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Individuals spend 80–90% of their day indoors and elderly subjects are likely to spend even a greater amount of time indoors. Thus, indoor air pollutants such as bioaerosols may exert a significant impact on this age group. The aim of this study was to characterize fungal contamination within Portuguese elderly care centers. Fungi were measured using conventional as well as molecular methods in bedrooms, living rooms, canteens, storage areas, and outdoors. Bioaerosols were evaluated before and after the microenvironments' occupancy in order to understand the role played by occupancy in fungal contamination. Fungal load results varied from 32 colony-forming units CFU m^{-3} in bedrooms to 228 CFU m^{-3} in storage areas. *Penicillium* sp. was the most frequently isolated (38.1%), followed by *Aspergillus* sp. (16.3%) and *Chrysonilia* sp. (4.2%). With respect to *Aspergillus* genus, three different fungal species in indoor air were detected, with *A. candidus* (62.5%) the most prevalent. On surfaces, 40 different fungal species were isolated and the most frequent was *Penicillium* sp. (22.2%), followed by Aspergillus sp. (17.3%). Real-time polymerase chain reaction did not detect the presence of *A. fumigatus* complex. Species from *Penicillium* and *Aspergillus* genera were the most abundant in air and surfaces. The species *A. fumigatus* was present in 12.5% of all indoor microenvironments assessed. The living room was the indoor microenvironment with lowest fungal concentration and the storage area was highest.

Indoor air quality in elderly care centers (ECC) is an emerging important issue arising in the last decade because of the increase in humans of (1) life expectancy, (2) number of individuals residing in ECC, and (3) population aging, which is correlated with the inversion of the age pyramid (United Nations, 2012; GEP/MSSS, 2010). In developed countries individuals spend 80–90% of their day indoors and elderly subjects are likely to spend even more time indoors (Almeida-Silva et al., 2014; Saksena et al., 2003). Thus, indoor air pollutants may play a critical for this age group (Simone et al., 2003; Mendes et al., 2013).

Due to the importance of indoor air quality (IAQ), an increasing number of studies concerning IAQ characterization and consequent health impacts have been conducted focusing predominantly on children exposed to indoor air pollutants (Veigas et al., 2010; Almeida et al., 2011; Pegas et al., 2010, 2011a, 2011b; Canha et al., 2010, 2011, 2012). However, given the magnitude of the ECC population that is aging and the considerable amount of time spent by elders within ECC, information linking contamination of ECC by viable particles and exposure of elders continues to be sparse (Zhen-Feng et al., 2011; GEP/MSSS, 2010).

The concentration of several pollutants may be markedly higher indoors than outdoors (Books et al., 1991). In addition, the presence

of indoor pollutants, even at low concentrations, may exert important biological impacts related to chronic exposure periods (Coelho et al., 2005). Among indoor physical and chemical pollutants, it is necessary to consider bioaerosols that consist of airborne particles that either contain living organisms such as bacteria, viruses, and fungi or originate from living organisms. Bioaerosols are ubiquitous, highly variable, complex, natural or synthetic in origin, and contribute to approximately 5–34% of indoor air pollution (Srikanth et al., 2008; Bio-aerosols, 2007).

Several studies positively correlated indoor exposure to microorganisms and microbial components with adverse health manifestations, including headache and respiratory symptoms (Douwes et al., 2003). Considering specifically fungi, their spores are complex agents that may contain multiple hazardous components. Health hazards may differ across species because fungi may produce different allergens and mycotoxins. Moreover, some species also infect humans (Eduard and Halstensen, 2009). Most infections occur in immunocompromised hosts or as a secondary infection, following inhalation of fungal spores or the toxins produced by them (Srikanth et al., 2008). The most common infectious fungus is *Aspergillus*. Although more than 250 species of *Aspergillus* genus have been described, only about 40 are considered to be clinically relevant, but this is an increasing in number (Klich, 2009). *Aspergillus* species that grow indoors include *Aspergillus fumigatus* and *Aspergillus flavus*, and these can produce nosocomial infections (Verma et al., 2003), allergic bronchial–pulmonary aspergillosis (ABPA), and sinusitis (Srikanth et al., 2008). Further, *Aspergillus fumigatus*, the most damaging pathogen of the genus *Aspergillus* (Kupfahl et al., 2007), belongs to the group of indicator microorganisms typical of moisture-damaged buildings and was shown to produce toxic substances when grown on common building material (Nieminen et al., 2002). The Elderly Exposure to Air Pollutants (EEtAP) Project was defined to evaluate the elderly daily exposure to different air pollutants and the Elderly Care Centers (ECCs) contamination to chemical and biological pollutants. This project started to study 10 ECCs and 384 old people living on these sites (Almeida-Silva et al., 2014). The aim of this study was to characterize fungal contamination within Portuguese ECC using conventional and molecular methods.

