ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2017, 55, 233–236
doi: 10.1093/mmy/myw064
Advance Access Publication Date: 23 August 2016
Brief Report

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High frequency of pathogenic *Aspergillus* species among nonsporulating moulds from respiratory tract samples

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Received 13 April 2016; Revised 22 June 2016; Accepted 1 July 2016

Abstract

Nonsporulating moulds (NSM) represent an identification challenge for clinical laboratories. Data on the prevalence of pathogenic species among NSM are lacking. We prospectively investigated consecutive thermotolerant (36°C) clinical NSM isolates from respiratory tract samples. A total of 123 isolates were identified by DNA sequencing and phenotypically characterized. Of those, 13 (11%) were pathogenic species (*Aspergillus fumigatus*, n = 10; *A. flavus*, n = 1; *A. hiratsukae,* n = 1*; Schizophyllum commune,* n = 1). Presumptive identification of *Aspergillus* species among NSM can be achieved by simple phenotypic testing.

Key words: Nonsporulating mould, *Aspergillus, Schizophyllum commune*, Basidiomycetes.

Culture remains the corner stone for laboratory diagnosis of fungal diseases. Once grown, moulds are usually identified by morphotyping, mostly based on fruiting bodies microscopic features. Nonsporulating moulds (NSM) are therefore very difficult to identify, yet they are commonly encountered in clinical laboratories.[1] While NSM were long considered as nonpathogenic basidiomycetous fungi, the pathogenicity of *Schizophyllum commune* and other filamentous basidiomycetes is increasingly recognized.[2] In addition, atypical strains of pathogenic species, including dysgonic *Aspergillus fumigatus*, that fail to sporulate on culture media are common in the laboratory and have been reported repeatedly.[3–6]

There is still very limited data regarding the relative frequency of pathogens among these isolates and how clinical microbiology laboratories should approach their identification. In a study comprising 52 NSM clinical isolates, 32% were potentially pathogenic.[7] However, many of those isolates were yielded from superficial samples which may carry contaminating organisms not related to disease. It is advised that definitive identification by molecular methods be pursued with isolates associated with significant clinical

disease. Unfortunately, clinical correlation is not always possible and sequenced-based identification is unavailable for many clinical laboratories. Benomyl susceptibility assay has been reported as a useful test to distinguish dysgonic *A. fumigatus* isolates from basidiomycetous fungi.[3] Microscopic features such as clamp connections may help identifying basidiomycetes,[8] but clinical isolates are often monokaryons and lack those structures.[2,9]

The main objective of this study was to evaluate the relative frequency of pathogenic fungi among clinical thermotolerant NSM isolates from respiratory samples. We also sought to define a simple presumptive phenotypic identification scheme for clinically important fungi. Since the vast majority of invasive mould infections involve the lower respiratory tract and that growth at ≥36 degrees Celsius (°C) is a prerequisite for deep tissue infections, we focused on thermotolerant NSM isolated from respiratory samples.

Isolates were collected at Laboratoire de santé publique du Québec (LSPQ), a provincial public health laboratory and reference mycology laboratory for hospital laboratories across the Province of Québec. Consecutive thermotolerant NSM isolates from respiratory tract samples received between October 31 2012 and August 30 2013 at LSPQ were included in this study. Patients' clinical data were not available. NSM was defined as a filamentous fungus failing to produce reproductive structures upon a one-week incubation on modified Sabouraud Emmons dextrose agar (SDA; BBL, Becton, Dickinson and Co.) and Potato dextrose agar (PDA; BBL), at 30°C (±2°C) and 36°C (± 1°C). Isolates that fulfilled those criteria were kept frozen at −80°C for further batch testing. For each isolate, a small piece (≈ 0.5 cm^3) of a colony was dissected then submerged into one milliliter of Sabouraud dextrose broth (BBL) with 10% glycerol in a cryotube.

Isolates were characterized phenotypically with a panel of four tests. Growth was evaluated at 42°C (±1°C) and 49°C (±1°C) on PDA. Urease activity was assessed using Christensen urea agar (BBL). Susceptibility to cycloheximide and benomyl was determined on Mycosel agar (BBL; 0.04% cycloheximide) and Leonian agar supplemented with 0.01% Benomyl (Sigma-Aldrich Co. LLC), respectively.[3] All four tests performed were evaluated after an incubation period of 7 days.

