International Journal of Environmental Health Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/cije20

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To cite this article: Carla Viegas , Beatriz Almeida , Liliana Aranha Caetano , Anani Afanou , Anne Straumfors , Cristina Veríssimo , Paulo Gonçalves & Raquel Sabino (2020): Algorithm to assess the presence of *Aspergillus fumigatus* resistant strains: The case of Norwegian sawmills, International Journal of Environmental Health Research, DOI: 10.1080/09603123.2020.1810210

To link to this article: https://doi.org/10.1080/09603123.2020.1810210

Published online: 19 Aug 2020.

INTERNATIONAL JOURNAL OF ENVIRONMENTAL HEALTH RESEARCH
https://doi.org/10.1080/09603123.2020.1810210

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ARTICLE

Algorithm to assess the presence of *Aspergillus fumigatus* resistant strains: The case of Norwegian sawmills

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ABSTRACT

Association between selection pressure caused by the use of azole fungicides in sawmills and the development of fungal resistance has been described. The aim of this study was to implement an algorithm to assess the presence of *Aspergillus* section *Fumigati* resistant strains in sawmills.

Eighty-six full-shift inhalable dust samples were collected from eleven industrial sawmills in Norway. Different culture media were used and molecular identification to species level in *Aspergillus* section *Fumigati* was done by calmodulin sequencing and TR_{34}/L98H and TR_{46}/Y121F/T289A mutations were screened by real-time PCR assay and confirmed by *cyp*51A sequencing. Six *Fumigati* isolates were identified as *A. fumigatus* sensu stricto and two of these grew on azole-supplemented media and were further analyzed by real-time PCR. One was confirmed to be a TR_{34}/L98H mutant.

The obtained results reinforce the need to assess the presence of *A. fumigatus* sensu stricto resistant isolates at other workplaces with fungicide pressure.

ARTICLE HISTORY
Received 8 March 2020
Accepted 11 August 2020

KEYWORDS
Section *Fumigati*; *Aspergillus fumigatus* resistant isolates; occupational exposure; cross-resistance; fungicide pressure

Introduction

With 37% of the country's area is covered by forest, Norway has a large forest industry. Although wood products such as cellulose-, fiber- or particleboard also are processed, the Norwegian forest industry has primarily been focused on raw materials, processing timber mainly from spruce (*Picea abies*) and pine (*Pinus sylvestris*). The Norwegian sawmill industry includes over 200 sawmills around the country and employs about 5000 workers (Straumfors et al. 2018). Workers in the wood industry also include around 70 000 carpenters, who are all exposed to wood dust and its components, including microorganisms that are potentially pathogenic to humans (Straumfors et al. 2018).

Studies performed on the occupational environment of sawmills have reported associations between fungal exposure and different respiratory disorders (Mandryk et al. 2000; Adhikari et al.

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2013). Personal exposure to fungal particles in sawmills is dominated by large fungal fragments (Afanou et al., 2018), and one-third of the workers may be exposed to levels of spores that exceed the suggested effect level of 10^5 spores/m^3 (Straumfors et al. 2018). According to ITS2-metabarcoding, fungi belonging to the order Eurotiales are the second most frequently found in the inhalable mycobiome of the sawmills (Straumfors et al. 2019). Included in this order, and representing 4.6% of the total reads, were five different *Aspergillus* spp., of which *A*. section *Fumigati* represented 16.6% of the reads (or 0.7% of the total reads) (unpublished).

Aspergillus section *Fumigati* is an opportunistic pathogen that can cause a wide spectrum of health effects including allergic syndromes, chronic pathologies (chronic pulmonary aspergillosis) and invasive aspergillosis (IA) which is characterized as one of the most severe forms of mycosis (Chowdhary et al. 2013), challenging to manage (Verweij et al. 2009) and with a high mortality rate (Verweij et al. 2013). According to recent data of LIFE organization, invasive infections caused by *Aspergillus* spp. present a high fatality rate that can reach 99% if not treated. The best outcomes are in leukaemia patients (30%), but outcomes are worse in other blood malignancies and immunodeficiencies. (http://www.life-worldwide.org/fungal-diseases/invasive-aspergillosis, accessed on 15 May 2020)

The management of diseases caused by *A. fumigatus* sensu stricto is complicated by the emergence of azole-resistant isolates. Association between selection pressure, caused by the use of azole fungicides in agriculture and floriculture, and the development of fungal resistance in *A. fumigatus* has been described in recent years (Verweij et al. 2012; Schoustra et al. 2019).

