Azole-resistance screening in occupational exposure assessments to mycobiota

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ABSTRACT
Exposure to azole-resistant fungal species in workplaces represents a health risk for workers. This study describes the prevalence of azole-resistant mycobiota in three occupational environments, namely, Bakeries, Waste industry, and Swine farms, and it proposes complementary sampling methods for the evaluation of occupational exposure to azole-resistant mycobiota at critical worksites. Azole-resistant species, including Aspergillus sp. and Mucorales order, were identified in the three occupational settings in 40 out of 91 (44%) samples collected by passive methods in 15 out of 17 assessed units. Further studies comprising both culture-based methods and molecular analysis of azole-resistant target species should be developed in order to improve the assessment of occupational exposure to resistant mycobiota and characterization of risk factors for workers. Thus, different sampling methods and analyses approaches contribute for the acquisition of more detailed information enabling industrial hygienists to perform better risk characterization.

KEYWORDS: Azole-resistance; occupational exposure; Bakeries; Waste industry; Swine; *Aspergillus*; Mucorales

1. INTRODUCTION
In the last decades, there has been an increasing concern related to the emergence of microbial drug resistance, a well-known threat to public health (Nucci et al., 2005). Although bacterial resistance to antibiotics is extensively described, scientific knowledge is generally scarce about fungal drug resistance and its effects.

A large number of fungal species can cause severe infections, especially among immunocompromised patients and/or patients with granulocytopenia and diabetes (Springer et al., 2016). Increasing resistance to antifungal drugs leads to fewer therapeutic options, and is now a major concern for *Candida* and *Aspergillus* infections (Nature Microbiology, 2017), and also for Mucorales order (Springer et al., 2016).

Development of resistant fungal species in the environment is commonly suspected to be associated to fungal exposure to azole fungicides, often designated as DMI (14-alpha demethylase inhibitors), trough selection pressure mechanisms. Cross-resistance to antifungal drugs can develop, since their molecular structure is similar to medical triazoles (Jeanvoine et al., 2017). Azole fungicides are used in crop and plant protection, preservation of materials, livestock production, and to prevent postharvest spoilage. Thus, employees from these occupational environments may be at risk of being exposed to fungal resistant strains (Viegas et al., 2017).

The genus *Aspergillus*, including *Aspergillus* section *Fumigati*, is ubiquitous in nature and one of the most prevalent in crops and cereals, such as used in baking industry (Gisi, 2013), and in several highly contaminated occupational environments, such as waste treatment (Hameed et al., 2007; Viegas et al., 2015a) and animal production (Sabino et al., 2012; Viegas et al., 2013).

It is currently discussed whether azole-resistance in environmental strains of *Aspergillus* section *Fumigati* can be caused by fungal selection pressure exerted by agricultural triazole fungicide use, such as in crop protection (Verweij et al., 2009), due to the structure similarity of clinical triazoles with triazole fungicides (Caetano et al., 2017). It is also likely that azole-resistance could develop in environments where azoles and organic matter coexist, such as in waste piles, or even in animal production facilities were feed of cereal origin are used or composting practices are performed, as *Aspergillus* section *Fumigati* isolates cultured from soil and compost have already been described to be cross-resistant to azole fungicides, and genetically related to clinical resistant isolates (Verweij et al., 2009).

The presence of azole-resistant strains of *Aspergillus* section *Fumigati* in patients and indoors, such has dwellings and occupational environments, has already been described (Lavergne et al., 2016; Caetano et al., 2017). In a recent study conducted in Eastern France, azole-resistant *A.* section *Fumigati* isolates were identified in sawmills, reinforcing an emerging concern also in the occupational health perspective (Jeanvoine et al., 2017).

Strategies to limit the development of azole-resistant strains, such as the development and marketing of new antifungal drugs or improved protocols for fungicide application, must be implemented, in order to prevail against this emergent public health and occupational issue (Viegas et al., 2018).

