International Journal of Environmental Health Research

ISSN: 0960-3123 (Print) 1369-1619 (Online) Journal homepage: https://www.tandfonline.com/loi/cije20

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To cite this article: Daniela Simões, Liliana Aranha Caetano, Cristina Veríssimo, Carla Viegas & Raquel Sabino (2019): *Aspergillus* collected in specific indoor settings: their molecular identification and susceptibility pattern, International Journal of Environmental Health Research, DOI: 10.1080/09603123.2019.1650903

To link to this article: https://doi.org/10.1080/09603123.2019.1650903

INTERNATIONAL JOURNAL OF ENVIRONMENTAL HEALTH RESEARCH
https://doi.org/10.1080/09603123.2019.1650903

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ARTICLE

Aspergillus collected in specific indoor settings: their molecular identification and susceptibility pattern

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ABSTRACT

Exposure to *Aspergillus* conidia is an increased risk factor for the development of respiratory symptoms. The emergence of azole resistance in *Aspergillus fumigatus* is a major concern for the scientific community. The aim of this study was to perform the molecular identification of *Aspergillus* species collected from different occupational and non-occupational indoor settings and to study the azole susceptibility profile of the collected *Fumigati* isolates. The selected *Aspergillus* isolates were identified as belonging to the sections *Fumigati, Nigri Versicolores, Terrei, Clavati* and *Nidulantes*. All the *Aspergillus fumigatus* were screened for azole resistance using an agar media supplemented with itraconazole, voriconazole and posaconazole. None of the tested isolates showed resistance to those azoles. Knowledge of *Aspergillus* epidemiology in specific indoor environments allows a better risk characterization regarding *Aspergillus* burden. This study allowed the analysis of the molecular epidemiology and the determination of the susceptibility pattern of *Aspergillus* section *Fumigati* found in the studied indoor settings.

ARTICLE HISTORY
Received 8 May 2019
Accepted 29 July 2019

KEYWORDS
Indoor air; occupational exposure; Aspergillus; azole resistance

Introduction

Bioaerosols consist in very small airborne biological particles (0.001–100 μm), ubiquitous in the environment (Kim et al. 2018) and composed by pollen, mites, virus, bacteria, fungi, endotoxins and mycotoxins. Some occupational environments are rich in bioaerosols like the ones found in waste industry, poultries and farms (Eduard 2009; Viegas et al. 2014).

Fungi are abundant and essential to the natural environment and only a small fraction of all fungal species are pathogenic for humans (Benedict et al. 2017). However, the exposure to bioaerosols containing fungi increases the risk of developing respiratory symptoms (Sabino et al. 2014). Both occupational and non-occupational exposures to fungi are important factors for human health, especially due to the amount of time spent indoors (Almeida-Silva et al. 2014). Some fungal species found in bioaerosols belong to *Aspergillus* genus, which may constitute an increased risk factor for the development of respiratory symptoms (Kim et al. 2018).

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Aspergillus is a widely distributed genus, found in diverse natural and artificial habitats, such as water, air, soil and vegetation (Hubka et al. 2017). These fungi can produce mycotoxins and a high amount of small conidia easily dispersed in the air. *Aspergillus* species are associated with several health problems, like mycotoxicosis, allergies, asthma, allergic bronchopulmonary aspergillosis, aspergilloma, necrotizing aspergillosis and invasive pulmonary aspergillosis (Dudakova et al. 2017). The frequency of these fungal respiratory infections has been more frequently reported during the last decades because of an increasing life expectancy of immunocompromised patients, cystic fibrosis patients (Nasri et al. 2019). The genus *Aspergillus* has been reported as one of the most prevalent fungi in asthmatic patients' houses, as well as in several highly contaminated occupational environments (like waste industry, poultries and plant production), increasing the potential risk of developing respiratory symptoms (Sharpe et al. 2015; Viegas et al. 2017).