Genomic DNA extraction was performed from 3 to 7 day-old colonies grown on SDA, using ZR Fungal/Bacterial DNA Miniprep (Zymo Research Corp.). Internal transcribed spacer (ITS; primers ITS1/4), D1/D2 and/or beta-tubulin (*benA*) genes were amplified and sequenced according to current guidelines.[10] Sequences were compared to reference sequences in GenBank, CBS-KNAW Fungal Biodiversity Centre and others if errors in databases were suspected.[11]

Table 1. Relative frequencies and classification of different species.

Identification	Phylum	Pathogen[a]	N	%
Aspergillus flavus	Ascomycota	Yes	1	0.8
Aspergillus fumigatus	Ascomycota	Yes	10	8.1
Aspergillus hiratsukae	Ascomycota	Yes	1	0.8
Bjerkandera adusta	Basidiomycota	Yes[b]	3	2.4
Cerrena unicolor	Basidiomycota	No	1	0.8
Coprinellus radians	Basidiomycota	No	1	0.8
Cryptosphaeria sp.	Basidiomycota	No	1	0.8
Hyphodermella rosae	Basidiomycota	No	1	0.8
Irpex lacteus	Basidiomycota	Yes[b]	57	45.6
Mycoacia fuscoatra	Basidiomycota	No	1	0.8
Oxyporus corticola	Basidiomycota	No	28	22.4
Panus neostrigosus	Basidiomycota	No	3	2.4
Perenniporia maackiae	Basidiomycota	Yes[b]	1	0.8
Phanerochaete sordida	Basidiomycota	No	6	4.8
Polyporus brumalis	Basidiomycota	No	2	1.6
Psathyrella candolleana	Basidiomycota	No	1	0.8
Schizophyllum commune	Basidiomycota	Yes	1	0.8
Trametes hirsuta	Basidiomycota	No	1	0.8
Trametes versicolor complex	Basidiomycota	No	2	1.6
Trametes trogii	Basidiomycota	No	1	0.8

[a]Implicated in human pulmonary infections.
[b]Only reported in a limited number of cases.

During the study period, 1853 fungal isolates were submitted to the LSPQ for identification, of which 162 (8.7%) were thermotolerant (36°C) NSM as defined per our study protocol. Thirty-nine isolates were excluded from subsequent analyses because of viability loss (n = 23) or inconsistent growth at 36°C after thawing (n = 16), leaving 123 isolates. A complete list of isolates with sequence data (GenBank accession numbers) and phenotypic characteristics is provided in Supplementary Table 1.

Sequence-based identification achieved species-level resolution in 120 isolates, comprising 18 different species. For two isolates, the species remained uncertain (*Trametes versicolor* species complex), while another could only be identified at the genus level (*Cryptosphaeria* species). The relative frequencies of different genera and species are presented in Table 1. Most isolates belonged to basidiomycetes (n = 110), including many Polyporales (n = 79). The most frequent species were *Irpex lacteus* (n = 57) and *Oxyporus corticola* (n = 28). *Aspergillus* was the third most commonly identified genus representing 12 (10%) isolates (*Aspergillus fumigatus*, n = 10; *Aspergillus flavus*, n = 1; *Aspergillus hiratsukae*, n = 1). One isolate of *Schyzophyllum commune* was found. *S. commune* and *Aspergillus* spp. were the only currently well-recognized pathogenic species, although *Irpex lacteus, Bjerkandera adusta* and *Perenniporia* spp. have been recently implicated in human diseases.[2]

