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Assessment of exposure to the *Penicillium glabrum* complex in cork industry using complementing methods

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Cork oak is the second most dominant forest species in Portugal and makes this country the world leader in cork export. Occupational exposure to *Chrysonilia sitophila* and the *Penicillium glabrum* complex in cork industry is common, and the latter fungus is associated with suberosis. However, as conventional methods seem to underestimate its presence in occupational environments, the aim of our study was to see whether information obtained by polymerase chain reaction (PCR), a molecular-based method, can complement conventional findings and give a better insight into occupational exposure of cork industry workers. We assessed fungal contamination with the *P. glabrum* complex in three cork manufacturing plants in the outskirts of Lisbon using both conventional and molecular methods. Conventional culturing failed to detect the fungus at six sampling sites in which PCR did detect it. This confirms our assumption that the use of complementing methods can provide information for a more accurate assessment of occupational exposure to the *P. glabrum* complex in cork industry.

KEY WORDS: *fungal exposure; molecular methods; PCR; Portugal; suberosis*

Cork is produced from the bark of the cork oak (*Quercus suber* L), which grows in the Mediterranean (1). Worldwide, cork oak plantations occupy close to 2.2 million hectares, 33 % of which in Portugal and 23 % in Spain (2). In 2012, 64.7 % of all cork was produced in Portugal, followed by Spain with 16.0 % (3). Portugal's cork industry employs about ten thousands workers (4).

This type of industry has already been associated with exposure to several fungal species, most commonly with the *Penicillium glabrum* complex and *Chrysonilia sitophila* (5-13). The presence of the *P. glabrum* complex in this industry involves the risk of respiratory diseases such as suberosis, a type of hypersensitivity pneumonitis that is one of the most prevalent diseases among cork workers (8-15).

Conventional methods may underestimate this fungal burden, as the detection of clinically relevant species with lower growth rates such as the *P. glabrum* complex may be hampered in samples with fast-growing fungi such as *C. sitophila* (9, 16-19).

We therefore decided to complement conventional methods with real-time polymerase chain reaction (PCR) in assessing fungal contamination in three cork plants in suburban Lisbon, hoping that the higher sensitivity of PCR compared to the conventional methods will give us a clearer idea of the potential health risk posed by this fungus.

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MATERIALS AND METHODS

Plants assessed

This pilot cross-sectional study included three cork plants. Plant A employs 26 workers and produces cork boards for further processing by other industries. Plant B employs 93 workers and mainly produces natural bottle corks. Plant C employs 150 workers and specialises in several cork-derived articles such as cork tiles, paper, and textile. All three plants provide respiratory protection devices to their workers, but they do not use them on a regular basis. All the plants work five days a week in two eight-hour shifts.

Samples were taken from the workplaces where workers spend most of their time.

Sample collection

Samples for both conventional and PCR methods were collected in January and February 2014 as shown in Table 1.

Conventional sampling methods

Air samples (50-100 L) were collected from 10 indoor sections (agglomeration, sanding, cutting, selecting, baking, tracing, grinding, pressing, mixing, and sawing) using an impactor with a flow rate of 140 L min^{-1} (Millipore air Tester, Millipore - Billerica, Massachusetts, USA) onto malt

Table 1 *Number of samples collected*

Plants	Conventional Methods		Molecular Biology	
	Indoor Air Samples	Surface Samples	Indoor Air Samples	Surface Samples
Plant A	5	5	4	NP
Plant B	3	3	3	NP
Plant C	5	5	5	NP

NP-not performed

extract agar supplemented with chloramphenicol (0.05 %). Samplers were placed at the breathing level of 0.6-1.5 m above the floor and as close as possible to workers. An outdoor sample was also collected for reference.

For a proper fungal characterisation from the assessed occupational environments, surface samples were also collected by swabbing the same indoor sites with a 10 by 10 cm square stencil, disinfected with 70 % alcohol solution between samplings, in line with the ISO 18593 requirements (20). The obtained swabs were then plated onto malt extract agar.

All the collected samples were incubated at 27 °C for 5 to 7 days. The fungal species were quantified (CFU m^{-3} for air samples and CFU m^{-2} for surface samples) and identified as described by Hoog et al. (21).

