

Morphological and Molecular Identification of Filamentous Fungi Isolated from Cosmetic Powders

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ABSTRACT

*Seven fungi were isolated from 50 samples of cosmetic powders. Morphological analyses and ribosomal DNA Internal Transcribed Spacers sequencing were performed which allowed the discrimination of the isolated fungi as *Aspergillus fumigatus*, *Penicillium sp.*, and *Cladosporium sp.* which could have, among their species, potentially pathogenic microorganisms.*

Key words: Microbial contamination, ribosomal DNA, cosmetics

INTRODUCTION

Cosmetics market requires constant product releases to ensure the company's competitiveness. New trends and consumer needs require agility in developing the new products with quality. Products, such as cosmetic and some food, are not expected to be sterile, however, they must be free of microbial pathogens and the total number of aerobic microorganisms per gram must be low (Behravan et al. 2005). The contamination of these products can result in their conversion into products hazardous for the consumers (Brasil 1999, Orus and Leranoz 2005, Sousa 2008). These contaminations could spoil the formulas in manufacturing plants and consumer's homes at ambient temperatures, if satisfactory measures of preservation are not instituted (Brasil 1999). Studies have reported the development and validation of rapid detection of microbial

contamination in the cosmetic and pharmaceutical formulations (Mislivec et al. 1993, Tran and Hitchins 1994, Anelich and Korsten 1996, Álvarez-Lerma et al. 2008, Lundov and Zachariae 2008). Fungi identification and classification lie on the traditional analyses of macroscopic and microscopic structures (Larone 2002, Watanabe 2002), and their characteristics may change considerably depending on the environment and the conditions they are exposed to. Therefore, molecular methods are tools that help to differentiate the microorganisms (Jimenez et al. 1999, Mirhosseini et al. 2011). Ribosomal DNA (rDNA) is an interesting target to fungal phylogeny studies as it encodes the highly polymorphic 600-800 bp ITS region (Internal Transcribed Spacers), being used for the differentiation of many yeast and fungal species (Larena et al. 1999, Anderson and Cairney 2004, Pinheiro 2004, Anderson and Parkin 2007).

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Because of the growing market of Brazilian cosmetics, the concern about product quality and consumer safety has increased in the recent years. This study aimed to detect the contamination and to characterize the filamentous fungi present in the cosmetic powders commercialized in Southern Brazil.

MATERIAL AND METHODS

Samples

Fifty Brazilian cosmetic powders from three brands (A, B, and C) were purchased.

Isolation and Morphological Identification

For the microorganism isolation, the cosmetic powders were diluted in Nutrient Broth (peptone 5 g.l⁻¹; beef extract 3 g.l⁻¹), plated in Sabouraud Dextrose Agar (peptone 10 g.l⁻¹; glucose 40 g.l⁻¹; agar 15 g.l⁻¹) and incubated at 26 °C for five days. The grown colonies were transferred to Malt extract agar slants (malt extract 30 g.l⁻¹; peptone 3 g.l⁻¹; agar 15 g.l⁻¹) and incubated at 26 °C for five days. For the macroscopic analysis, the colonies were observed daily for color, size, texture, and exudates formation. Reproductive structures were microscopically observed by the microcultivation technique in malt extract agar, which was adapted from the Brazilian National Health Surveillance Agency (ANVISA) methodology.

DNA Extraction and PCR amplification

Fungi were cultivated in the flasks containing malt extract broth, at 26 °C and 170 rpm. The pellets were washed with TE Buffer (1 M Tris-Cl, 0.5 M EDTA pH 8.0), added of 500 µl of lysis buffer and 10 µl of SDS 10% and maintained for 10 minutes at room temperature and then at 60 °C for 10 minutes. Glass beads and 250 µl of phenol: chloroform: isoamyl alcohol (25:24:1) were added to the pellets, homogenized and centrifuged at 13000 x g. One milliliter of ethanol was added to the supernatant and centrifuged. One milliliter of 80% ethanol was added to the precipitate and homogenized with subsequent centrifugation (13000 x g). Ethanol was completely dried at 40 °C; the extracted DNA was resuspended in 30 µl of deionized water and stored at 4 °C until use (Guimarães et al. 2006). All the solutions used were as described by Xufre *et al.* (2000). The PCR

reactions were carried out in 25 µl volume using 4 to 10 µl of the template DNA and ITS1-F (5' CTT GGT CAT TTA GAG GAA GTA A 3') and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3') primers (Anderson and Cairney 2004). The thermocycler programme used was an initial denaturation (94 °C for 4 min), 30 cycles of denaturation (94 °C for 1 min), annealing (40 °C - increasing 0.5 °C per second during 30 s), and extension (72 °C for 1 min) (Anderson and Cairney 2004). The genetic materials were electrophoresed on 1% agarose gel in the TAE buffer 1x (40 mM Tris acetate; 1 mM EDTA), and gels were stained with ethidium bromide and observed in UV detector (Sambrook et al. 1989). Approximated molecular sizes of the amplicons were determined using molecular weight marker 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, California, USA).