Since data related to azole-resistance in occupational environments in Portugal is barely known, the main goal of the present study was to describe the presence of azole-resistant fungal species in different occupational settings: Bakeries, Waste industry, and Swine farms.

2. MATERIALS AND METHODS
Different samples were collected by passive methods from three distinct occupational settings around Lisbon (Portugal) between January and July 2017 (Table 1).

Settled dust samples were obtained from 10 bakeries by collecting the floor dust with the help of a sterilized bag, as previously described (Caetano et al., 2017). Electrostatic dust cloths (EDCs), effective at collecting dust, were also collected from bakeries, by allowing dust to settle for, at least, 15 days (Caetano et al., 2017; Viegas et al., 2018 *a*). Air conditioning filters from 16 fork lifters operating in waste industry were collected and analyzed after processed and extracted with distilled water (Viegas et al., 2017).

Bedding material and feed samples from 5 swine farms, and raw materials from the bakeries, were also collected and processed as follows: 4.4 g of each (not oven-dried prior to processing, thus retaining natural water content) were extracted and 150 μL of this suspension was inoculated (Caetano et al., 2017; Viegas et al., 2018b).

The prevalence of azole resistance was determined for all samples in azole-supplemented media by seeding 150 μL of the wash suspension on Saboraud agar supplemented with 4 mg/L itraconazole, 1 mg/L voriconazole, or 0.5 mg/L posaconazole, according to the EUCAST guidelines (The European Committee on Antimicrobial Susceptibility Testing, 2017).

After incubation at 27 °C for 5 to 7 days, fungal densities (colony-forming units (CFU) per 1 m^2 of filter/EDC area, or per 1 gram of settled dust/raw material/bedding/feed) were calculated, and fungal species were identified microscopically using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures.

3. RESULTS

Azole-resistant species, including *Aspergillus* sp. and Mucorales order, were identified in the three occupational settings in 40 out of 91 (44%) samples collected by passive methods in 15 out of 17 assessed units, as follows: in 15 EDC (including one contaminated with *Aspergillus* section *Circumdati)* and in 2 raw materials (contaminated with *Rhizopus* sp. and *Mucor* sp.) from 8 bakeries; in 15 forklifters air-conditioning filters (including 10 contaminated with *Aspergillus* sp. and 2 contaminated with Mucorales) from the 2 waste treatment units; in 3 bedding material (including one contaminated with *Rhizopus* sp.) and in 5 feed samples from all the assessed swine farms (Table 1). No azole-resistance was detected in settled dust samples from bakeries.

Among target species (*Aspergillus* sp. and Mucorales) most isolates identified in azole-supplemented media were non-susceptible to 1 mg/L voriconazole, the most prevalent being *Rhizopus* sp. (24,931 isolates in bakeries; 500 isolates in fork lifters; 1 isolate in swine farms), followed by *Aspergillus* section *Nigri* (2,009 isolates in fork lifters filters) and *Aspergillus* section *Circumdati* (50 isolates in bakeries). Additionally, isolates of *Aspergillus* sections *Nigri* (3,418 isolates), *Circumdati* (17 isolates), *Candidi* (14 isolates) and *Aspergilli* (1 isolate), and of *Syncephalastrum racemosum* (500 isolates) were identified as non-susceptible to 4 mg/L itraconazole in fork lifters filters.

Of note, *Aspergillus* section *Nigri* was isolated in three different azole-supplemented media, suggesting a possible cross-resistance mechanism to azoles present at fork lifters operation setting.

