosuppressed [86], and/or poor specificity that create delays in diagnosis [87].

Our group has been working toward identifying and validating volatile biomarkers of *Coccidioides* infections using untargeted volatile metabolomics analyses of in vitro cultures, murine model lung infections, and respiratory specimens from humans with Valley fever. In an analysis of in vitro volatilomes, we cultured six strains of each species, *C. immitis* and *C. posadasii*, under temperature and oxygen conditions to induce the two life forms: mycelia and spherules [56•]. We detected 353 *Coccidioides* VOCs from the headspace of the cultures and showed the *Coccidioides* volatilome is strongly dependent on life form, i.e., the saprobic mycelia vs. parasitic spherules, but independent of species. Next, we investigated the volatile biomarkers of Valley fever that arise from host-pathogen interactions using a murine lung infection model and identified a set of 36 VOCs significantly correlated to cytokine abundance, which cluster mice by disease severity [57•]. As observed with *Aspergillus* and *Candida*, we found very little overlap between the VOC profile in vitro and in the mouse model; only one compound, decanal, was identified in both studies. We are now analyzing the volatile metabolomes from respiratory specimens, including BALF and breath, to identify VOCs that discriminate Valley fever from other common causes of community-acquired pneumonia in endemic regions of the USA. Combined, these studies suggest that *Coccidioides* spp. and the host produce volatile metabolites that may yield biomarkers for a Valley fever breath test.

Environmental Fungi

Trichoderma species are opportunistic fungi found in a variety of environmental settings [88, 89]. While cases of human infection are rare, nine species are currently noted as opportunistic human pathogens, the most common being *Trichoderma longibrachiatum* [89, 90]. The only currently available diagnostic of a *Trichoderma* species infection requires the histopathological identification of hyaline septate hyphae, a feature morphologically similar to *Aspergillus* species, possibly resulting in greater misdiagnosis [89, 91]. An in vitro study by Hermosa et al. noted significant differences in the volatilomes of four *Trichoderma* species—*T. asperellum*, *T. atroviride*, *T. afroharzianum*, and *T. longibrachiatum*—when grown on potato dextrose agar, but only partial separation in the volatilomes when grown on soil [59]. Analysis of the eight different volatile profiles suggested the observed species separation was mostly due to differences in VOC abundance, which were drastically reduced in all species when grown in soil. The study emphasized the role that growth environment plays in the volatilome and noted it may be possible to differentiate between *Trichoderma* species in vitro using VOC abundance profiles.

The fungal genus *Fusarium* is found worldwide in soil, water, and plants [92]. Many species are common phytopathogens, but over 20 species have been identified as opportunistic human pathogens [93]. Diagnosis involves a combination of methods including culture, chest CT, 1,3-β-D-glucan test, PCR, and histopathology. Yu et al. reported on the in vitro volatilomes of four common pathogenic *Fusarium* species, identifying unique sesquiterpene profiles for each [58].

Alternaria species are saprobic fungi primarily associated with allergies and asthma [94]. However, *Alternaria* species can cause opportunistic human infections, including cutaneous and subcutaneous infections, oculomycosis, rhinosinusitis, and onychomycosis [94]. Current diagnostic methods include culture, histopathology, and direct microscopy [95]. Weikl et al. characterized the fungal volatilomes of *Alternaria alternata* and *Fusarium oxysporum* under various conditions [33]. The authors observed distinct volatilomes for both species, noting a high sesquiterpene emission from *A. alternata* in comparison to *F. oxysporum.*

Overall, these studies demonstrate the potential for the identification of species-specific fungal volatile signatures, which would be useful to differentiate infectious etiologies in rare cases of human infection.