DNA sequencing and data analysis

Amplicons were sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing kit (GE Healthcare Life Sciences – Buckinghamshire, UK) and the automatic DNA sequencer ABI Prism 377 (Applied Biosystems, Brazil). The rDNA amplicons were sequenced twice, aligned using the BioEdit Sequence Alignment Editor to obtain the consensus sequence, and compared through the CLUSTALW (<http://www.ebi.ac.uk/tools/clustalw/>) (Hall 1999). Blastn searches were performed at the NCBI GenBank data library (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al. 1997) and compared between themselves using the CLUSTALW (<http://ebi.ac.uk/tools/clustalw/>). The sequence data were deposited in the GenBank Nucleotide Database.

RESULTS AND DISCUSSION

Seven filamentous fungi were isolated from the cosmetic powders but none developed exudate. Filamentous fungi 5, 9, 11, 12 and 13 showed green colonies of cotton appearance. The filamentous fungus 4 differed from the others in color; the filamentous fungus 10 differed in appearance presenting brown colored colonies and powder aspect (Fig. 1).

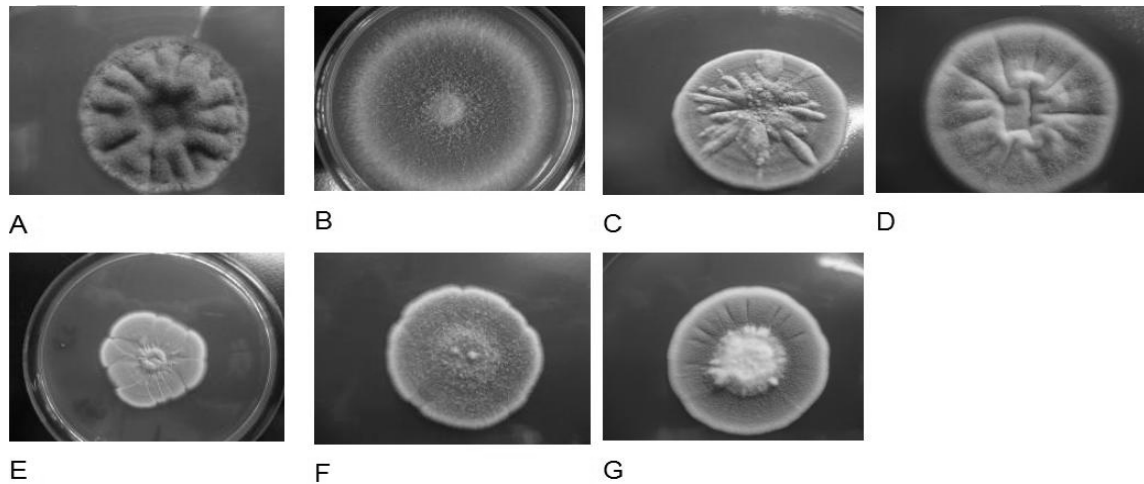


Figure 1 - Filamentous fungi growth in Malt extract agar. 10^6 spores of each filamentous fungus were incubated for five days at $26\text{ }^\circ\text{C} \pm 2\text{ }^\circ\text{C}$. A: Filamentous fungus 4 – *Cladosporium* sp.; B: Filamentous fungus 10 – *Aspergillus fumigatus*; C: Filamentous fungus 5 – *Penicillium* sp.; D: Filamentous fungus 9 – *Penicillium* sp.; E: Filamentous fungus 11 – *Penicillium* sp.; F: Filamentous fungus 12 – *Penicillium* sp.; G: Filamentous fungus 13 – *Penicillium* sp.