More than 350 *Aspergillus* species were already described and organized in sections (Hubka et al. 2018). The section *Fumigati* covers about 50 to 60 potentially pathogenic species for humans (Hubka et al. 2017). In fact, species belonging to this section (especially *A. fumigatus* sensu stricto) are the most frequently associated with respiratory problems due to the small size of its conidia (1–4 μm), easily inhaled and with the ability to reach the lower respiratory tract (Sabino et al. 2014). The identification of this genus to species level should be based on molecular methods since cryptic species have similar morphology but distinct antifungal susceptibility profiles (Sabino et al. 2014). These cryptic species may have an intrinsic resistance to specific antifungals, like azoles (Van Der Linden et al. 2011; Vermeulen et al. 2015). The most frequently used medical azoles are voriconazole, itraconazole and posaconazole (Dudakova et al. 2017). However, new combinational therapies with clinically licensed drugs against fungal infections are being tested as potential alternatives (Fakhim et al. 2018). The most recent concern about *Aspergillus*, especially *A. fumigatus* sensu stricto, is the emergence of acquired azole-resistance due to the high usage of azoles fungicides in agriculture and in an extensive range of construction materials, generating a positive selective pressure of azole-resistant strains (Vaezi et al. 2018b), which may lead to therapeutic fails in case of infection. Thus, the indoor exposure to *Aspergillus* and especially to less susceptible or resistant isolates may pose a difficult medical problem in the management of patients with aspergillosis (Verweij et al. 2009; Snelders et al. 2012; Kleinkauf et al. 2013; Prigitano et al. 2014; Perlin et al. 2017).

The aim of this study was to perform the molecular identification of *Aspergillus* species collected from different occupational and non-occupational environments and to understand the azole susceptibility pattern of isolates identified as *Fumigati* (section) and collected from those different indoor settings.

Materials and methods

Sampling in indoor environments

Sampling was conducted in different indoor environments located in the Lisbon district, as part of an enlarged exploratory study aiming to establish protocols to assess exposure to mycobiota in several occupational environments. Active and passive methods of sampling were applied to this study. Air samples (active sampling) were collected through an impaction method with a flow rate of 140 L/min (Millipore air Tester, Millipore, Billerica, MA, USA) onto each plate according to manufacturer's instructions. Passive methods [(surfaces, electrostatic cloth devices (EDC), air conditioning filters and feed samples)] were applied to each indoor environment, as previously described (Viegas et al. 2018a, 2018b, 2018c). Samples were collected from the following environments: taxis' air conditioning filters (N = 19; 28 personal vehicles used as controls), fork lifters' air conditioning filters (N = 11) of a waste industry, dairy farm (N = 1), veterinary clinic (N = 1), dwellings (N = 28), faculty environment (N = 1) and bakeries (N = 13) (Figure 1).

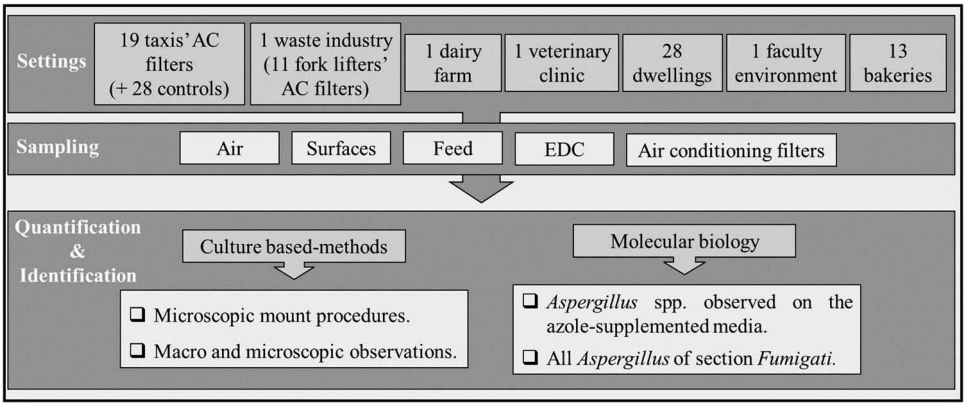

Figure 1. Illustration of settings and methods applied to sampling, to quantify and to identify the isolates, as well the selection criteria to molecular identification. (AC: air conditioning).