MATERIALS AND METHODS

Elderly Care Centers

This investigation was conducted in four ECC, situated in Loures, in the main outskirts of Lisbon (Figure 1). Considering the particular characteristics of the surrounding environment, ECC were classified as urban or suburban. ECC 1, 2, and 3 are located in a suburban area and ECC 4 in an urban area. Only ECC 1 was equipped with a heating ventilation and air conditioning (HVAC) system; however, this system was rarely used. The cleaning maintenance frequency varied from once a day to once a week, with living rooms (LR) the microenvironment having the highest cleaning frequency. This fact is due to the large number of elders that spend more than 8 h/d in this microenvironment.

Samples Collection, Preparation, and Analyses

Air fungal contamination was studied by conventional and molecular methods. Fungi contamination was assessed in four ECC in five different microenvironments: bedrooms (BR),

FIGURE 1. Localization of the sampling sites.

LR, canteens, storage areas, and outdoors, at 1 m height.

Conventional Methods Using conventional methods, air samples were collected through an impaction method by a microbiological air sampler (MAS-100TM) with an airflow rate of 140 L/min onto malt extract agar (MEA) supplemented with the antibiotic chloramphenicol (0.05%). In the same indoor environments, surfaces were swabbed using a 10 by 10 cm square stencil that was disinfected with 70% alcohol solution between samples according to the International Standard ISO 18593 (ISO 2004). The obtained swabs were then plated into MEA media. All collected samples were incubated at $27 \pm 2°C$ for 5 to 7 d. After lab processing and incubation of collected samples, quantitative and qualitative results were obtained with identification of the isolated fungal species. In order to understand the possible influence of human occupancy on fungal contamination, an additional approach was applied. Fungal contamination was assessed before and after occupancy in two different indoor microenvironments: BR and LR. The air samples were collected as described previously.

Molecular Analysis For molecular analysis, 20 air samples of 250 L were collected from the 4 ECC using the Coriolis µ air sampler (Bertin Technologies), at 300 L/min airflow rate. Each air sample was collected into a conic sterile tube containing 10 ml sterile phosphate-buffered saline and 0.05% Triton X-100. Five milliliters from the collection liquid was centrifuged at $2500 \times g$ for 10 min and supernatant was removed to leave a 250-µl pellet that was subsequently used for DNA extraction. DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) according to the manufacturer's recommendations. Molecular identification of *Aspergillus fumigatus* was achieved by real-time polymerase chain reaction (RT PCR) using the Rotor-Gene 6000 qPCR Detection System (Corbett) under specific cycling conditions and with specific primers and probes (Table 1). Reactions included $1\times$ iQ Supermix (Bio-Rad), 0.5 µM of each primer, and 0.375 µM of TaqMan probe in a total volume of 20 µl. Amplification followed a three-step

PCR reaction: 40 cycles with denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s. The specificity of the primers and probe set was confirmed by testing these primers in DNA extracted from pure cultures of different species from the same genus (*A. fumigatus, A. flavus, A. terreus, A. niger, A. versicolor*, and *A. ochraceus*) and 14 other species corresponding to different fungal genera (data not shown).

