Aspergillus species as emerging causative agents of onychomycosis

Aspergillus spp. agents émergents responsables d’onychomycose


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Summary
Background. — Onychomycosis is a common nail infection caused by dermatophytes, non-dermatophyte molds (NDM), and yeasts. Aspergillus species are emerging as increasing causes of toenail onychomycosis. The purpose of this study was species delineation of Aspergillus spp. isolated from patients with onychomycosis.

Methods. — During a period of one year (2012–2013), nail samples were collected from patients clinically suspected of onychomycosis and subjected to microscopic examination and culture. Species identification was performed based on macro- and micro-morphology of colonies. For precise species identification, PCR-amplification and sequencing of the beta-tubulin gene followed by BLAST queries were performed where required.

Results. — A total of 463/2,292 (20.2%) tested nails were diagnosed with onychomycosis. Among the positive specimens, 154 cases (33.2%) were identified as saprophytic NDM onychomycosis, 135 (29.2%) of which were attributable to Aspergillus. Aspergillus species isolated from the infected nails included Aspergillus flavus (77.3%, n = 119), Aspergillus niger (n = 4), Aspergillus tubingensis (n = 4), Aspergillus terreus (n = 3), Aspergillus sydowii (n = 2), Aspergillus spp. (n = 2), and Aspergillus candidus (n = 1). Among the patients diagnosed with onychomycosis due to Aspergillus (average patient age, 47.4 years), 40 had fingernail and 95 toenail involvement. The large toenails were most commonly affected.
Introduction

*Aspergillus* species are ubiquitous environmental molds frequently isolated from soil, air, water, and vegetation. They cause a wide range of diseases in humans, including invasive or allergic aspergillosis, aspergilloma, sinusitis, onychomycosis, and keratitis [4,29,31]. Onychomycosis is a common infection of the nail with worldwide distribution caused by dermatophytes, yeasts, and some species of non-dermatophyte molds (NDM) [25,36]. It represents 30% of superficial fungal infections and 50% of all nail disorders [28]. The incidence of onychomycosis due to non-dermatophyte (saprophytic) molds is increasing [11,33,37]. Factors contributing to this increase are damage of the nail surface during nail grinding or manicuring, use of artificial nails, advanced age, peripheral vascular diseases, diabetes, smoking and regular swimming [22,28]. Saprophytic onychomycosis can be caused by various fungi, including *Scopulariopsis brevicaulis*, *Aspergillus* spp., *Fusarium* spp., *Acremonium* spp., and *Scytalidium* spp. [6,7,10,16,18,28,37]. Some epidemiological studies indicate that *Aspergillus* is an emerging cause of toenail onychomycosis, and after *Scopulariopsis* it is the most common cause of non-dermatophyte mold onychomycosis [13]. Over the recent years, onychomycosis caused by different *Aspergillus* species is increasing, evidenced by case reports and epidemiological studies [2,25,26,39]. While identification of unknown *Aspergillus* isolates would be important to clinicians for informed therapeutic decisions, prognosis, and epidemiological purposes [5], many closely related *Aspergillus* species are identified using morphological criteria only in diagnostic laboratories. Therefore, in this study, our aims were to determine the prevalence of *Aspergillus* species isolated from patients with mycotic nail infections in Tehran, Iran, as well as to carry out precise molecular species delineation of rare aspergilli. This study could potentially herald the beginning of a suite of molecular epidemiological studies of superficial fungal nail diseases in Iran.

Materials and methods

Clinical samples

Over a period of one year (2012 to 2013), clinical specimens (nail scrapings) were collected from patients referred to two medical mycology laboratories in Tehran. The samples were taken from deep cuttings of the affected nail along the part bordering the healthy part of the nail. One part of each collected nail fragment was examined by potassium hydroxide (KOH 20%) preparation for the presence of fungal...
elements, and another part was cultured onto Sabouraud dextrose agar (Difco, Detroit, MI, USA) with chloramphenicol by inoculation of three nail fragments onto the agar plate and incubation for up to four weeks at 25–28 ºC. Diagnosis of NDM Aspergillus onychomycosis was made based on dystrophic nail appearance, microscopy-confirmed presence of fungal elements characteristic of saprophytes in KOH preparations, lack of dermatophyte or yeast colonies in culture, and growth of identical Aspergillus colonies in all triplicate culture inoculums. Samples having characteristic saprophytic hyphal elements observed by direct microscopy and significant growth of Aspergillus in culture were considered for species identification based on macro-/microscopic criteria of colonies including slide cultures.