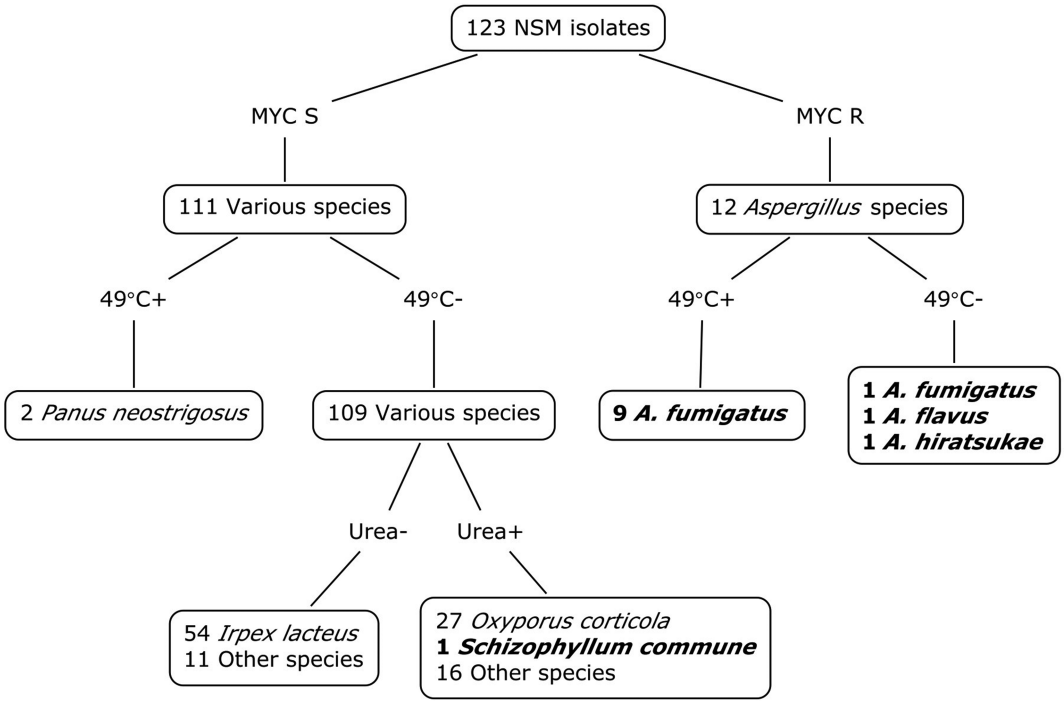

Figure 1. Phenotypic identification algorithm of 123 thermotolerant NSM isolates included in this study. MYC = Mycosel agar (0.04% cycloheximide); S = susceptible; R = resistant.

Susceptibility to cycloheximide and benomyl were the most discriminative characteristics for distinguishing *Aspergillus* species from other organisms. All tested *Aspergillus* isolates were resistant to cycloheximide and susceptible to benomyl, while opposite results were observed with all other organisms, except a *Cryptosphaeria* isolate that was also susceptible to benomyl. Thermotolerance at 49°C was observed in all but one *A. fumigatus* isolates, while it was an uncommon feature among other species, as seen only in two *Panus neostrigosus* isolates. Urease test and growth at 42°C were poorly discriminative. A simplified identification algorithm derived from phenotypic data is presented as Figure 1.

To our knowledge this is the largest study assessing the definite molecular identification of consecutive clinical NSM isolates. Our data revealed a high frequency (11%) of pathogenic species among thermotolerant NSM isolated from respiratory tract samples, including mostly *Aspergillus* species (10%). This is an important finding as NSM may be overlooked and considered as insignificant airway colonizers or laboratory contaminants by many clinical laboratories.

The high prevalence of *Aspergillus* species and low prevalence of *S. commune* contrasted with previous studies[1,7,8] and may be explained by distinct selection criteria. Also, it is worth noting that in recent reviews, only 7 of 114 (6%) *S. commune* infection cases were from North America, while 87 (76%) were from Asia, suggesting that geographic variations may be at play.[2,12] We reviewed all isolates identified at LSPQ between 1988 and 2014 (n = 38,814) and found only 7 (0.02%) *S. commune* (from 5 patients), corroborating its rare occurrence in our territory, although isolates may have been missed. As a reference laboratory in mycology, we recognize that we may have overestimated the prevalence of pathogenic species. However, we believe that referral bias was limited because most local laboratories send us systematically all their NSM isolates.

We also showed that *Aspergillus* species could be set apart by simple and inexpensive phenotypic tests such as susceptibility to cycloheximide and thermotolerance. This could prove to be a useful screening tool for routine identification. Further validation by other observers is warranted as other species may exhibit similar characteristics.

In conclusion, a significant proportion of NSM may represent pathogenic fungi including *Aspergillus* species, of which *A. fumigatus* could be presumptively identified by simple phenotypic testing.

Acknowledgments

We thank Marie-France Sirois, Thérenne Desmeules, and Éric Fournier for their technical assistance.

Funding

This work was supported by an investigator-initiated study grant from Pfizer Canada Inc. (to S.F.D.).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary Material

Supplementary data are available at *MMYCOL* online.

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