Potential pathogenic fungi assessment through molecular biology in cork industry

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ABSTRACT
Portugal has been the world leader in the cork sector in terms of exports, employing ten thousands of workers. In this working activity, the permanent contact with cork may lead to the exposure to fungi, raising concerns as potential occupational hazards in cork industry. A study was developed aiming at assessing fungal contamination due to *Aspergillus fumigatus* complex and *Penicillium glabrum* complex by molecular methods in three cork industries in the outskirt of Lisbon city. The chosen fungal species are the ones most frequently associated with respiratory problems in workers from these industries. Twelve air samples of 250L were collected in order to identify fungal species, at the molecular level, by Real Time PCR. *Aspergillus fumigatus* complex was not detected in any of the samples collected. However, *Penicillium glabrum* complex was detected in ten out of twelve samples, corroborating the high prevalence of this species in cork industries. This species is an indicator of respiratory diseases such as Suberosis, the most prevalent disease among workers from cork industries. These results, assessed by molecular methods, suggest that preventive and protective measures should be applied in order to minimize occupational exposure to *Penicillium glabrum*.

Keywords: Pathogenic fungi; assessment; *Penicillium glabrum* complex; *Aspergillus fumigatus* complex; cork industry

1. INTRODUCTION
Cork comes from the bark of the cork oak (*Quercus Suber* L), a tree with very special characteristics that grows in Mediterranean regions (Patacho, 2012). Worldwide, cork oak occupies an area very close to 2.2 million hectares, with the Iberian Peninsula accounting for 56.0% of the total area, from which 33% belong to Portugal and 23% to Spain (Pestana and Tinoco, 2009). World cork production was 201,000 tons in 2012. Portugal has been the world leader in the cork sector in terms of exports. In 2012, Portugal took a share of 64.7 % followed by Spain with 16.0 % (APCOR, 2013), employing ten thousands workers (Ministério do Trabalho e da Solidariedade Social, 2009).
Several fungal species have already been found in this industry (Danesh et al., 1997; Oliveira et al., 2003; Santos et al., 2005), being *Chrysonilia sitophila* and *Penicillium glabrum* complex the most prevalent (Winck et al., 2004; Pereira et al., 2000; Serra and Peterson 2008; Basílio et al., 2006; Cruz, 2003; Oliveira, 2011), followed by *A. fumigatus* complex (Villar et al., 2009). Importantly, the presence of *P. glabrum* complex in this type of industry is an indicator of respiratory diseases such as suberosis (Pimentel et al., 1973; Villar et al., 2009; Winck et al., 2004). In fact, side-by-side with occupational asthma, suberosis is the most prevalent disease among workers from cork industries (Wink et al., 2004).
The application of molecular tools is crucial in this setting, since fungal species with faster growth rates, such as *C. sitophila* (Francuz et al., 2010) may hide other species with clinical relevance, such as species belonging to *P. glabrum* and *A. fumigatus* complexes (Pereira et al., 2000; Francuz et al., 2010; Malta-Vacas et al., 2012; Viegas et al., 2012; Viegas et al., 2014).
This study was developed aiming at assessing fungal contamination due to *Aspergillus fumigatus* complex and *Penicillium glabrum* complex by molecular methods in three cork industries in the outskirt of Lisbon city, since these fungal species are the ones more frequently associated with respiratory problems in workers from this occupational setting.

2. MATERIALS AND METHODS
Air samples of 250L were collected using the impinger Coriolis µ air sampler (Bertin Technologies), at 300 L/min airflow rate. Samples were collected onto 10ml sterile phosphate-buffered saline with 0.05% Triton X-100, and the collection liquid was subsequently used for DNA extraction using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions.
Five millilitres of the collection liquid were centrifuged at 2500 × g for 10 min, supernatant was removed and DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) according to the manufacturer's recommendations. Molecular identification of *P. glabrum* complex and *A. fumigatus* complex was achieved by Real Time PCR (RT-PCR) using the Rotor-Gene 6000 qPCR Detection System (Corbett). Reactions included 1× iQ Supermix (Bio-Rad), 0.5 µM of each primer (table 3), and 0.375 µM of TaqMan probe in a total volume of 20 µl. Amplification followed a three-step PCR: 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. A non-template control was used in every PCR reaction. As positive controls DNA samples were obtained from reference strains from the Mycology Laboratory from the National Institute of Health Doutor Ricardo Jorge (INSA).

3. RESULTS AND DISCUSSION

Twelve air samples were collected from different working areas of the three plants analysed. *Aspergillus fumigatus* complex was not detected in any of the 12 samples collected. However, in ten of those samples *Penicillium glabrum* complkex was detected, corroborating the high prevalence of this species in cork industries (Table 1).

Table 1 – Molecular detection of *P. glabrum*

Sampling sites	Real Time PCR *P. glabrum* (Cq– Cycle threshold)
Tracing - Plant A	-
Cutting - Plant A	+ (37,07)
Baking - Plant A	+ (35,07)
Selecting - Plant A	+ (35,30)
Rectification - Plant B	+ (36,45)
Grinding of raw materials - Plant B	+ (34,71)
Grinding in mills - Plant B	+ (35,76)
Agglomeration - Plant C	+ (35,21)
Mixing - Plant C	-
Sawing - Plant C	+ (36,51)
Pressing - Plant C	+ (38,73)
Sanding - Plant C	+ (35,58)

The fact that the DNA from *P. glabrum* complex was amplified at quite high Cq indicates that the fungal load is likely to be low. It is important to note that these values are within the limit of detection and are specific as no amplification occurs when samples from other fungi (e.g. A. *fumigatus* complex and *A. flavus* complex) are amplified with this pair of primers.