Aspergillus resistance to azoles is mainly driven by selective pressure, either by treatment or prophylaxis of patients with aspergillosis with clinical azoles or by exposure of *A. fumigatus* to azole fungicides in the environment. These mechanisms result in the acquisition of mutations in the *cyp*51A gene, usually tandem repeats (TR) in the promoter region combined with single or multiple point mutations in the coding region (TR_{34}/L98H; TR_{53}; TR_{46}/Y121F/T289A, TR_{120}) (Hare et al. 2019; Schoustra et al. 2019).

In addition to environmental use of azole fungicides for protection of spruce and pine fields, other activities such as wood processing, preservation and maintenance also employ azole fungicides (Jeanvoine et al. 2017). Sawmills processing resinous woods frequently use azole fungicides to protect wood from phytopathogenic fungi that cause wood deterioration making wood unworkable (Gisi 2014). Propiconazole and tebuconazole are the principal azole molecules used in sawmill environments. In fact, these two molecules are among the five 14α-demethylase inhibitors (DMIs) related to clinical azoles and causing the increase of azole antifungal resistance (Snelders et al. 2012). Wood is typically treated by sinking boards into a mixture of several fungicides. Although the recommendations for their correct management may be followed, azole residues are still spread through the environment and can pollute all the environmental compartments (water, air and soil). These azole molecules have an important negative impact through their toxicity and persistence in the environment (Chowdhary et al. 2013). Additionally, the presence of azole pressure in this occupational environment may boost the persistence of azole-resistant fungal strains (Snelders et al. 2012; Verweij et al. 2013).

So far, there are no studies regarding the frequency of azole resistance in *A. fumigatus* sensu stricto in occupational environments from Norway. The aim of this study was to implement an algorithm to assess the presence of *A. fumigatus* resistant isolates in sawmills in Norway.

Materials and methods

Assessed sawmills

Eleven large – and medium-sized industrial sawmills (sawing, sorting, kiln drying and planning companies) in Norway that process spruce and/or pine were included in the study, which is part of a larger longitudinal study on respiratory health of Norwegian sawmill workers. Exposure to wood dust, endotoxins, fungi, resin acids and terpenes in these industries has been published previously

(Afanou et al., 2018; Straumfors et al. 2018, 2020), and the inhalable sawmills' mycobiome has been characterized (Straumfors et al. 2019).

The impregnation facilities of the sawmills used either azole-containing chemicals (such as Wolsit, containing 5-chloro-2 methyl-2 H-isothiazole-3-on and 2-methyl-2 H-isothiazol-3-on) or Cu-containing chemicals (such as Wolmanit, no azole). Timber treatment is done with either azole-containing primer (1-(4-chlorophenyl)-4,4-dimethyl-3-1,24-triazole(-1-ylmethyl)pentane-3-ol C7-C9-alkyl-3-[3-(2 H-benzotriazol-2yl)]-5-(1,1-dimethyl)-4-hydroxyphenyl propionates) or paint (hydoxyphenylbenzotriazol-derivatives).

Sampling and characterization of viable mycobiota

Eighty-six full-shift inhalable dust samples were collected between 2013 and 2014. The samples were collected with 37 mm conical inhalable sampling (CIS) cassettes with a conical inlet hole of 8 mm (Casella Solutions, Kempston, UK), loaded with a polycarbonate filter (pore size 1 μm) and using an airflow of 3.5 L/min. The airflow rate was calibrated and recorded using a digital flow meter (Defender, SKC Inc., Eighty-Four, PA, US) before and after sampling. Exposed filters were transferred to 15 ml tubes containing 5 ml PBS with 0.1% BSA and sonicated for 5 min, to remove microbial contamination from the filter surfaces, followed by shaking for 25 min at 500 rpm. The suspension was poured into a new tube, and the extraction process repeated with 2 ml PBS-BSA before the suspensions were pooled together. Aliquots of 1 ml of the sample suspensions were stored at −20°C until analysis.

