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Brief Report

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Profiling of volatile organic compounds produced by clinical *Aspergillus* isolates using gas chromatography–mass spectrometry

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Abstract

Volatile organic compounds (VOCs) in exhaled breath may identify the presence of invasive pulmonary aspergillosis. We aimed to detect VOC profiles emitted by *in vitro* cultured, clinical *Aspergillus* isolates using gas chromatography–mass spectrometry (GC-MS). Three clinical *Aspergillus* isolates and a reference strain were cultured while conidiation was prevented. Headspace samples were analyzed using a standardized method. Breath samples of patients from which the cultures were obtained were checked for the presence of the VOCs found *in vitro*. Each *Aspergillus* isolate produced a distinct VOC profile. These profiles could not be confirmed in exhaled breath *in vivo*.

Key words: gas chromatography-mass spectrometry, *Aspergillus*, headspace analysis, exhaled breath analysis, invasive pulmonary aspergillosis.

Clinical Trial registry no: NCT02106117

Exhaled breath analysis has potential as a noninvasive diagnostic tool for detection of invasive pulmonary aspergillosis (IPA). Our group showed that patients with IPA and neutropenic controls could be discriminated using an electronic nose.[1] Analytical chemistry techniques, such as gas chromatography–mass spectrometry (GC-MS), are required to determine which volatile organic compounds (VOCs) drive this signal.[2]

Various studies described VOCs detected in headspaces of *Aspergillus* isolates *in vitro* and in breath from patients with IPA.[3–8] Both 2-pentylfuran and a combination

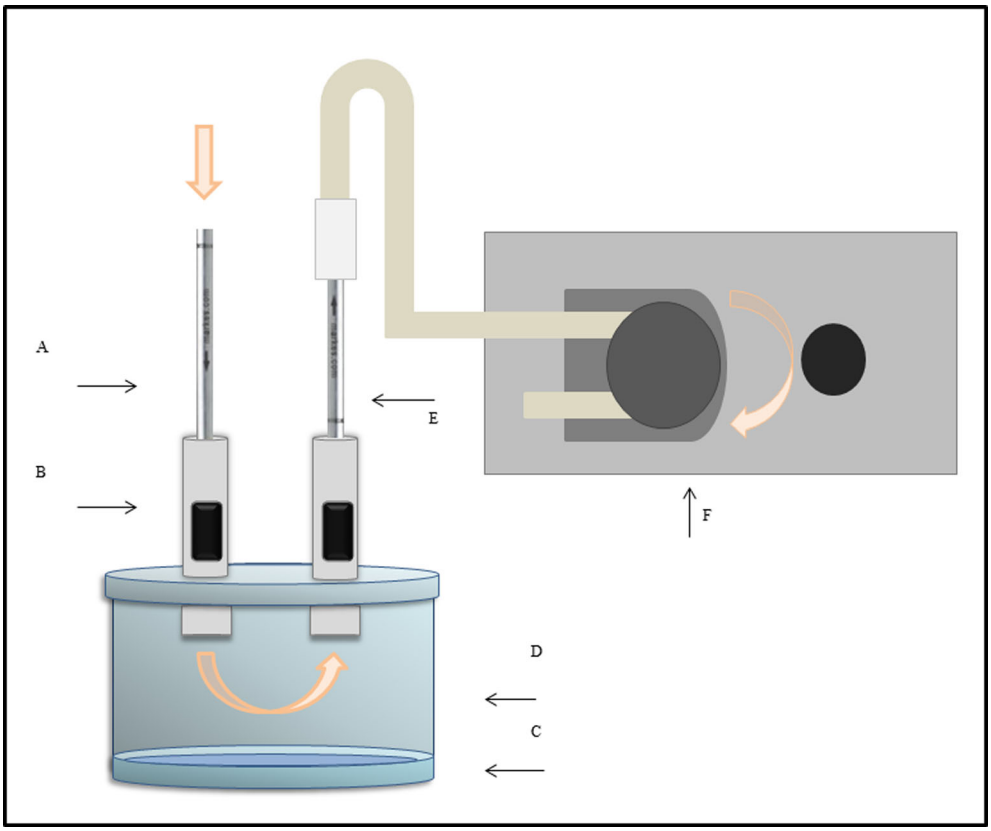

Figure 1. Overview of the set-up used for the *in vitro* experiments. A: Inlet Tenax tube (filter); B: valve; C: *Aspergillus* sandwich culture; D: headspace; E: outlet Tenax tube; F: peristaltic pump.