Sampling for PCR

For PCR identification we collected 250-litre air samples through impinger method using a Coriolis µ air sampler (Bertin Technologies, Montigny-le-Bretonneux, France) at the airflow rate of 300 L min^{-1}. Samples were collected onto 10-mL sterile phosphate-buffered saline with 0.05 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). The collection liquid was used for DNA extraction with the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

Five millilitres of the liquid were centrifuged at 2500 *g* at room temperature for 10 min, supernatant was removed, and DNA extracted.

The *P. glabrum c*omplex was identified with the real-time polymerase chain reaction (qPCR) using the Rotor-Gene 6000 qPCR Detection System (Corbett, Quiagen, Valencia, Califórnia, USA). Reactions included 1× iQ Supermix (Bio-Rad, Berkeley, California, USA), 0.5 µM of each primer (Table 2), and 0.375 µM of TaqMan probe (Table 3) in a total volume of 20 µL. Amplification followed three steps: 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. For positive control we used DNA samples obtained from the reference strain provided by the Mycology Laboratory of the National Institute of Health Dr Ricardo Jorge. We sequenced the internal transcribed spacers (ITS) from rDNA as well as the genes that codify the calmodulin and beta tubulin proteins. The obtained sequences matched 100 % the sequence of the reference *P. glabrum* complex.

RESULTS

Fungal identification and load determined with conventional methods

The conventional identification of fungal species in air confirmed the presence of countless colonies of *C. sitophila* in Plant A. In Plant B *C. sitophila* was also prevalent, whereas Plant C presented a larger diversity of fungal species, among which the most prevalent were *Penicillium* genus (76.5 %) and *Geotrichum* (11.8 %) (Table 3). In addition to these two genera, *Aureobasidium* sp., *Chrysonilia* sp., *Cladosporium* sp. and species from *Aspergillus niger* complex were also identified.

The distribution of fungal species in the surface samples of Plants A and B was similar, with isolates from the *A. fumigatus* complex being the only ones found in addition to *C. sitophila*. In Plant C the most prevalent genera were *Trichoderma* and *Penicillium* (52.9 %; 29.4 %). *Aureobasidum* sp. and species belonging to *A. fumigatus* complex were also isolated.

All three plants had higher fungal load indoors than outdoors.

Fungal detection using PCR

Real-time PCR identified the *P. glabrum* complex in 10 out of the 12 air samples, that is, in six more sampling sites than the conventional method did (Table 4). The fact that the DNA was amplified at quite high quantitation cycles

Table 2 *Sequence of primers and TaqMan probes used for Real Time PCR (from* http://www.epa.gov/microbes/moldtech.htm#penicillium*)*

P. glabrum	**Sequence**
Primer Forward	
Primer Reverse	5'-CATTACTGAGTGAGGGCCCTCT-3' 5'-CGTGAGGCGGGAGCA-3'
Prob	5'-CCAACCTCCCACCCGTG-3'

Table 3 *Most prevalent fungal distribution in three cork production plants*

Fungi species	Plant A		Plant B		Plant C	
	Air (CFU m^{-3})	Surfaces (CFU m^{-2})	Air (CFU m^{-3})	Surfaces (CFU m^{-2})	Air (CFU m^{-3})	Surfaces (CFU m^{-2})
C. sitophila	Countless	Countless	Countless	Countless	40	ND
Penicillium sp.	ND	ND	ND	ND	780	$5x10^4$
A. fumigatus complex	ND	$2x10^4$	10	$20x10^3$	ND	$1x10^4$
Geotrichum sp.	ND	ND	ND	ND	120	ND
Trichoderma sp.	ND	ND	ND	ND	ND	$9x10^4$

ND-not detected

(Cq) suggests that the fungal load was low. It is important to note that these values are above 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. The fact that the Cq does not reflect colony-forming units might indicate that qPCR detects fungal particles that have not germinated under the growth conditions used. For example, in the air sample collected from the raw material grinding section in Plant B we did not detect any *Penicillium* colonies (Table 4), but the Cq value obtained by qPCR was the lowest, suggesting a higher DNA content. In other words, we might have detected a sporulated form of the fungus that did not germinate in our media.

DISCUSSION

The studied plants were all microbiologically contaminated but with different levels of fungal load. All air samples with countless colonies can safely be regarded as exceeding threshold values for occupational exposure proposed by ACGIH and the World Health Organization (WHO) (22-24). Since it was impossible to assess real contamination, for health protection purposes it would be wise to assume the most critical contamination scenario (25).