The observation of reproductive structures by the optical microscopy confirmed that the filamentous fungi 5, 9, 11, 12 and 13 presented different reproductive structures from filamentous fungi 4 and 10 (Fig. 2). The combined analysis showed that the filamentous fungi 5, 9, 11, 12 and 13 were *Penicillium* sp., filamentous fungus 4 was *Cladosporium* sp. and filamentous fungus 10 was *Aspergillus* sp.. The ITS amplicons ranging from 650 to 700 bp were in agreement with the other

reports (Larena et al. 1999, Anderson and Parkin 2007, Cardoso et al. 2007, Manter and Vivanco 2007). The GenBank accession numbers are indicated in the brackets. The filamentous fungus 4 was identified as *Cladosporium* sp. (GU270579), filamentous fungi 5, 9, 11, 12 and 13 were identified as *Penicillium* sp. (GU270574, GU270576, GU270575, GU270577 and GU270578) and filamentous fungus 10 was identified as *Aspergillus fumigatus* (GQ499183).

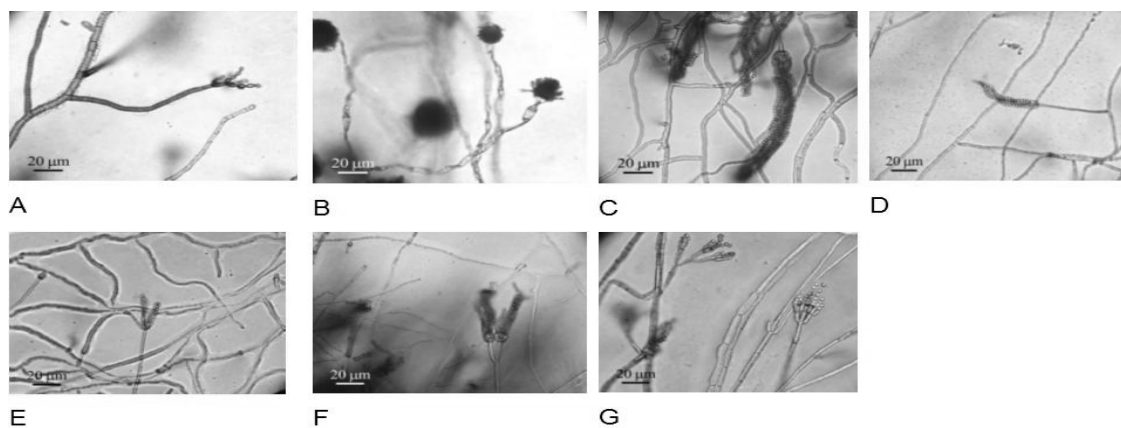


Figure 2 - Development of reproductive structures in Malt Extract agar. Microscopical analyses were performed after incubation for five days at $26\text{ }^\circ\text{C} \pm 2\text{ }^\circ\text{C}$. A: Filamentous fungus 4 – *Cladosporium* sp.; B: Filamentous fungus 10 – *Aspergillus fumigatus*; C: Filamentous fungus 5 – *Penicillium* sp.; D: Filamentous fungus 9 – *Penicillium* sp.; E: Filamentous fungus 11 – *Penicillium* sp.; F: Filamentous fungus 12 – *Penicillium* sp.; G: Filamentous fungus 13 – *Penicillium* sp.

There was high similarity between the ITS sequences of the filamentous fungi identified as *Penicillium* sp., with the filamentous fungus 13 showing the lowest percentage of similarity among the other *Penicillium* sp. (87 to 89%).

The taxonomy of *Penicillium* is complex due to its large number of species which have very few differences. Despite that the classification systems of organisms are based on the observable characteristics, many species classified as *Penicillium* are morphologically similar, and this method of identification remains difficult (Cardoso et al. 2007). This was observed in the present results, since the morphological analyses showed all the isolated fungi belonging to *Penicillium* genus with green colonies of cotton appearance. The comparison of nucleotide sequence of ITS region between these fungi also did not reveal a satisfactory discrimination since there was very low degree of ITS variability, as previously reported by Cardoso et al. (2007).

Therefore, seven filamentous fungi isolated from cosmetics powders and identified them as *Aspergillus fumigatus*, *Penicillium* sp., and *Cladosporium* sp. could have among their species potentially pathogenic microorganisms (Behravan et al. 2005, Orus and Leranoz 2005). These results are valuable due to the fact that the use of contaminated cosmetic products, even within the limits established by the Brazilian legislation, could cause serious damage to the health especially in the people who already have poor health condition.

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