Table 1. Azole-resistant fungal burden in samples collected by passive methods from all the assessed units

Setting	Samples collected		Number of samples with azole-resistant isolates		
	Sample type	N	Total	*Aspergillus* sp.	Mucorales
Bakeries (10 units)	Settled dust	7	0	0	0
	EDC	27	15	1	2
	Raw materials	26	2	0	1
Waste industry (2 units)	Filters from forklifters	16	15	10	2
Swine farms (5 units)	Bedding material	5	3	0	1
	Feed	10	5	0	0
	Total	**91**	**40**	**11**	**6**

4. DISCUSSION

This study reports the presence of azole-resistant fungal species in three different occupational settings in Portugal; Bakeries, Waste industry, and Swine farms.

Although there is guidance for the identification of *Aspergillus* species in clinical settings, it lacks for occupational exposure purposes either in clinical or other occupational settings. Moreover, there is a lack of standardized protocols for the screening of azole-resistance in environmental samples, due to the heterogeneity of such matrices (Dudakova et al., 2017).

In this study, passive sampling methods (EDCs, air-conditioning filters, bedding material) were used to collect environmental samples in the different occupational settings, in order to collect contamination from a longer period compared with the active methods (Viegas et al. 2015b; Viegas et al. 2017). Fungal species not-susceptible to azole drugs were identified in three different azole-supplemented media, using the EUCAST clinical guidelines as reference due to the lack of specific guidelines for environmental samples.

The presence of azole-resistant fungal species in the three surveyed workplaces, in particular, azole-resistant *Aspergillus* sp. in the waste industry, and, to a lower extent, in bakeries, may potentially place these workers at high health risk, as exposure to resistant fungi may reach infectious levels within a confined space more readily. Noteworthy, azole-resistant *Aspergillus* sp. were identified in EDC samples from bakeries, and in air-conditioning filters from waste fork lifters, thus, highlighting the effectiveness of the applied passive sampling methods and sample matrices treatment to determine the resistant mycobiota in the environment at distinct workplaces.

The identification of Mucorales in all the analyzed settings is also of concern since invasive fungal diseases due to this order are increasing (Kontoyiannis et al., 2005; Bitar et al., 2009; Auberger et al., 2012). Mucorales are not susceptible to voriconazole, the first-line antifungal drug for invasive aspergillosis. Moreover, the dominant and fast growth of these species in voriconazole screening media may hinder the presence of *Aspergillus* genera and other species with clinical relevance (Springer et al., 2016; Caetano et al. 2017).

The presence of resistant mycobiota in air-conditioning filters and in bedding material seems to be closely associated with a need to replace filters, or bedding material, more frequently in fork lifters and at swine farms, respectively, in order to avoid the proliferation and reaerosolization of resistant mycobiota. The presence of resistant mycobiota in EDC suggest the need for improved cleaning procedures at bakeries.

Azole-resistant fungal species were also detected in raw materials (bakeries) and feed (swine farms). Global warming is increasing the prevalence of crop fungal pathogens, and may also increase the prevalence of fungal disease in humans as fungi adapt to survive in warmer temperatures. It is, therefore, of the outmost importance to characterize azole-resistance in specific workplaces where high fungal load and azole pressure might be expected (Nature Microbiology, 2017).

Therefore, fungal resistance to azole drugs should be addressed in exploratory studies to assess occupational exposure to total fungal burden, and to specific *Aspergillus* sp. and Mucorales order burden baseline at specific workplaces, in order to achieve an adequate risk characterization (Viegas et al., 2016; Viegas et al., 2017).

5. CONCLUSIONS

The EDC, air-conditioning filters, and bedding material proved to be sampling devices and matrices suitable for the assessment of occupational exposure to resistant mycobiota in Bakeries, Waste industry, and Swine farms, respectively.

The use of passive methods allows collecting contamination from a larger period in workplaces when compared with active air sampling, unveiling critical worksites.

Further studies comprising both culture-based methods and molecular analysis of azole-resistant target species should be developed in order to improve the assessment of occupational exposure to resistant mycobiota and characterization of risk factors for workers. Thus, different sampling methods and analyses approaches contribute for the acquisition of more detailed information enabling industrial hygienists to perform better risk characterization.

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