Furthermore, fungi found in the surfaces samples sites can also be aerosolised (26). The *Trichoderma* species were isolated only in the surface samples, highlighting the importance of analysing surfaces for a more accurate assessment of fungal contamination (27).

The *A. fumigatus* complex was present in all the plants. This saprophytic fungus is very common and may increase occupational health risk (28, 29) posed by the *P. glabrum* complex, since it has also been implicated in the development of suberosis (30, 31).

The *Penicillium* genus was identified only in plant C, and the same is true for the *Geotrichum* and *Trichoderma* genera. This is probably because countless colonies of *C. sitophila* could have obstructed an effective counting of other colonies in the collected samples (16, 18, 19). Thanks to qPCR, however, we were able to detect the *P. glabrum* complex in all three plants. The use of the two types of analytical methods in this study, culture analysis and PCR-based detection, has given a more comprehensive idea of fungal contamination in cork industry. On the one hand, with the culture analysis we were able to identify and quantify organisms posing a higher occupational risk from inhalation and compare their levels with legal and scientific

Table 4 *Conventional quantification of isolates belonging to* Penicillium *genera and molecular detection of* P. glabrum

Sampling sites	Air (CFU/m^3)	Surfaces (CFU/m^2)	Real Time PCR (Air) (Cq– Cycle threshold)
Tracing - Plant A	ND	ND	-
Cutting - Plant A	ND	ND	+ (37.07)
Baking - Plant A	ND	ND	+ (35.07)
Selecting - Plant A	ND	ND	+ (35.30)
Rectification - Plant B	ND	ND	+ (36.45)
Grinding of raw materials - Plant B	ND	ND	+ (34.71)
Grinding in mills - Plant B	ND	ND	+ (35.76)
Agglomeration - Plant C	20	$2x10^4$	+ (35.21)
Mixing - Plant C	60	ND	-
Sawing - Plant C	660	ND	+ (36.51)
Pressing - Plant C	40	ND	+ (38.73)
Sanding - Plant C	ND	$3x10^4$	+ (35.58)

+-detected; ND-not detected

guidelines. On the other hand, this conventional method is limited by several factors, including incubation conditions and competition between species (32), which might be the reason for not identifying *Penicillium* species in plants A and B. These limitations were overcome by the use of qPCR. This method is based on the amplification of genomic regions specific to certain fungal species, which increases sensitivity and removes interference by other species present in the sample (32). Although the primers and probes used in this study may amplify DNA of *P. lividum*, *P. pupurescens, P. spinulosum*, and *P. thomii*, none of these species has ever been described in the cork industry, and it is safe to assume that our detection is limited to the *P. glabrum* complex.

In addition, unlike PCR, the conventional methods can hardly distinguish between the *Penicillium* species. We therefore believe that these two methods should be used in parallel, as they complement each other to provide useful information for the assessment of occupational exposure to fungi (18, 19).

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Ocjena izloženosti kompleksu *Penicillium glabrum* u proizvodnji pluta s pomoću komplementarnih metoda

Hrast plutnjak druga je vrsta po učestalosti u portugalskim šumama, zbog čega je ta zemlja najveći izvoznik pluta na svijetu. Profesionalna je izloženost plijesnima *Chrysonilia sitophila* i *Penicillium glabrum* česta u proizvodnji pluta, a potonja vrsta povezana je s plućnom bolesti suberozom. Međutim, prilikom procjene izloženosti konvencionalnim se metodama često podcjenjuje prisutnost te vrste u radnom okolišu. Stoga je cilj ovog istraživanja bio provjeriti pretpostavku da polimerazna lančana reakcija (PCR) kao molekulska metoda dopunjuje nalaze konvencionalnih metoda i time daje bolji uvid u profesionalnu izloženost radnika u proizvodnji pluta. U tu smo svrhu istražili onečišćenje kompleksom *P. glabrum* u trima tvornicama pluta u okolici Lisabona oslanjajući se na konvencionalne i molekulske metode. PCR je otkrio prisutnost plijesni u šest uzoraka, u kojih konvencionalne metode nisu otkrile njihovu prisutnost. To potvrđuje našu pretpostavku da se primjenom komplementarnih metoda može steći bolji uvid i napraviti točnija procjena profesionalne izloženosti kompleksu *P. glabrum* u proizvodnji pluta.

KLJUČNE RIJEČI: *izloženost plijesnima; molekulske metode; PCR; Portugal; suberoza*