Processing of environmental samples

Cultures were performed as previously described (Madsen et al. 2012; Viegas et al. 2018a, 2018b, 2018c). Inoculated samples were inoculated onto Malt Yeast Agar Extract (MEA) (Oxoid, Hampshire, United Kingdom), Sabouraud Dextrose Agar (SDA) (Oxoid, Hampshire, United Kingdom) and Dichloran Glycerol Agar Base (DG18) (Oxoid, Hampshire, United Kingdom) and incubated at 27°C for 5–7 days in order to allow the growth of all fungal species present in the samples. Fungal quantification was determined by the calculation of the number of colony forming units (CFU) per m^3 (air) or m^2 (surfaces). Species were identified microscopically using tease mount or Scotch tape mount and lactophenol cotton blue. Morphological identification was achieved through macro and microscopic characteristics, according to de Hoog et al., 2016.

To ascertain which of the collected *Aspergillus* isolates were able to grow in the presence of azoles and, from those, which were *A. fumigatus* azole-resistant, azole-supplemented media were used (Morio et al. 2012). The wash suspension (150 µL) from EDC and filters was directly seeded onto both Sabouraud dextrose agar without chloramphenicol (SDA) and onto SDA supplemented with 4 µg/mL of itraconazole, 1 µg/mL of voriconazole and 0.5 µg/mL of posaconazole, according to the EUCAST guidelines (EUCAST 2017).

Selection of Aspergillus isolates for molecular identification

Aspergillus isolates were selected for further analyses following two criteria: 1) all the *Aspergillus* isolates that grew directly on the azole-supplemented media; 2) all *Aspergillus* isolates identified as belonging to section *Fumigati* that grew in non-supplemented media (Figure 1).

Processing the Aspergillus isolates

A spore suspension (in phosphate saline buffer) of each selected *Aspergillus* isolate (following the previously mentioned criteria) was stored at −80°C and then plated onto malt extract agar (MEA) with chloramphenicol (0.05%) and incubated at 27°C, for 7 days to proceed to further studies. The identification and purity of these isolates were confirmed by macro and micro morphologic analysis. DNA of those isolates were extracted using ZR Fungal/Bacterial DNA MiniPrep™, Zymo Research (California, USA), according to manufacturers' instructions.

Molecular identification

Molecular identification to species level was achieved by sequencing the calmodulin or β-tubulin genes, using the primers indicated in Table 1.

To amplify the calmodulin gene and β-tubulin gene, 1.75 µL of each primer (10 µM) was used, together with one unit of Illustra™ puReTaq Ready-To-Go™ *PCR* Beads, GE Healthcare Life Sciences (Buckinghamshire, UK), 18 µL of ultrapure water and 4 µL of DNA (Sabino et al. 2015). Amplifications of calmodulin and β-tubulin genes were carried out as described by Sabino et al., 2015. PCR products were analyzed by electrophoresis in a 2% agarose gel and the resultant PCR amplicons were purified using illustra™ ExoProStar™, GE Healthcare Life Sciences (Buckinghamshire, UK).

Sequencing of both strands was performed with the BigDye terminator v1.1 Cycle sequencing kit, Applied Biosystems (California, USA) in the thermal cycler. For calmodulin sequencing were used the same primers as those used in the PCR amplification. For β-tubulin sequencing, another set of primers was used (Table 1). Sequencing reactions were done according to Sabino et al., 2015. Nucleotide sequences were edited using the program Chromas Lite, Technelysium (South Brisbane, Australia), and then compared with the databases CBS (http://www.westerdijkinstitute.nl) and NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolates belonging to section *Fumigati* were identified as *A. fumigatus* sensu stricto when the obtained calmodulin or β tubulin gene sequences showed coverage or homology values >98%. The remaining are mentioned as *Aspergillus fumigatus*. The obtained sequences were submitted to the GenBank database and the correspondent accession numbers are listed in Table S1.