RESULTS AND DISCUSSION

Several studies confirmed that individuals older than 65 yr spend a significant number of hours in indoor environments compared to young people (Simone et al., 2003; Klepeis et al., 2001). This finding strengthens the importance of indoor air quality (IAQ) studies in the elderly, who are especially susceptible to effects of low concentrations of pollutants associated with underlying chronic diseases (Simone et al., 2003). Moreover, air contains a significant number of microorganisms, acting as a medium for their transmission or dispersal. Inhalation, ingestion, and dermal contact are the routes of human exposure to airborne microorganisms, with inhalation being the predominant route (Srikanth et al., 2008).

Fungal Assessment—Conventional Methods

Fungal Load Figure 2 shows the total fungal load for all microenvironments assessed in the four ECC. The results ranged from 32 colony-forming units (CFU) m^{-3} in the BR of ECC 1 to 228 CFU m^{-3} in the storage room of ECC 4. On average, the LR and storage area were the two microenvironments with lowest and highest fungal load, 58 CFU m^{-3} and 118 CFU m^{-3}, respectively. This may be attributed to the fact that all LR have a cleaning frequency of once per day while the storage room is full of nutrients that favor fungal growth (Ekhaise et al., 2008).

ECC 1 and ECC 3 presented a higher outdoor fungal load compared with indoor microenvironments, 88 CFU m^{-3} and 92 CFU

TABLE 1. Specific Primers and TaqMan Probes Used in Real-Time PCR for DNA Amplification of Isolates Belonging to *Aspergillus fumigatus* Complex

	Reaction conditions	
Sequence	Concentration (μM)	Ann T (°C)
F: CGCGTCCGGTCCTCG R: TTAGAAAAATAAAGTTGGGTGTCGG P: FAM-TGTCACCTGCTCTGTAGGCCCG-TAMRA	0.375	52

Note. For *A. fumigatus* (Cruz-Perez et al., 2001).
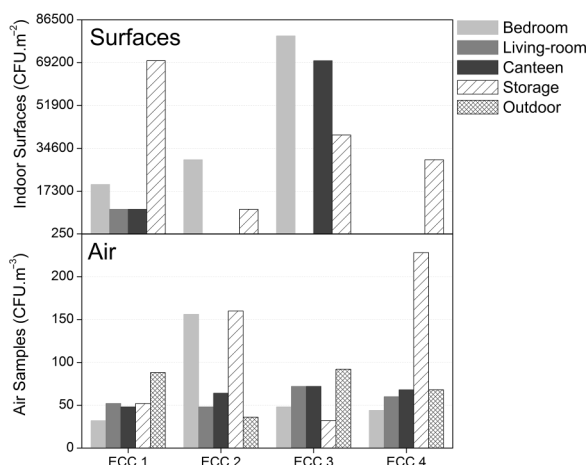
FIGURE 2. Air and surfaces fungal load in four ECCs (values in CFU m^{-3} and CFU m^{-3}, respectively).

m^{-3}, respectively. Data thus suggest an influence of outdoor air on IAQ related to fungal penetration (Goyer et al., 2001). Both ECC are located in a suburban area with a large green area in the surroundings. On the other hand, in all indoor microenvironments of ECC 2 the indoor/outdoor (I/O) ratio was higher than 1. Storage of ECC 4 was the other microenvironment with an I/O ratio above 1, suggesting fungal contamination from inside (Nevalainen, 2007; Rao et al., 1996).

None of the microenvironments assessed exceeded the reference value defined by Portuguese law of 500 CFU m^{-3} (NT-SCE-02, 2009). The storage area of ECC 1, where 228 CFU m^{-3} was isolated, revealed the highest fungal load. However, the national legislation does not consider the occupants' susceptibility, since it is applied to several types of establishments such as schools, offices, and hospitals, among others. Considering this fact, the results

were also compared with a more demanding hospital threshold, and it was observed that 6.3% of indoor microenvironments assessed exceeded the threshold defined by Krzysztofik in 1992 of 200 CFU m^{-3} (Augustowska and Dutkiewicz, 2006).