Future Directions

Fungal pathogens are an increasing concern for public health due to numerous factors, including the growth of at-risk populations, expanding geographical ranges of endemic pathogens, and the rise of multidrug-resistant

species [5]. Current diagnostic measures often rely on obtaining a biospecimen (e.g., sputum, blood, and biopsy), which can be invasive and, at times, fail to obtain a viable biological sample. Alternatively, fungal infections are diagnosed via serology, but these tests lack sensitivity in immunosuppressed persons, who are most susceptible to opportunistic mycoses. Consequently, the non-invasive and culture-independent aspect of breath-based tests offer an advantage for clinical diagnostics. However, while the volatile profiles of a few fungal genera (*Aspergillus*, *Candida*, and *Coccidioides*) have been characterized in vivo, the majority of fungal volatilomes remain uncharacterized in human breath samples. It is imperative that breath-based diagnostic methods are also explored for all major worldwide endemic mycoses, e.g., blastomycosis (*Blastomyces*), histoplasmosis (*Histoplasma*), and paracoccidioidomycosis (*Paracoccidioides*), emerging mycoses, e.g., talaromycosis (*Talaromyces marneffei*), adiaspiromycosis (*Emmonsia*), and emergomycosis (*Emergomyces*), and opportunistic fungal pathogens, e.g., lomentosporosis (*Lomentospora prolificans*), scedosporiosis (*Scedosporium* species), and cryptococcosis (*Cryptococcus neoformans*).

As described herein, there is sufficient data showing the feasibility of breath-based diagnostics for respiratory fungal infections. To move beyond pilot studies, it is necessary to increase the size of human breath sample cohorts, ensure translatability through independent laboratory studies, and expand biomarker discovery research to include additional fungal genera. Currently, the lack of standardization for collection and analysis of breath samples is a major limitation for the development of breath-based biomarkers [96]. Many studies utilize different sampling, pre-concentration, and analysis methodologies, thus reducing the ability to compare results between independent investigations. Furthermore, the effect of the host's immune system, co-morbidities, and antifungal treatment on breath volatilome profiles has not been fully described. Since breath analysis offers real-time information about the physiological state of an organism, breath-based testing could assess patient response to medications, even early in the course of treatment. Prior studies by Hertel et al. and Koshy et al. demonstrated feasibility, both noting VOC differences after fungal treatment [42, 54]. Additionally, as many fungal species (*Aspergillus*, *Candida*, etc.) also act as commensal organisms, it will be essential to be able to differentiate between natural colonization and infection [97, 98]. Gao et al. demonstrated it is feasible for breath tests to non-invasively distinguish between colonization and infection for patients infected with the bacterium *Acinetobacter baumannii*, noting differences in the abundance of eight putative biomarkers [99]. However, advancing similar studies to fungal infections will require a larger proportion of the total infectious disease research funding portfolio to go toward mycoses in order to address the current research gap. Ideally, breath-based methods will become part of the diagnostic arsenal and in certain cases may act as a pre-screening tool. Utilizing current GC-MS instrumentation [100], offline breath-based testing can be easily implemented within clinical diagnostic laboratories, thus requiring minimal additional training or capital expenditures. The long-term goal of developing real-time clinical breath tests is feasible through the continued development of online breath analysis instruments, including direct-injection mass spectrometry and sensor-based VOC detection technologies.

While fungal infections are a significant public health burden for humans, they are also a major threat for non-human animal hosts [101, 102]. Analyses of infectious fungal disease trends revealed increases in fungal alerts and cases of animal fungal infections worldwide [103]. Of the major classes of infectious agents, fungi were noted as the greatest threat to animal hosts due to being responsible for the majority (~ 72%) of species extinction and regional extirpation events [103]. Due to their large impact on numerous animal species, increased monitoring of fungal infections is essential. In companion animals (dogs, cats, horses, etc.), fungal infections can range from a self-limiting respiratory tract infection to dissemination and death [104, 105]. Consequently, similar challenges to human medicine are encountered by veterinarians due the close resemblance of fungal infection symptoms to numerous other diseases and a lack of definitive diagnostic methods [106]. The feasibility of animal breath tests has been established [107–109], with noted advantages such as the ease of breath collection; however, further investigation is needed to establish the baseline volatilomes of healthy animals, identify fungal biomarkers within specific populations, and improve the accessibility and standardization of instrumentation and analysis.

Conclusions

Breath-based diagnostics are a rapidly expanding field that can be utilized to address the rising public health threat of fungal disease. Early detection of fungal infections is critical in reducing disease mortality. It is necessary to develop novel techniques for available, affordable, and accurate fungal diagnostics worldwide. Increased biomarker research in humans and animals, optimization of VOC collection and analysis techniques, and comprehensive clinical and veterinary studies are needed to develop breath-based methods to detect fungal disease.

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