of four different sesquiterpenes were suggested as exhaled IPA biomarkers.[6,7] These studies show large variations between volatiles found *in vitro* and *in vivo*; however, no direct comparisons have been made. We hypothesized that GC-MS analysis allows identification of VOCs in headspace samples of *Aspergillus* isolates obtained from neutropenic patients with IPA or *Aspergillus* colonization. Next, we explored whether VOCs found *in vitro* could be detected in breath samples taken from these same patients.

We obtained two *Aspergillus fumigatus (A. fumigatus 1, A. fumigatus 2)* isolates from BAL fluid of patients with probable IPA, and an *Aspergillus niger (A. niger)* isolate from a patient with sputum colonization.[9] Af293 was used as reference strain. For species identification, the ITS region and parts of the β-tubulin and calmodulin genes were amplified and sequenced (Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands).[10]

Aspergillus spp. usually invade the lung tissue without conidiation. Therefore, sandwich cultures were performed.[11] A perforated polycarbonate membrane (5–10 µm thickness, 6×10^8 pores cm^{-1}, pore size 0.1 µm, Profiltra, Netherlands) was placed on 15 ml minimal medium (6 g l^{-1} $NaNO_3$, 1.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.2 ml l^{-1} Vishniac; pH 6.0, 1.5% agar), containing 25 mM xylose, in a glass Petri dish. The plate was heated to 60°C and 2 ml 1.25% agarose was added. After cooling down, 2 µl conidia suspension (concentration $10 \times e^8$) was inoculated and topped with a second membrane. Cultures were incubated under aerated conditions at 37°C.

After 92 h each sandwich culture was placed in a glass cuvette (290 ml) and sealed airtight with a Viton-lined glass lid containing an inlet and outlet equipped with valves (Fig. 1).[12] Filtered air was flushed through the inlet for 2 min after which the valves were closed. The cuvettes were incubated at 37°C for 5 h. For headspace sampling, two adsorption tubes (Tenax™ GR 60/80, Interscience, Breda, Netherlands) were placed on the inlet and outlet, the latter trapping VOCs using a peristaltic pump (500 ml, flow 250 ml min^{-1}). Each experiment consisted of an isolate cultured in quintuple, and a negative control: an uninoculated sandwich culture. Experiments were replicated within 2 weeks.

After transportation to an automated thermal desorption unit (Markes TD100, Cincinnati, OH, USA), tubes were analyzed on a quadrupole mass spectrometer (GC-MS-QP2010, Shimadzu, Den Bosch, Netherlands) using a standardized method.[13]

Table 1. Median abundance of the identified volatile organic compounds produced by the four *Aspergillus* isolates and the negative controls.

				Median relative abundance VOCs per isolate				
No.	Rt (s)	Formula	Trivial name	*A. fumigatus 1*	*A. fumigatus 2*	*A. niger*	Af293	NC
1	148	C5H8	1,3-pentadiene	**14 708***	70	0.4	**695***	112
2	155	C4H10O	2-methyl-2-propanol	497	366	0	**3158***	64
3	259	C5H6O	2-methylfuran	**158***	**34***	0.7	25	11
4	272	C4H10O	2-methyl-1-propanol	0	0	**386***	0	0
5	302	C5H12O	2-methyl-2-butanol	111	28	25	**906***	25
6	383	C6H12O	UIC	**117***	21	7	**126***	41
7	440	C5H10O	UIC	1	0.5	**34***	0.5	2
8	916	C8H18O	2-ethyl-1-hexanol	0	**1498***	**422***	0	0
9	998	C12H26O	UIC	0.4	**192***	4	131	3
10	1125	C11H20O	2-methylisoborneol	16	12	**144***	27	12

The median relative abundance ($\times 10^3$) after de-noising, baseline correction and retention time correction is displayed.
*VOCs with significant higher abundance in *Aspergillus* isolates then in the negative controls (NC), in both the initial and the replicate experiment.
Rt (s), retention time in seconds; UIC, unidentified component; VOCs, volatile organic compounds.