Different culture media were used in order to enhance the selectivity for fungal growth: malt extract agar (MEA) supplemented with chloramphenicol (0.05%), dichloran-glycerol agar (DG18), Sabouraud dextrose agar (SDA) and SDA supplemented with either 4 mg/L itraconazole (ITR), 1 mg/L voriconazole (VOR) or 0.5 mg/L posaconazole (POS) (adapted from EUCAST 2018).

After incubation at 27°C for 5 to 7 days, fungal burden densities (colony-forming units, CFU/m^2) were calculated. Fungal species were identified microscopically using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures. Morphological identification was achieved through macro and microscopic characteristics as noted by De Hoog (De Hoog 2016).

After identification of section *Fumigati* in one of the media (MEA, DG18, SDA or azoles supplemented agars), only one isolate from one of the media of each sample was used; it was selected the one with higher possibility to obtain a culture of the *Fumigati* isolate without contamination.

Identification of azole-resistant Aspergillus *section* Fumigati

Inhalable dust samples and *A*. section *Fumigati* isolates were further analyzed for identification of antifungal resistance, following the procedures on the implemented algorithm (Figure 1). This included spreading the isolates onto SDA media supplemented with either 4 mg/L ITR, 1 mg/L VOR or 0.5 mg/L POS (protocol adapted from Arendrup et al. 2011).

Molecular identification and detection of resistance genes in Aspergillus *section* Fumigati

Molecular identification to species level in *A*. section *Fumigati* was done by calmodulin sequencing. Amplifications were performed in a 25 μl volume reaction of Illustra PureTaq Read-to-Go PCR beads (GE Healthcare, Buckinghamshire, UK), containing 15 pmol of the primers Cmd5 and Cmd6 and 20 to 50 ng of *Aspergillus* genomic DNA (Hong et al. 2005). Amplifications were carried out with an initial denaturation at 95°C for 10 min, followed by 38 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and a last final extension step of 72°C for 7 min. PCR products were analyzed by electrophoresis through 2% agarose gels and the resultant PCR amplicons were purified using the ExoSAP-IT enzyme system (USB Corporation, Cleveland,

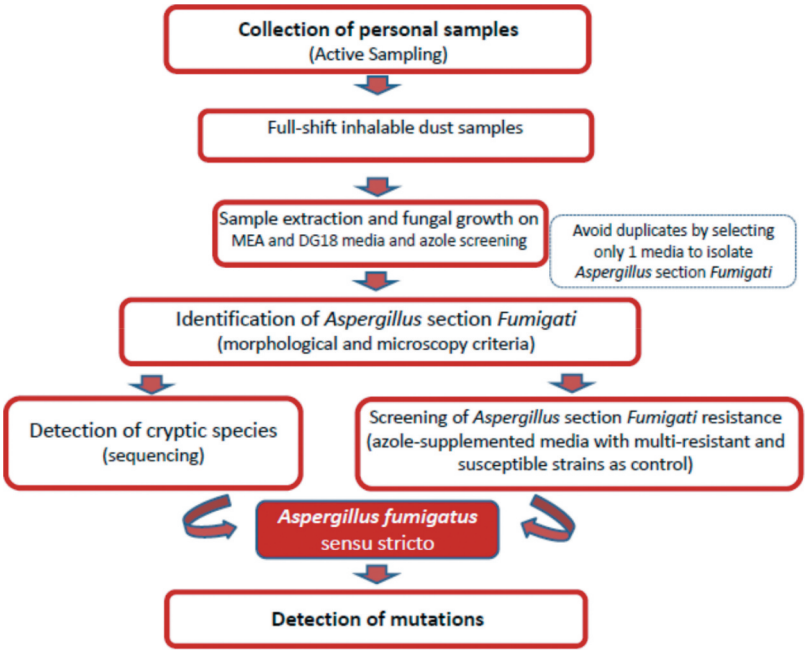

Figure 1. Algorithm applied to assess the presence of *Aspergillus fumigatus* sensu stricto resistant strains on sawmills.

