



## Brief Report

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

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## 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.<sup>1</sup> While NSM were long considered as nonpathogenic basidiomycetous fungi, the pathogenicity of *Schizophyllum commune* and other filamentous basidiomycetes is increasingly recognized.<sup>2</sup> 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.<sup>3–6</sup>

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.<sup>7</sup> 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.<sup>3</sup> Microscopic features such as clamp connections may help identifying basidiomycetes,<sup>8</sup> but clinical isolates are often monokaryons and lack those structures.<sup>2,9</sup>

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  $\geq 36$  degrees Celsius ( $^{\circ}\text{C}$ ) 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  $30^{\circ}\text{C}$  ( $\pm 2^{\circ}\text{C}$ ) and  $36^{\circ}\text{C}$  ( $\pm 1^{\circ}\text{C}$ ). Isolates that fulfilled those criteria were kept frozen at  $-80^{\circ}\text{C}$  for further batch testing. For each isolate, a small piece ( $\approx 0.5\text{ cm}^3$ ) 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  $42^{\circ}\text{C}$  ( $\pm 1^{\circ}\text{C}$ ) and  $49^{\circ}\text{C}$  ( $\pm 1^{\circ}\text{C}$ ) 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.<sup>3</sup> 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.<sup>10</sup> Sequences were compared to reference sequences in GenBank, CBS-KNAW Fungal Biodiversity Centre and others if errors in databases were suspected.<sup>11</sup>

**Table 1.** Relative frequencies and classification of different species.

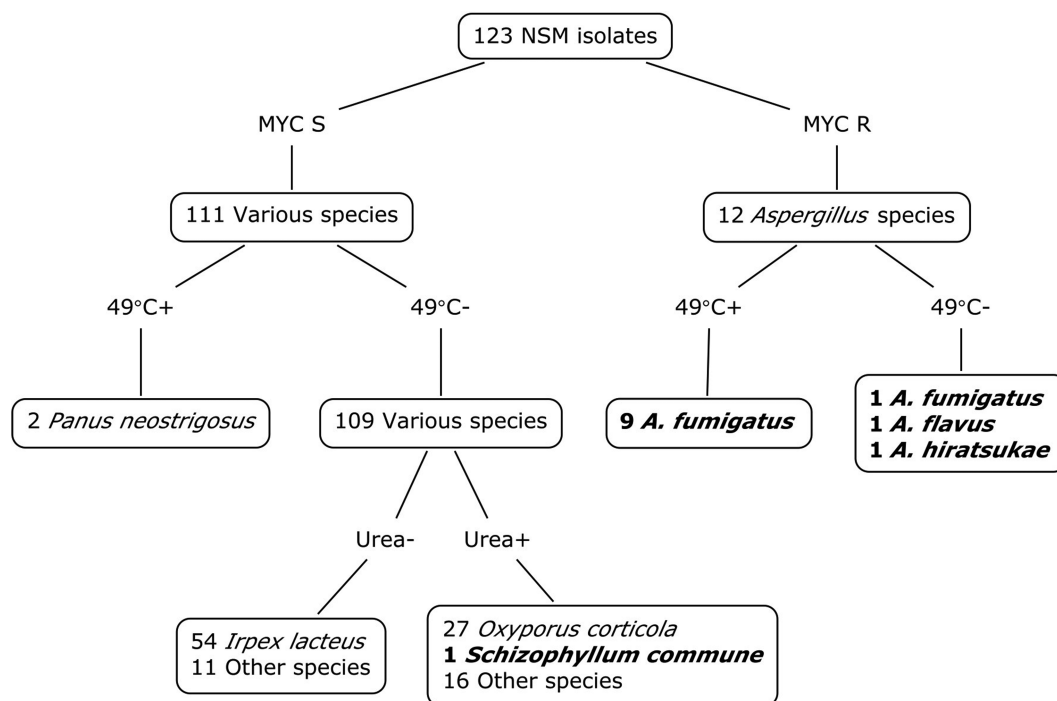
Identification	Phylum	Pathogen <sup>a</sup>	N	%
<i>Aspergillus flavus</i>	Ascomycota	Yes	1	0.8
<i>Aspergillus fumigatus</i>	Ascomycota	Yes	10	8.1
<i>Aspergillus hiratsukae</i>	Ascomycota	Yes	1	0.8
<i>Bjerkandera adusta</i>	Basidiomycota	Yes <sup>b</sup>	3	2.4
<i>Cerrena unicolor</i>	Basidiomycota	No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

<sup>a</sup>Implicated in human pulmonary infections.

<sup>b</sup>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 ( $36^{\circ}\text{C}$ ) 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  $36^{\circ}\text{C}$  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 *Schizophyllum 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.<sup>2</sup>



**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<sup>1,7,8</sup> 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.<sup>2,12</sup> 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.

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## 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](http://www.mmycol.org) online.

## References

- Garcia-Hermoso D, Alanio A, Cabaret O et al. High diversity of non-sporulating moulds in respiratory specimens of immunocompromised patients: should all the species be reported when diagnosing invasive aspergillosis? *Mycoses*. 2015; **58**: 557–564.
- Chowdhary A, Kathuria S, Agarwal K et al. Recognizing filamentous basidiomycetes as agents of human disease: A rev *Med Mycol*. 2014; **52**: 782–797.
- Summerbell RC. The benomyl test as a fundamental diagnostic method for medical mycology. *J Clin Microbiol*. 1993; **31**: 572–577.
- Callister ME, Griffiths MJ, Nicholson AG et al. A fatal case of disseminated aspergillosis caused by a non-sporulating strain of *Aspergillus fumigatus*. *J Clin Pathol*. 2004; **57**: 991–992.
- Balajee SA, Lindsley MD, Iqbal N et al. Nonsporulating clinical isolate identified as *Petromyces alliaceus* (anamorph *Aspergillus alliaceus*) by morphological and sequence-based methods. *J Clin Microbiol*. 2007; **45**: 2701–2703.
- Zhang C, Kong Q, Cai Z et al. The newly nonsporulated characterization of an *Aspergillus fumigatus* isolate from an immunocompetent patient and its clinic indication. *FG & B*. 2015; **81**: 250–260.
- Pounder JI, Simmon KE, Barton CA et al. Discovering potential pathogens among fungi identified as nonsporulating molds. *J Clin Microbiol*. 2007; **45**: 568–571.
- Singh PK, Kathuria S, Agarwal K et al. Clinical significance and molecular characterization of nonsporulating molds isolated from the respiratory tracts of bronchopulmonary mycosis patients with special reference to basidiomycetes. *J Clin Microbiol*. 2013; **51**: 3331–3337.
- Sigler L, de la Maza LM, Tan G et al. Diagnostic difficulties caused by a nonclamped *Schizophyllum commune* isolate in a case of fungus ball of the lung. *J Clin Microbiol*. 1995; **33**: 1979–1983.
- CLSI. *Interpretative Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guidelines*. CLSI Document MM18-A. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
- Jung PE, Fong JJ, Park MS et al. Sequence validation for the identification of the white-rot fungi *Bjerkandera* in public sequence databases. *J Microbiol Biotech*. 2014; **24**: 1301–1307.
- Chowdhary A, Randhawa HS, Gaur SN et al. *Schizophyllum commune* as an emerging fungal pathogen: a review and report of two cases. *Mycoses*. 2013; **56**: 1–10.