PCR identification of Aspergillus species: For molecular tests, about 10–20 cubic millimeters of the fresh colonies were added to 200 µL distilled water in a 1.5 mL tube. Two hundred microliters of glass beads (0.5 millimeter in diameter) were added, shaken or vortexed rigorously for about 5 min, centrifuged at 8000 rpm for 1 min, after which 200 µL of the supernatant was submitted to DNA extraction by a commercial kit (GeneAll, Korea) according to the instructions of the manufacturer. A 1-µL aliquot of template DNA, 0.4 µM of each forward Bt2a (5’-GGT AAC CAA ATC GGT GCT GCT TTC-3’) and reverse Bt2b (5’-ACC CTC AGT GTA GTG ACC CTT GGC-3’) primer [14], 12.5 µL of premix (Amplicon, Denmark), and enough water to produce a final volume of a 25 µL were used for PCR amplification of the beta tubulin gene, using the following PCR conditions: Initial denaturation for 5 min at 94 ºC, followed by 35 cycles of amplification, consisting of denaturation for 30 sec at 94 ºC, annealing for 30 sec at 58 ºC, and extension for 1 min at 72 ºC, followed by an ultimate extension step at 72 ºC for 10 min. PCR products were electrophoresed on a 1.2% agarose gel containing 0.5 µg/mL ethidium bromide for about one hour in TBE (Tris 0.09 M, Boric acid 0.09 M, EDTA 2 mM) buffer. PCR products were purified using a purification kit (Bioneer, Korea) and sequenced bidirectionally using the forward and reverse primers employed in the PCR. Each sequence chromatogram was analyzed and confirmed manually, using the Geneious software (http://www.geneious.com). Final species delineation was based on comparison of the sequences with relevant reference sequence in GenBank, using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast) and/or considering the micro/macromorphology of the cultures.

### Table 1 Frequency distribution of onychomycosis by causative agent and infection location.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Yeasts</th>
<th>Dermatophytes</th>
<th>NDM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fingernail</td>
<td>154</td>
<td>12</td>
<td>45</td>
<td>211</td>
</tr>
<tr>
<td>Toenail</td>
<td>23</td>
<td>120</td>
<td>109</td>
<td>252</td>
</tr>
<tr>
<td>Total</td>
<td>177</td>
<td>132</td>
<td>154</td>
<td>463</td>
</tr>
</tbody>
</table>

NDM: non-dermatophyte mold.

### Table 2 Frequency of non-dermatophyte molds identified as the causative agents of onychomycosis in this study.

<table>
<thead>
<tr>
<th>Non-dermatophyte mold species</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus</td>
<td>119 (77.3)</td>
</tr>
<tr>
<td>A. niger</td>
<td>4 (2.6)</td>
</tr>
<tr>
<td>A. tubingensis</td>
<td>4 (2.6)</td>
</tr>
<tr>
<td>A. terreus</td>
<td>3 (2)</td>
</tr>
<tr>
<td>A. sydowii</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>A. candidus</td>
<td>1 (0.7)</td>
</tr>
<tr>
<td>Aspergillus spp.</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>Fusarium spp.</td>
<td>9 (5.8)</td>
</tr>
<tr>
<td>Cladosporium spp.</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>Scopulariopsis</td>
<td>4 (2.6)</td>
</tr>
<tr>
<td>Penicillium</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>Chrysosporium</td>
<td>1 (0.7)</td>
</tr>
<tr>
<td>Trichoderma</td>
<td>1 (0.7)</td>
</tr>
<tr>
<td>Total</td>
<td>154 (100)</td>
</tr>
</tbody>
</table>