Fungal Identification Twenty-one different fungal species in indoor air were detected in a total of 1573 isolates. Table 2 also shows that *Penicillium* sp. was the species most frequently isolated (38.1%), followed by *Aspergillus* sp. (16.3%), and *Chrysonilia* sp. (4.2%) in indoor air samples. These results were similar to those obtained in a study conducted in child care centers (Zuraimi et al., 2009). Besides these fungal genera, other fungi were also identified: *Acremonium* sp., *Cladosporium* sp., *Neoscytalidium* sp., *Chrysosporium* sp., *Geotrichum* sp., *Alternaria* sp., *Scopulariopsis* sp., *Beauveria* sp., *Ulocladium* sp., *Aureobasidium* sp., and *Paecilomyces* sp. Other studies assessing fungal contamination also found *Penicillium* sp., *Aspergillus* sp., *Cladosporium* sp., and *Alternaria* sp. were the most prevalent fungi detected (Zhen-Feng et al., 2011; Ren et al., 2011; Zuraimi et al., 2009).

With respect to *Aspergillus* genus, three different fungal species in indoor air were detected in a total of 256 isolates. *Aspergillus candidus* was the species most frequently isolated (62.5%), followed by *A. fumigatus* (14.8%) and *A. niger* (13.2%). With regard to qualitative assessment of fungal contamination, it is postulated that among other species, *Aspergillus fumigatus, Aspergillus versicolor,* and *Penicillium* species, all of which were isolated in this study, need to be considered as indicators of humidity problems and/or a potential risk to

TABLE 2. Most Frequent Fungi Genus Isolated Indoors

Indoor surfaces samples	Frequency (n; %)
Penicillium sp.	90000; 22.2
Aspergillus sp.	70000; 17.3
Chrysosporium sp.	50000; 12.3
Cladosporium sp.	40000; 9.9
Chrysonilia sp.	40000; 9.9
Others	115000; 28.4
Indoor air samples	**Frequency (n; %)**
Penicillium sp.	600; 38.1
Aspergillus sp.	256; 16.3
Chrysonilia sp.	224; 4.2
Others	493; 41.4
Outdoor air samples	**Frequency (n; %)**
Penicillium sp.	84; 26.4
Aspergillus sp.	64; 20.1
Chrysonilia sp.	48; 15.1
Others	122; 38.4

health. According to the American Industrial Hygiene Association (AIHA, 1996), determination of biological contamination in environmental samples with the species *Stachybotrys chartarum, A. versicolor, A. flavus, A. fumigatus,* and *A. niger* requires implementation of corrective measures (NT-SCE-02, 2009; AIHA, 1996). In this study, the four species of *Aspergillus* already mentioned were identified in the bedroom and living room from ECC 1 for *A. versicolor,* living room from ECC 1 and ECC 2 for *A. fumigatus*, living room from ECC 2 for *A. niger,* and canteens from ECC 3 for *A. flavus*.

Despite of the fact that the two genera most isolated outdoors were also detected indoors, namely, *Penicillium* sp. (26.4%) and *Aspergillus* sp. (20.1%), in all the ECC fungal species that were isolated were different between indoors and outdoors areas. Some of them were only present in indoor areas, suggesting fungal contamination from indoors (Nevalainen 2007; Rao et al., 1996). Nevertheless, when fungal levels, are considered, it needs to be taken into account that indoor and outdoor environments are quite different, which on its own justifies the diversity of species between different microenvironments. Concentrations of indoor airborne fungi are also characterized by their generation and rate of air exchange (AER). This last factor plays different roles contributing

either to increase or to reduction of fungal exposure indoors (Zuraimi et al., 2009).

Aspergillus fumigatus was found in two indoor spaces—living room from ECC 1 and living room from ECC 2—and in one outdoor sample. Considering the occupants' susceptibility, hospital guidelines for ECC fungal contamination evaluation were adopted, since these are more demanding than Portuguese law. Faure et al. (2002) used the acceptability threshold for hospital settings >2 CFU room^{-1} without *A. fumigatus*. This threshold was used to extrapolate air results and perform corrective measures in the contaminated areas. Considering this threshold, 12.5% of the indoor air samples presented species from *A. fumigatus*.