OH), according to the manufacturer's instructions. Sequencing of one strand (forward) was performed with the BigDye terminator v 1.1 Cycle sequencing kit (Applied Biosystems) in the thermal cycler using the primer cmd5 used in the amplification PCR with the following conditions: an initial denaturation at 96°C for 5 sec, followed by 30 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, followed by one cycle of 72°C for 5 min. The resultant nucleotide sequences were edited using the program GeneStudio ™ Professional Edition version 2.2.0.0 and aligned with the program MEGA version 10.0.5. The obtained sequences were compared with sequences deposited in the GenBank (Bethesda, MD, USA) in order to achieve the identification to species level.

All isolates identified as *A. fumigatus sensu stricto* that grew on SDA azole-supplemented media were tested by a multiplex real-time PCR, which screens for TR_{34}/L98H and TR_{46}/Y121F/T289A mutations that have been reported worldwide in patients without previous clinical azole exposure and associated with environmental exposure. These mutations are found in the *cyp*51A gene and in its promoter. This PCR was performed using the AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) on the Qiagen RotorGene Q instrument (Qiagen, Hilden, Germany) following the manufacturer's instructions. To confirm the real-time PCR results, the *cyp*51A gene and its promoter were sequenced according to Prigitano et al. (2014) and Mellado et al. (2001). Nucleotide sequences were edited and aligned as described above.

Statistical analysis

Data were analyzed in SPSS 22.0 statistical software for Windows. Frequency analysis (n; %) and the calculation of the minimum and maximum median were performed for the qualitative and quantitative data, respectively.

Results

Mycobiota and Aspergillus *section* Fumigati *characterization*

In the eighty-six dust samples analysed, fungal contamination in MEA ranged from 0 CFU/m^3 to 2.7 × 10^5 CFU/m^3 (in a sample from the sorting of green timber – spruce and pine – in winter), and from 0 CFU/m^3 to 1.3 × 10^5 CFU/m^3 (in the parting department of a pine wood mill in winter) in DG18.

The most common fungal species found in MEA samples were *Chrysonilia sitophila* (65.20%), followed by *Mucor* sp. (23.86%) and *Rhizopus* sp. (10.75%) (Table 1). In DG18, the most common species was *C. sitophila* (99.57%), followed by *Penicillium* sp. (0.26%) and *Aspergillus* sp. (0.14%) (Table 1).

In MEA, section *Fumigati* was found in three samples (n = 3; 5.67 × 10^2 CFU/m^3 isolates 8278, 8812 and 8814). In DG18, four different *Aspergillus* sections were detected: *Circumdati* (n = 5; 9.52 × 10^2 CFU/m^3) found in four different samples; *Candidi* (n = 4; 7.58 × 10^2 CFU/m^3) found in one sample; *Fumigati* (n = 2; 5.09 × 10^2 CFU/m^3 isolates 8009 and 8007) found in two samples and *Nigri* (n = 1; 1.73 × 10^2 CFU/m^3) found in one sample.

Aspergillus isolates were also obtained from non-supplemented SDA inoculated directly with the dust sample extracts. Two sections were found: *Fumigati* (n = 2; 3.57 × 10^2 CFU/m^3) in two samples (isolates 8370 and 8458); *Aspergilli* (2.07x10^2 CFU/m^3) found in one sample. No *Aspergillus* isolates were found in azole-supplemented media.

Screening of antifungal resistance

Among the seven *Fumigati* isolates identified (MEA, DG18 and SDA, each one of them from different samples), six were able to be recovered in pure culture for further analysis, two of which were able to grow in the presence of one or two medical triazoles when using the azole-supplemented media: one (isolate 8007) was detected only in itraconazole-supplemented media and the other (isolate 8278) grew in both itraconazole – and posaconazole-supplemented media.

Molecular identification and detection of resistance genes in Aspergillus *section* Fumigati

The six *Fumigati* isolates were identified as *A. fumigatus sensu stricto* by calmodulin sequencing (GenBank Accession numbers: MT521737 to MT521742). Two of these, the ones that grew on azole-supplemented media, were further analyzed by real-time PCR for the detection of TR_{34}/L98H or TR_{46}/Y121F/T289A mutations. Isolate 8007 did not show any of the detectable mutations. Isolate 8278 (that grew in the presence of itraconazole and posaconazole) was found to be a TR_{34}/L98H mutant, confirmed by sequencing of the *cyp*51A gene and its promoter [GenBank Accession numbers: MT521736 (promotor) and MT521735 (gene)].

Table 1. Fungal distribution in the sawmills' samples.