Azole-resistance screening of all Aspergillus fumigatus isolates

Screening media were used to study the azole-resistance profile of all *A. fumigatus* selected according to the two criteria previously mentioned. A spore suspension of each isolate, corresponding to 0.5 McFarland, was inoculated with a swab in each azole-supplemented medium and in an SDA plate without antifungal added (to serve as growth control). Plates were incubated at 27ºC for 7 days. As negative and positive controls, a reference susceptible strain (ATCC 204305) and a pan-azole resistant strain (TR_{34}/L98H) were used, respectively. To confirm doubtful growth in the screening media, a gradient test – ETEST®, bioMérieux (Marcy-l'Étoile, France) was used in order to determine the minimal inhibitory concentration (MIC). Endpoints were raised to the nearest two-fold dilution value that matched the CLSI (Clinical & Laboratory Standards Institute) concentration ranges to facilitate minimal inhibitory concentrations comparisons. *Aspergillus fumigatus* is considered resistant to itraconazole and voriconazole when the MIC is higher than 2 µg/mL and to posaconazole when MIC is higher than 0.25 µg/mL (EUCAST 2017).

Results

Among the 45 *Aspergillus* spp. isolates studied, 22 of them were obtained from samples collected in non-occupational environments (dwellings) and 23 from occupational settings [veterinary

Table 1. Primers used to amplify and sequencing calmodulin and β-tubulin genes.

Amplified region	5′ → 3′	Primer	Sequence	Reference
Calmodulin gene	Forward	Cmd5	5′-CCGAGTACAAGGAGGCCTTC-3′	(Hong et al., 2005)
	Reverse	Cmd6	5′-CCGATAGAGGTCATAACGTGG-3′	
β-tubulin gene (amplification)	Forward	Bt2a	5′-GGTAACCAAATCGGTGCTGCTTTC-3′	(Glass and Donaldson, 1995)
	Reverse	Bt2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′	
β-tubulin gene (sequencing)	Forward	Btub1	5′-AATTGGTGCCGCTTTCTGG-3′	(Balajee et al., 2005)
	Reverse	Btub4	5′-AGCGTCCATGGTACCAGG-3′	(Alcazar-Fuoli et al., 2008)

clinic (N = 1), faculty environment (N = 5), waste industry (N = 8), dairy farms (N = 3), bakeries (N = 2) and taxis (N = 4)] (Table 2). Concerning *Aspergillus* air load, the range varied from 62 CFU/m^3 (58.4% prevalence) on a dairy farm to 80 CFU/m^3 (0.3% prevalence) on bakeries. From the 45 *Aspergillus* spp. isolates 12 were obtained from azole-supplemented media and 33 obtained from culture media without azole supplement: MEA (air samples), SDA and DG18 (EDC samples). To guarantee that there were no duplications, only one isolate was used from each sample. Eleven *Aspergillus* section *Nigri* and one section *Fumigati* were isolated from the azole-supplemented media and 33 from the non-supplemented media; all were identified through molecular tools (Table 2). From the isolates collected in non-supplemented media, 30 isolates were identified as *A. fumigatus*, corroborating the previous identification. However, three isolates were misidentified: morphological identification lead to their classification as belonging to section *Fumigati* but after sequencing, they were identified as *A. sydowii, A. terreus* sensu stricto and *A. clavatus* sensu stricto (Table 2).

Ten of the studied *Aspergillus* isolates were identified as cryptic species, detected in three different indoor environments (dwellings, veterinary clinic and waste industry). Cryptic species found were: *A. sydowii* (n = 1), *A. welwitschiae* (n = 2) and *A. tubingensis* (n = 7) (Table 2). From the 11 isolates that grew directly in azole-supplemented media, nine (81.8%) were cryptic species (Table 2).

No azole-resistance was found in the 31 *A. fumigatus* isolates tested. Neither growth was observed in the media with posaconazole nor in the one with itraconazole. Eleven isolates, however, showed residual growth in the SDA media supplemented with voriconazole. Classification of residual growth was given when (1) several small colonies grew, or when (2) few, but larger, colonies grew onto the media. For this reason, two isolates with these two differential residual growths were selected and further tested to confirm their susceptibility pattern. The MIC values for voriconazole were determined by Etest for the two isolates both resulting in a value of 0.125 μg/mL for both. According to EUCAST breakpoints to voriconazole, these results corroborate the fact that the residual growth has no significance in voriconazole-screening media for resistance.