Fungal contamination by *P. glabrum* was detected in the three plants suggesting that there might be an increased risk of development of fungi-related diseases, such as hypersensitivity pneumonitis (interstitial lung disorder), later called Suberosis (Morell et al., 2003). It is also important to point out that the discrimination of *Penicillium* species from each other is difficult when using conventional methods, based mainly on morphological features. Instead, with the PCR detection, specific probes can be designed for each species allowing differential detection of species (Viegas et al., 2012; Viegas et al., 2014).

4. CONCLUSIONS

With this study, it was possible to assess occupational fungal exposure due to *P. glabrum* in this setting by molecular detection through real-time PCR. Therefore, it is recommended the application of preventive and protective measures in order to minimize fungal exposure preventing the workers from developing *P. glabrum* related diseases, such as Suberosis.

5. ACKNOWLEDGMENTS

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6. REFERENCES

APCOR. (2013). Estudo de caracterização setorial.

Basílio, M., Gaspar, R., Pereira, C. & Romão, M. (2006). Penicillium glabrum cork colonising isolates – preliminary analysis of their genomic similarity. *Revista Iberoamericana de Micologia*; 23: 151-154.

Cruz, J. (2003). Doença Respiratória dos Trabalhadores da Indústria da Cortiça [dissertation]. Porto: Faculdade de Medicina da Universidade do Porto.

Francuz, B., Year, H., Geraut, L., Bensefa-Colas, L., Nghiem, Z.H. & Choudat, D. (2010). Occupational asthma induced by Chrysonilia sitophila in a worker exposed to coffee grounds. Clinical and Vaccine Immunology, 17(10): 1645-1646.

Malta-Vacas, J., Viegas, S., Sabino, R. & Viegas, C. (2012). Fungal and microbial volatile organic compounds exposure assessment in a waste sorting plant. *Journal of Toxicology and Environmental Health*, 48: 22-23.

Ministério do trabalho e da Solidariedade Social. (2009). Boletim do Trabalho e do Emprego 45/2009

Morell, F., Roger, A., Cruz, M.J., Muñoz, X. & Rodrigo, M.J. (2003). Clinical Study and New Etiologic Agents in a Series of Eight Patients. *Occupational and environmental lung disease*, 124: 1145-1152.

Oliveira, A., Peres, C., Pires, J., Pereira, C., Vitorino, S., Marques, I., Crespo, M. & San-Romão, M. (2003). Cork stoppers industry: defining appropriate mould colonization. *Microbiological Research*, 158: 117-124.

Oliveira, B. (2011). Identification and fingerprinting of cork fungi: a phenetic approach [dissertation]. Lisboa: Universidade de Lisboa.

Patacho, D. (2012). O sobreiro - Green Cork. Retrieved July 2012, from: http://www.greencork.org/index8.php?idlink=7&idbanner=7&idlingua=1&abriu=sim&idcontador=27093.

Pereira, R., Pires, A., Valle, M.J., Boas, L.V., Marques, J.J.F. & Romão, M.V.S. (2000). Role of Chrysonilia sitophila in the quality of cork stoppers for sealing wine bottles. *Journal of Industrial Microbiology and Biotechnology*, 24: 256-261.

Pestana, M. &Tinoco, I. (2009). A indústria e o comércio da cortiça em Portugal durante o século XX. *Silva Lusitana*; 17(1): 1-26.

Pimentel, J.C. & Avila, R. (1973). Respiratory disease in cork workers ("suberosis"). *Thorax*, 28: 409-423.

Santos, M., Bragança, M. & Casimiro, P. (2005). Microrganismos associados à cortiça em diferentes fases da sua fileira. *Silva Lusitana*, 13(1): 75-93.

Serra, R., Peterson, S. &Venâncio, A. (2008). Multilocus sequence identification of Penicillium species in cork bark during plank preparation for the manufacture of stoppers. *Mycrobiology*; 159: 178-186.

Viegas, C., Malta-Vacas, J. & Sabino, R. (2012). Molecular biology versus conventional methods – Complementary methodologies to understand occupational exposure to fungi. *International Symposium on Occupational Safety and Hygiene*, 478-479.

Viegas, C., Malta-Vacas, J., Sabino, R., Viegas S. & Veríssimo, C. (2014) Accessing indoor fungal contamination using conventional and molecular methods in Portuguese poultries. *Environmental Monitoring and Assessment*, 186(3): 1951 – 1959.

Villar, A., Munõz, X., Cruz, M.J. & Morell, F. (2009). Hypersensitivity Pneumonitis Caused by Mucor Species in a Cork Worker. *Archivos de Bronconeumología*, 45(8): 405-407.

Winck, JC., Delgado, L., Murta, R., Vanzeller, M. & Marques, J.A. (2004). Cork workers' occupational asthma: lack of association with allergic sensitisation to fungi of the work environment. *International Archives of Occupational and Environmental Health*, 77: 296-300.

Winck, JC., Delgado, L., Murta, R., Lopez, M. & Marques, J. (2004). Antigen characterization of major cork moulds in Suberosis (cork worker's pneumonitis) by immunoblotting. *Allergy*, 59: 739-745.