R (V3.2.1) was used for statistical analysis. Peak detection, de-noising, and retention time correction were performed using the XCMS-package, resulting in an ion-fragment peak table.[13,14] Compounds were reconstructed from fragments by performing a principal component analysis on fragments within a retention time frame of 5 s. The added intensities of the fragments in principal component 1 with an absolute loading above 0.1 formed the compound abundancy.

Per isolate, the resulting components of the initial experiment were compared to the pooled negative controls. This was repeated for the replicate experiment. The compounds with significantly higher abundance in the *Aspergillus* cultures in both the initial and replicate experiment were selected for *in vitro Aspergillus* identification. Differences were determined with a Mann–Whitney *U* test ($P < .05$), median abundancies were reported. Resulting compounds were manually checked in the chromatograms. Identification was performed as described previously.[13]

Presence of *in vitro* identified components was checked in breath samples of the three patients from which the isolates were obtained, using chemical standards. Four neutropenic patients without IPA, based on a negative BAL (galactomannan, culture), and serum galactomannan, were selected as controls. All patients participated in a prospective trial performed in the Academic Medical Center, Amsterdam and the University Medical Center, Utrecht (clinicaltrials.gov NCT02106117) and gave written informed consent before inclusion. The study was approved by the local institutional review board.

Patients inhaled maximally through a three-way nonrebreathing valve connected to an inspiratory VOC filter (A2, Honeywell, France) and exhaled a full expiratory vital capacity into a Tedlar bag (SKC Inc, Eighty Four, PA, USA). Within 30 min 500 ml of breath was sampled from the bag into a Tenax tube (flow 250 ml min^{-1}). VOCs were considered possible biomarkers if detected in breath from the patient of which the isolate was obtained but not in neutropenic controls.

The *in vitro* compound list consisted of 249 components, mostly originating from the medium. Ten compounds had significantly higher abundance in the *Aspergillus* headspace then in negative controls in both replicates (Table 1). These 10 VOCs were compared with the VOCs in exhaled breath of the selected patients *in vivo*. All breath samples, including those of the neutropenic controls, contained 1,3-pentadiene and 2-ethyl-1-hexanol. 2-methyl-1-propanol was found in all but one. None of the other components were detected in breath.

This is the first study to perform a direct comparison of VOCs found in breath samples of patients with IPA, with those found in headspaces of the same isolates *in vitro*. Our methods for headspace sampling, GC-MS analysis, and compound identification were carefully standardized.

We confirmed presence of several metabolites produced by *Aspergillus* spp. that were previously detected in other studies, such as 1,3-pentadiene, 2-methyl-1-propanol (*A. niger*) and 2-methylisoborneol (*A. versicolor)*.[3,4] The three fungal components identified in breath samples were already found in breath from healthy subjects.[15]

The absence of 2-pentylfuran in breath samples and cultures is in accordance with published data.[5] It was argued that 2-pentylfuran might originate from hemoglobin (blood agar *in vitro*, pulmonary hemorrhage *in vivo*), rather than from the fungus itself.[8] We did not detect sesquiterpenes in *Aspergillus* cultures or breath samples. This might be explained by differences in GC-MS methodologies,

sampling techniques, growth media and/or isolates: for example, it was shown that sesquiterpenes could only be detected when adding elastin to culture medium.[5] These difficulties reproducing VOC profiles between studies is a common phenomenon also observed with bacteria.[16]

A direct comparison of isolates *in vitro* and *in vivo* is important and might allow identification of a possible IPA breath marker. However, several issues complicate this comparison. Despite efforts to mimic conditions in the human lung by preventing conidiation, circumstances *in vitro* might still be quite different. This could lead to an altered fungal metabolism resulting in a changed VOC profile.[5,17] Furthermore, the host response might be responsible for differences in VOCs observed *in vivo*.[18] VOC production might also be dependent on growth phase or availability of nutrients in culture media. Finally, it cannot be excluded that the VOCs identified *in vitro* are present in breath of IPA patients but remain under the detection limit, or that sampling techniques (glass cuvettes vs Tedlar bags) affected VOC profiles.

These data suggest that VOCs allow identification of *Aspergillus* isolates in culture. These VOC profiles could not be confirmed *in vivo*, possibly due to differences in growth conditions and sampling methods. These results underline the difficulties of translating *in vitro* experiments to *in vivo* conditions.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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