Indoor environments	Collected samples	Culture media	*Aspergillus* species (Number of obtained colonies)
Dwellings	Air	MEA	*A. fumigatus* (n=6); *A. fumigatus* sensu stricto (n=3); *A. sydowii* (n=1); *A. terreus* sensu stricto (n=1).
	EDC	Azole-supplemented	*A. welwitschiae* (n=2)
		SDA	*A. fumigatus* sensu stricto (n=9)
Veterinary clinic	EDC	Azole-supplemented	*A. tubingensis* (n=1)
Faculty environment	Air	MEA	*A. fumigatus* (n=1); *A. fumigatus* sensu stricto (n=3)
	EDC	SDA	*A. fumigatus* sensu stricto (n=1)
Waste industry	Air conditioning filters from fork lifters	Azole-supplemented	*A. tubingensis* (n=6); *A. fumigatus* sensu stricto* (n=1); *A. niger* sensu stricto (n=1)
Dairy farm	Air	MEA	*A. fumigatus* sensu stricto (n=3)
Bakeries	Air	MEA	*A. fumigatus* sensu stricto (n=1)
	EDC	DG18	*A. clavatus* sensu stricto (n=1)
Taxis	Air conditioning filters	SDA	*A. fumigatus* sensu stricto (n=2) *A. fumigatus* (n=1)
		Azole-supplemented	*A. niger* sensu stricto (n=1)

* This *Aspergillus fumigatus* sensu stricto was isolated from SDA medium supplemented with voriconazole.
MEA-Malt Yeast Extract Agar
SDA – Sabouraud Dextrose Agar
DG18- Dichloran Glycerol Agar Base

Discussion

Some environments are rich in bioaerosols that include potentially pathogenic fungi, like *Aspergillus*. Farmers are known to be a risk-group prone to develop respiratory illness caused by the exposure to bioaerosols, but there are other non-farmer workers who are also exposed to the high concentration of bioaerosols, such as the waste industry workers. Furthermore, non-occupational exposure to fungi should be considered as an important factor regarding respiratory illness, taking into account the high amount of time spent inside houses (Hoppe and Martinac 1998; Eduard 2009; Kim et al. 2018).

The exposure to environments contaminated with *Aspergillus* may be the cause of respiratory problems or may enhance them. In fact, globally, *Aspergillus* is estimated to be responsible for causing health issues to millions of people annually (Bongomin et al. 2017; Antunes 2018). Due the emergence of *A. fumigatus* isolates resistant to azoles, the European Center for Disease Control published a technical report with recommendations that included the improvement of epidemiological surveillance and the investigation of the environmental origin of resistant isolates through environmental and laboratory studies, including in non-agricultural environments (Kleinkauf et al. 2013).

From the *Aspergillus* isolates identified by molecular tools, 31 belonged to the section *Fumigati*, corroborating previous morphological identification. No cryptic species (of section *Fumigati*) were detected, but eight isolates from this section were identified only as *A. fumigatus* due to the low coverage and/or homology of the DNA sequences (not higher than 98%, which may not be discriminatory enough to distinguish a sensu stricto from a cryptic species). Three (6.7%) of the analyzed isolates were misidentified (as section *Fumigati*), and the molecular tools allowed a correct identification. Eleven isolates belonging to the section *Nigri* and one isolate belonging to the section *Fumigati* grew in the azole-supplemented media. Some of the *Nigri* species (e.g.: *A. tubigensis*) are described as less susceptible to azoles (Vermeulen et al. 2015; Badali et al. 2016), and the epidemiological cutoff (ECV) for these species are one step higher than for *A. fumigatus* therefore, there is a higher probability to grow in azole-supplemented media. Furthermore, *A. welwitschiae* and *A. tubingensis* are cryptic species, both described with low susceptibility to all medical azoles (Alcazar-Fuoli et al. 2009; Hashimoto et al. 2017). *Aspergillus tubingensis* is described as the less azole-susceptible species from the section *Nigri* (Alcazar-Fuoli et al. 2009; Hashimoto et al. 2017). This may explain why 7 out of 12 isolates that grew in azole-supplemented media were identified as *A. tubingensis*. It is therefore important to use molecular tools in order to identify the *Aspergillus* species collected in the environment, aiming to distinguish if it is a sensu stricto or a cryptic species. The knowledge about the epidemiology and susceptibility of cryptic species is less understood than the one about the sensu stricto species, thus reinforcing the importance of these studies. A higher exposure to cryptic species may be a potential risk to human health, due to their intrinsic resistances, leading to possible treatment failure in case of infection (Sabino et al. 2014).