### Results

Among the 2292 nail samples, a total of 463 (20.2%) were diagnosed with onychomycosis. Details on clinical sites of infection and causative fungal agent are summarized in Table 1. Among the positive specimens, 154 (33.2%) cases were identified as NDM onychomycosis. Direct microscopic examination showed irregular saprophytic septate hyphae associated with single or grouped conidia in a few samples. Table 2 displays NDMs isolated from nails with onychomycosis in our study. The causative agents in 135/154 cases of NDM onychomycosis (29.2%) of the total number

![Figure 1](Image 303x201 to 541x335)
of onychomycosis cases) were diagnosed as *Aspergillus* spp. Among these, only four cases had a negative direct smear; however, because of dystrophic nail features and growth of identical *Aspergillus* in all triplicate culture inoculums, these four cases were considered as onychomycosis. A total of 35 *Aspergillus* isolates, including 15 non-*flavus* species, were subjected to sequence analysis of the beta tubulin gene, the most commonly used target for molecular species identification of *Aspergillus*. Amplification of the beta tubulin gene by the primer set Bt2a/Bt2b yielded single bands of approximately 550 bp on agarose gel electrophoresis (Fig. 1). The most common *Aspergillus* species isolated from NDM onychomycosis was *Aspergillus flavus* (77.3%, \( n = 119 \)), followed in prevalence by *A. niger* (2.6%, \( n = 4 \)), *Aspergillus tubingensis* (2.6%, \( n = 4 \)), *Aspergillus terreus* (2%, \( n = 3 \)), *Aspergillus sydowii* (1.3%, \( n = 2 \)), *Aspergillus candidus* (0.7%, \( n = 1 \)), and *Aspergillus* spp. (1.3%, \( n = 2 \)). Examples of microscopic features characteristic of each species of *Aspergillus* isolated in the study are shown in Fig. 2. The number of *Aspergillus* isolates in fingernail and toenail lesions were 40 (29.62%) and 95 (70.38%), respectively. Two patients had a mixed infection of *Candida* and *Aspergillus* (one patient had *A. niger* and the other had *A. flavus*). The youngest patient was 19 years old, and the oldest was 87 years old — both of them with their toenails affected — and the overall mean patient age was 47.4 years. Various clinical forms of onychomycosis due to *Aspergillus* species, including distal subungual onychomycosis, lateral subungual onychomycosis, distal and lateral subungual onychomycosis, and total dystrophic onychomycosis were seen and the examples are shown in Fig. 3.

**Discussion**

Recently we have experienced a high number of onychomycosis due to NDM — particularly *Aspergillus* species — in our laboratory. The occurrence of 135 cases of *Aspergillus* nail infection in one year at the two medical mycology laboratories in Tehran is remarkable, and together with previous

![Figure 2](image.png)
studies on onychomycosis in Tehran and in some other parts of the country, our data suggest that *Aspergillus* spp., mainly *A. flavus*, are somewhat commonly involved in NDM onychomycosis in Iran [8, 10, 18, 27].

Unlike almost all studies performed in Iran, in our survey, the frequency of onychomycosis due to yeasts and NDMs exceeds the frequency of nail infections caused by dermatophytes [18]. This high occurrence was similar to that of Scherer et al. who found a shift from dermatophytes to saprophytes as agents of nail infections [33]. A number of risk factors may contribute to this situation, of which "change of lifestyle" may be the most logical explanation, including improvement in the level of hygiene, aging communities, and a decrease in the risk of contracting infections such as tinea unguium. Concordant with this fact, the most recent epidemiological survey of dermatophytosis in Iran showed a decrease in the frequency of nail infection by dermatophytes [1]. Instead, the phenomenon of aging led to an increase in some disorders such as peripheral vascular disease of nails, diabetes, repeated nail trauma, and immune function disorders, which are all noted to be risk factors of nail infections by molds [6, 23]. The high frequency of nail infections due to *A. flavus* can be attributed to the fact that this species is the most frequent *Aspergillus* isolated from the environment in Iran [9] and this species is one of the common pathogenic fungi to humans [19].