Table 2 also shows that in indoor surfaces samples, 14 different fungal species were isolated in a total of 405,000 isolates. Species from *Penicillium* genera were the ones most frequently isolated (22.2%), followed again by *Aspergillus* sp. (17.3%), *Chrysosporium* sp. (12.3%), and *Cladosporium* sp. and *Chrysonilia* sp. (9.9%). Other fungi were also isolated, including *Acremonium* sp., *Scopulariopsis* sp., *Scytalidium* sp., *Geotrichum* sp., *Alternaria* sp., *Syncephalastrum* sp., and *Paecilomyces* sp. Among *Aspergillus* genus, *A. candidus* was also found on surfaces (14.3%), with *A. niger* (85.7%) the most isolated species.

Occupancy Influence on Fungal Contamination

The role of human occupancy as a source of fungi is poorly understood. Therefore, an additional approach was applied in one ECC in order to better understand the possible influence of human occupancy on fungi contamination. Bedroom and LR were studied on three consecutive days during the occupied and nonoccupied periods. According to Figure 3, the indoor fungal load was always lower in the bedroom before occupancy than after occupancy. In LR the highest values were measured before occupancy, with an exception for the LR (c), which presented a high fungal load after occupancy. However, in that case outdoor fungi
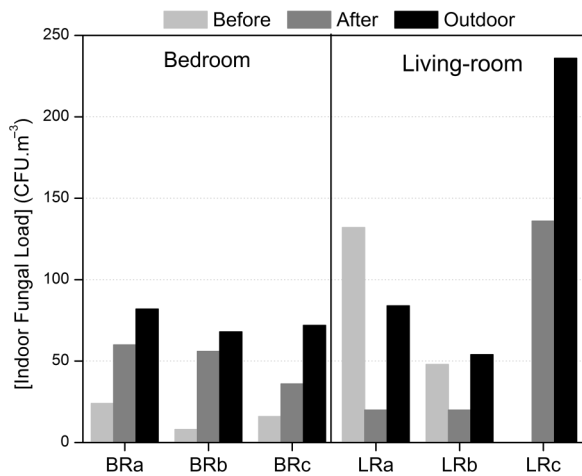
FIGURE 3. Air fungal load assessed before and after elderly occupancy (values in CFU m^{-3}).

concentration also presented at high levels, which may explain this phenomenon. Outdoor fungal load was always higher than indoor concentration, with an exception for LR (a) before occupancy (132 CFU m^{-3}).

Figure 4 shows the most frequent fungi isolated before and after elderly occupancy in BR and LR. In bedroom, *Chrysonilia* sp. (26.3%) was the most common, followed by *Cladosporium* sp. (21.1%) before occupancy. *Penicillium* sp. and *Chrysonilia* sp. were the most identified after occupancy, both with 36.4%. In LR *Penicillium* sp. (33.3%) was also the most isolated before occupancy and
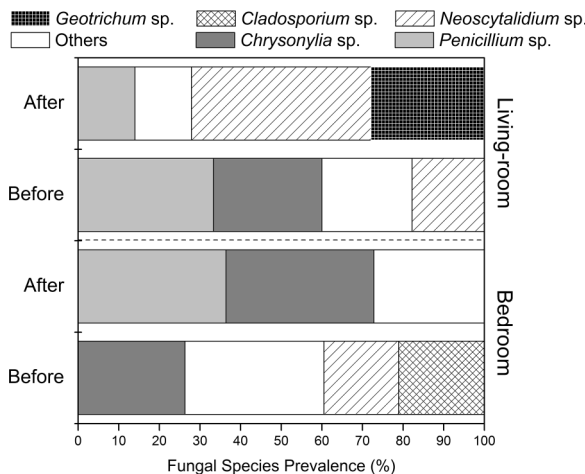

FIGURE 4. Most frequent fungi isolated before and after elderly occupancy.