None of the *A. fumigatus* isolates tested by screening media was azole-resistant. However, some false positives in SDA media supplemented with voriconazole were found. Thus, we recommend the use of a higher concentration of voriconazole (2 or 4 μg/mL) in voriconazole-screening media in order to eliminate false-positive results. No environmental *A. fumigatus* azole-resistant isolates were found in Portugal yet, and the obtained results corroborate this fact. The first case of an azole-resistant *Aspergillus fumigatus* described in Portugal was a clinical isolate detected in the Northern region (only in 2018) harboring the mutation TR_{34}/L98H – the predominant mutation in some countries, like the Netherlands, Belgium and Turkey (Monteiro et al. 2018; Pinto et al. 2018). This mutation is described in strains from azole-naive patients and in the environment, leading to the hypothesis that it could be related to the use of azole fungicides in agriculture (Snelders et al. 2008, 2009; Verweij et al. 2009, 2016; Jeanvoine et al. 2017). Actually, azole fungicides are globally used and in different environments, like farms and industry. This means that a fungicide selection pressure could occur in different countries and in different

environments (Jeanvoine et al. 2017), and a change in the susceptibility profiles, with the emergence of resistant strains, may arise (Vaezi et al. 2018a). Thus, despite we did not find azole-resistant *Aspergillus fumigatus* in the studied environments, this does not mean that there are no environmental azole-resistant isolates in these settings.

Since *A. fumigatus* is described as the major etiological fungal agent causing respiratory infections or allergies, persons exposed to high amounts of conidia may have an increased risk to develop respiratory symptoms. So, it is important to keep a good hygiene, ventilation and maintenance of houses and workspaces to decrease the amount of bioaerosols in the air and to improve the air quality, preventing respiratory allergic symptoms (Ercilla-Montserrat et al. 2017; Kim et al. 2018). Furthermore, it is crucial to continue studying the fungal biodiversity (especially regarding *Aspergillus* spp.) and analyzing their molecular epidemiology in both occupational (especially in the industry) and non-occupational environments, focusing on the surveillance of the antifungal susceptibility in *A. fumigatus* isolates.

There are some limitations in this study, including the use of screening media for passive sampling, wherein the number of obtained azole-resistant isolates belonging to the genus *Aspergillus* may be underestimated due to the dominance of other genera with faster growth rates (Degois et al. 2017), such as the isolates belonging to *Mucorales* order (especially in the waste industry). As *Mucorales* are, in general, less susceptible to voriconazole, the growth of these species in voriconazole-screening media, may hinder the presence of *Aspergillus* (Springer et al. 2016; Caetano et al. 2018).

Overall, it was possible to characterize the molecular epidemiology and the resistance profile of *Aspergillus* in specific occupational and non-occupational indoor environments. The knowledge of the *Aspergillus* epidemiology in specific indoor environments allows a better risk characterization regarding the exposure to the found *Aspergillus* burden. Further studies are needed to achieve the most suitable algorithm to be applied to environmental samples to characterize the resistance profile of the environmental fungal burden.

Acknowledgments

This work was supported by FCT – Fundação para Ciência e Tecnologia under the project EXPOsE – Establishing protocols to assess occupational exposure to microbiota in clinical settings (02/SAICT/2016 – Grant nº 23222).

Disclosure of interest

The authors report no conflict of interest.

Funding

This work was supported by the FCT – Fundação para Ciência e Tecnologia [02/SAICT/2016 – Grant nº 23222].

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