MEA			DG18		
Species	n (CFU/m^3)	%	Species	n (CFU/m^3)	%
C-hrysonilia sitophila	1.87x10^6	65.20	*C-hrysonilia sitophila.*	1.71x10^6	99.57
Mucor sp.	6.83x10^5	23.86	*Penicillium* sp.	4.38x10^3	0.26
Rhizopus sp.	3.08x10^5	10.75	*Aspergillus* sp.	2.39x10^3	0.14
Penicillium sp.	4.76x10^3	0.17	*Cladosporium* sp.	3.62x10^2	0.02
A. section *Fumigati*	5.67x10^2	0.02	*Chrysosporium* sp.	1.68x10^2	0.01
TOTAL	2.86x10^6	100	TOTAL	1.72x10^6	100

Discussion

This study presents the first attempt to assess azole resistance on occupational environments in Norway, namely, in Norwegian sawmills. Preliminary results in our study show the presence of *Aspergillus fumigatus* sensu stricto isolates with reduced susceptibility to medical azoles in approximately 7% of the samples. One isolate was resistant to two different triazoles and carried the TR_{34}/L98H mutation, which is described worldwide as related to the use of azole fungicides in agriculture (Snelders et al. 2008, 2009; Verweij et al. 2009, 2016).

Acquired resistance to triazoles in ascomycetes (e.g. *Aspergillus* and *Candida* species) is described to be involved in the emergence of antifungal drug resistance, menacing the effective use of medical triazoles used in the treatment of fungal diseases worldwide (Verweij et al. 2016). While some fungal species present innate resistance to azoles, acquired azole resistance can also be detected in fungi from occupational environments, such as sawmills, where some selection pressure might be exerted on fungal populations by azole fungicides (14-alpha demethylase inhibitors, DMI) used for timber preservation.

Propiconazole and tebuconazole are the two main azole fungicides used for wood treatment in sawmills to protect wood from basidiomycete fungi (Gisi 2014; Jeanvoine et al. 2017). Both fungicides are short-tailed triazoles with similar molecular structure to broadly used medical triazoles, namely, voriconazole. DMI is used against phytopathogenic fungi and is thought to be an environmental source of secondary antifungal resistance worldwide (Verweij et al. 2009; Snelders et al. 2012; Chowdhary et al. 2013).

It has already been described that the fungicides applied have a more important role in the development of resistance than the wood processing practices (Jeanvoine et al. 2017). Two previous studies reported medical azole-resistant strains carrying the TR_{34}/L98H mutation with resistance to five DMIs (propiconazole, tebuconazole, epoxiconazole, bromuconazole, difenoconazole) (Chowdhary et al. 2012; Snelders et al. 2012). In this study, it was possible to detect two isolates able to grow in the presence of one or two medical triazoles when using screening media. These results support the theory that azole pressure is present in the environment in which fungicides are used while there is a growing concern that exposure of *A. fumigatus* to azole fungicides in the environment can cause cross-resistance to medical triazoles. All the isolates grown in at least one azole-supplemented media should be further tested by the reference microdilution method (EUCAST) to determine the minimal inhibitory concentration (MIC). Further analysis of the *cyp*51A gene should also be performed, in order to detect other possible mutations that confer resistance to azoles that may not be associated with environmental exposure.

This study emphasizes the need to assess fungal resistance profile in the environment of specific working environments. The azole resistance screening method used in this study unveils the need to monitor the fungal burden in high-load settings. Our results also show the importance of species identification and resistance profile characterization, so that *Aspergillus* presence in the environment and the potential risks of exposure to resistant mycobiota can be determined, and effective strategies developed to restrain the emergence of azole-resistant fungal strains.