Although our study is not a comprehensive epidemiological survey and we did not test all NDMs delivered to our laboratory, these random data demonstrate an increasing occurrence of onychomycosis due to non-dermatophyte molds, especially *Aspergillus* species. The prevalence of NDMs isolated from nail infections in various parts of the world range between 1.49 and 33.5% [6, 28, 29, 38]. According to the data in the present survey, the overall prevalence of NDM onychomycosis is 33.2% with *Aspergillus* spp. being the most common cause of NDM onychomycosis. This result is similar to those reported by Hilmioglupolat et al. [20], Hwang et al. [22], and Zaini et al. [37]; however, our results differ from those reported in some other studies, for example Bonifaz et al. [6] and Khosravi et al. [24], who reported *Scopulariopsis brevicaulis* and Tosti et al. [35] who reported *Fusarium* spp. as the principal etiologic agents. The most
common species of *Aspergillus* identified in our study was *A. flavus*, which is different to what was reported by Ng et al. [29], Gupta et al. [15], and Shoikohi et al. [34], who found *A. niger*, *A. sydowii*, and *A. terreus* to be the most common agents, respectively. In the present investigation, *A. fumigatus*, the infamous cause of invasive *Aspergillus* worldwide, was not isolated from any of the patients, but in some studies, this species was isolated from nail infections [3,13,29,37]. The *Aspergillus* species most frequently isolated from nail infections are *Aspergillus fumigatus*, *Aspergillus tamarii*, *Aspergillus niger*, and *A. flavus* [6], while by application of molecular techniques several rare *Aspergillus* species have been identified from nail infections, including *A. terreus*, *A. alliaceus*, *A. ochraceus*, *A. versicolor*, *A. sydowii*, *A. candidus*, and *A. nomius* [13,39]. In the present study, some uncommon *Aspergillus* species, including *A. sydowii*, *A. tubingensis*, and *A. candidus*, were isolated and identified by beta tubulin sequencing. To our knowledge, onychomycosis due to *A. tubingensis* and *A. sydowii* are here reported from Iran for the first time.

Morphological features are insufficient for precise identification of *Aspergillus* species. For example in Section *Nigri*, *A. brasiliensis*, *A. acidus*, *A. awamori*, *A. niger*, and *A. tubingensis* have similar morphological characteristics, and sequencing of a suitable DNA target is essential for differentiation of such closely related species [21,30,32]. In our study, all black aspergilli were initially reported as *A. niger* based on morphology, but after sequence analysis of beta tubulin genes it was revealed that half of isolates were in fact *A. tubingensis*.

In this study, toenail onychomycosis represented 70.38% of all cases of *Aspergillus* onychomycosis, which is similar to the results reported by Hilmiogluop et al. (67%) [20] and Gianni et al. (67%) [12], but lower than those reported by Bonifaz et al. (96%) [6], Tosti et al. (89.7%) [35], Hwang et al. (84.7%) [22], and higher than the results by Zaini et al. (54.3%) [37] and Hashemi et al. (33.7%) [18]. Bonifaz et al. reported that 50.0% of the onychomycosis patients had predisposing factors such as peripheral vascular disease; however, our patients were not significantly associated with predisposing disease or risk factor, except for being elderly [6] The presence of a black area in nails infected with *A. niger* is common [25]; likewise, onychomycosis due to *A. niger* and *A. tubingensis* were usually associated with black pigmentation in affected nails investigated in the present study (Fig. 3).

The drug choice for treatment of onychomycosis is associated with causative agent, the number of nail involved and the risks and benefits of different treatments [17]. The in vitro activity of terbinafine is more effective than itraconazole against different *Aspergillus* species, however therapeutic choices are very scarce [11].

In conclusion, accurate identification of *Aspergillus* species, which are increasing causes of onychomycosis, is necessary for clarifying the microbial and clinical epidemiology aspects of various causative agents of nail infections. As morphological criteria are insufficient for identification of uncommon *Aspergillus* species, molecular methods could be a suitable complementary approach to compensate for this limitation. In this study, we report a high occurrence of *A. flavus* as an emerging cause of saprophitic onychomycosis in Iran.

**Disclosure of interest**

The authors declare that they have no conflicts of interest concerning this article.

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**References**

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