Neoscytalidum sp. the most abundant species after elderly occupancy (44.2%). Further, other fungi were isolated including *Acremonium* sp., *Stachybotrys chartarum, Chrysosporium* sp., and *Geotrichum* sp. Among *Aspergillus* genus, *A. fumigatus* and *A. niger* were also identified.

Stachybotrus chartarum, which was isolated in BR, similar to *A. fumigatus*, grows in environments with high levels of humidity and produces mycotoxins, including macrocyclic trichothecenes and satratoxin G (Urvashi et al., 2011). Inhalation of *S. chartarum* has been associated with multiple symptoms including muscle aches, headaches, cough, pulmonary hemorrhage, dermatitis, and interstitial lung disease. Moreover, infant deaths from acute idiopathic pulmonary hemorrhage were postulated to be related in part to the presence of this fungus (Jamie et al., 2010). It is important to be aware of the fact that the fungal load found for this species (12 CFU m^{-3}) might be underestimated since this fungus is not easily aerosolized because of its sticky nature, thus becoming difficult to be detected by air sampling (Duchaine and Meriaux, 2001). In addition, *S. chartarum* has slow growth and thus is difficult to detect by conventional methods (Malta-Vacas et al. 2012; Cooley et al., 1998). The presence of these species, with also the identification of species belonging to *Aspergillus* genus, requires immediate intervention in order to avoid the health risk of elderly to infections (NT-SCE-02, 2009; AIHA, 1996).

Fungal Assessment—Molecular Methods

Culture methods have several disadvantages, including poor precision and a highly variable underestimation of exposure. The underestimation depends on sampling strains, microbial robustness, and size of aggregates that may grow only into one colony. Further, the number of CFU depends on culture conditions, nutrient medium, and presence of other species. Consequently, results based on cultivation are at best semiquantitative (Eduard and Halstensen, 2009; Eduard and Heederik, 1998). However, in the case of a possible fungal exposure through inhalation,

conventional methods offer the advantage of enabling identification and quantification only of viable microorganisms and, subsequently, the ones producing higher risk for occupant health (Samson et al., 2000). Therefore, conventional and molecular biological methods when applied together are complementary tools useful in the evaluation of microbiological contamination (Viegas et al., 2012; Malta-Vacas et al., 2012).

Molecular methods were applied in order to detect the presence of *Aspergillus fumigatus* complex. Species from this complex belong to the group of microorganisms considered indicators of moisture-damaged buildings (Samson et al., 1994). Their spores are easily spread in the air and therefore pose a high risk of exposure for both animals and humans (Land et al., 1987). Moreover, the conidia from these species are small enough to traverse the terminal respiratory airways and reach the pulmonary alveoli (Ben-Ami et al., 2010).

Real-time PCR did not detect the presence of *A. fumigatus* complex, probably due to the presence of environmental contaminants present indoors that inhibit PCR analyses, which may result in false-negative samples (Burton et al., 2008). It is also possible that the number of microorganisms present in the environment was below the threshold of amplification by real-time PCR. This is in agreement with the low levels of fungal load detected by conventional methods. One cannot exclude that there is underestimation of fungal load by conventional methods, as *Aspergillus* is a thermophilic species (Fulleringer et al., 2006) and all collected samples were incubated at 27°C.

CONCLUSIONS

Data demonstrated the characterization of fungal contamination in Portuguese ECC using conventional and molecular methods. Of the assessed indoor microenvironments examined, in 37.5% an I/O ratio higher than 1 was noted. The storage area of ECC 1 presented the highest fungal contamination, exceeding the hospital threshold of 200 CFU m^{-3}. Species from *Penicillium* and *Aspergillus* genera were the

most abundant in air and surfaces. The species *A. fumigatus* was present in 12.5% of all indoor microenvironments assessed. Fungal load in BR was higher after elderly occupancy, but LR presented lower amounts of fungi after occupancy. The presence of the identified species, namely, *Stachybotrys chartarum, A. fumigatus,* and *A. niger*, requires immediate intervention to avoid risk of elderly infection.

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