This study suggests an innovative approach regarding the application of an algorithm for the surveillance of azole-resistance among *Aspergillus* species in environmental samples. This algorithm can be applied in a broad range of environmental samples collected from the workplaces, using different sampling methods (active and passive) (Viegas et al. 2019, 2020) and different environmental matrixes such as the protection devices used by workers (Viegas et al. 2020). When possible, different sampling methods should be used in combination, to avoid having to rely on a single method and to represent a long-term time period–integrated scenario (Viegas et al. 2017, 2019, 2020; Leppänen et al. 2018). In the present study, an active full-shift (8 hrs) personal sampling was done, reflecting the inhalable exposure during a working day. Other active sampling methods (short-term air sampling) will reflect microbial load from a short period of time (mostly minutes) with large spatial and temporal variations (Hyvärinen et al. 2001; Viegas et al. 2019), whereas

passive methods will be used in future studies to unveil fungal contamination levels from a broad period of time (weeks to several months). Another benefit from this algorithm approach is the use of DG18 culture media that restricts the growth of some fungal species and consequently increases *Aspergillus* counts in DG18 in regard to MEA (Viegas et al. 2020). Preferably, for exposure assessments in occupational environments where high fungal contamination is expected, such as sawmills, the use of DG18 media should be used to restrict fast-growing fungi, such as Mucorales. Still, the prevalence of other fungi on both culture media *(C. sitophila* and Mucorales) emphasizes the challenging task that is to obtain an accurate exposure assessment and consequently, risk characterization, to clinical relevant fungi, such as *Aspergillus* (Viegas et al. 2017, 2020).

To our knowledge, this is the second study in which *Aspergillus fumigatus* sensu stricto resistant isolates bearing the mutation (TR_{34}/L98H) were found on sawmills. An enlarged study performed in 20 sawmills from France (Jeanvoine et al. 2017), with a wider pool of samples and comprising air and soil samples, also found 20 isolates with the same mutation. A major result of our study was the successful identification of one resistant *A. fumigatus* isolate harbouring the TR_{34}/L98H mutation by analyzing air samples at a low flow rate (3.5 L/min), something that was not possible in other studies (Jeanvoine et al. 2017). The air sample size and the flow rate used in this study were lower in comparison with other studies, where flow rate could reach 700 L of air for 20 min (van der Linden et al. 2013) and small aliquots from dust samples were used and frozen at −20 not ensuring 100% of viability of the isolates belonging to section *Fumigati* (Denning et al. 1992), representing limitations associated with sampling collection and storage. Taking into consideration the above-mentioned, our results are most certainly underestimated.

The discovered *A. fumigatus* isolate harbouring the TR_{34}/L98H mutation was from the department for sorting of green timber, i.e. before drying. In this department, freshly sawn timber was transported on tracks and manually sorted based on quality by use of docking saw, and subsequently automatically sorted into piles by dimension. The piles were stacked and dried at the next stage of the process line. The manual sorting implicates a closer contact by workers with the contaminated timber enhancing workers 'exposure to *Aspergillus* isolates.

Triazole fungicides used at sawmills and triazole drugs used for prophylaxis/treatment of mycosis (namely aspergillosis) have similar chemical structures and mechanisms of action. The fact that the azole-resistant genetic variant of *A. fumigatus* was detected at Norwegian sawmills, suggests that fungicides used at sawmills may decrease fungal sensibility to azole drugs, and represent potential sources of resistance for fungi causing invasive mycoses in humans. Our results also corroborate the added value of the proposed algorithm to support and provide guidance from fieldwork (sampling) to benchwork for exposure assessors. Thus, more occupational assessments are necessary to provide local epidemiologic data so that prevention measures can be implemented to tackle this public health menace (Aranha Caetano et al. 2018).

Conclusions

Azole-resistant *A. fumigatus* sensu stricto was detected in the inhalable aerosol at Norwegian sawmills, suggesting that sawmills using triazole fungicide represent potential sources of resistance for fungi. The emergence of environmental-derived resistant fungi may affect the management of fungal diseases.

Our findings reinforce the need to assess the presence of section *Fumigati* and, namely, *A. fumigatus* sensu stricto resistant strains at other workplaces with fungicide pressure applying the suggested algorithm. To what degree such workplaces represent reservoirs of resistant fungi, and occupational exposure represents a new source of infection should be further investigated.

Disclosure statement

None. I have full control of all primary data and permission is given to the journal to review the data if requested.

Funding

This work was supported by the Research council of Norway [218232/H20]; Instituto Politécnico de Lisboa [IPL/2018/WasteFRPD_ESTeSL]; Fundação para Ciência e Tecnologia [02/SAICT/2016 – Project nº 23222]. H&TRC authors gratefully acknowledge the FCT/MCTES national support through the UIDB/05608/2020 and UIDP/05608/2020.

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