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Different species of *Aspergillus* involved in ungual pathologies

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Abstract

In recent years non dermatophyte fungi (NDF) are often described as responsible for onychomycosis. The problems related to these fungi concern their identification and treatment. Nowadays a number of* Aspergillus *were observed in this kind of pathologies but the identification at level of species is frequently omitted. In this work we analysed 26 cases of onychomycosis caused by* Aspergillus*, belonging to 8 different species. The identification were based on micro- and macro-morphological characteristics; physiological characters; and molecular analysis (ß-tubulin gene). 3 species are very interesting:* A. persii*, a species recently described;* A.nomius*, never described before in onychomycosis, and* A.melleus*, likely a new opportunistic fungus involved in medical mycology.

Though the identification of these fungi is an additional workload for the laboratory of medical mycology, there is the need to increase the number of identified NDF in order to improve the management of onychomycosis.

Introduction

Onychomycoses are defined as an invasive fungal infection of the nails and represent about the 50% of nail disorders [1]. The estimated prevalence is more than 10% in the general population and reaches 40% in elderly individuals [1]. Usually, diseases are caused by yeasts or, more frequently by a group of keratinophilic fungi, known as dermatophytes. However, in recent years onychomycoses are often caused by non-dermatophyte fungi (NDF) and the percentages of these cases is increasing, accounting for 2% to 22% of all onychomycosis [1,2]. NDF are filamentous fungi, saprotrophs, thermophiles or thermotolerants, usually present in the environment (e.g.: in soil, in organic materials in advanced state of decomposition, in the air).
The NDF isolated from cases of onychomycosis generally belong to the genera *Scopulariopsis* Bainier, *Fusarium* Link, and *Acremonium* Link, with high variable incidence depending on the population and the studied geographic area [1-4]. In addition, thank to an increasing diagnostic effort, in recent years more *Aspergillus* onychomycosis cases have been identified and several *Aspergillus* P. Micheli ex Link species, earlier never or rarely described before as cause of onychomycosis, are now identified as nail infection agents [5-8]. In most cases, fungal identification requires procedures difficult to apply in the routine of hospital laboratories.
The aim of this study is to identify, at the species level, the strains of *Aspergillus* isolated from cases of onychomycosis due to NDF, and to estimate the occurrence of *Aspergillus* in the analyzed cases.

Materials and methods

This study (carried out from October 2006 to February 2010) concerns 44 cases of non-dermatophytic infections of foot fingernails related to 17 males and 27 females whose age ranges from 39 to 75 years. All the considered patients had no significant medical history. Nail fragments scraped and clipped from nail lesions were analyzed. The mycological diagnosis consisted of two stages: the direct examination with 30% potassium hydroxide, and the culture of the nail scrapings on modified Sabouraud medium (1.5% agar, 2% glucose, 1% neopeptone, supplemented with 250 mg/l of cycloheximide and 30 mg/l of chloramphenicol). The direct examination was considered positive when the observation revealed mycological structures (fungal filaments) in the nail specimen.
As concerns the culture, five nail pieces (multiple inoculation techniques) were plated on Petri dishes. These culture plates were incubated at 28°C for 4 weeks and cultures were checked every weeks. According to Gupta *et al.* [9], the culture and direct examination were repeated at least twice, though this protocol presents some problems, such as the need that the patient is observed repeatedly, the related costs, and the overall time spent.
The patients involved in the study met the following criteria: microscopic direct examination of nail scraping

positive for tree times; three repeated positive cultures for the same fungus; absence of other concomitant pathogenic or contaminant fungi.
For macro and micro-morphological fungal identification purposes, the isolated strain was sub-cultured on Czapek Yeast Agar (CYA) at 25°C/37°C, Malt Extract Agar (MEA), CYA20S and Czapek dox (CZ), according to the classical identification protocols for *Aspergillus* genus [10, 11].
After the identification, viable cultures were deposited at the Centraalbureau voor Schimmelcultures, Utrecht, The Nederlands (CBS), and at the Mycotheca Universitatis Taurinensis, Turin, Italy (MUT).
The sequence of β-tubulin gene was obtained in order to definitely identify the fungus. Total fungal genomic DNA was isolated by means of Qiagen DNeasy Plant Mini Kit® (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions. Amplification of β-tubulin gene was performed by using Bt2a and Bt2b primers [12]. PCR reaction was performed in a Biometra T3000 Thermocycler (Biometra biomedizinische Analytik GmbH, Goettingen Germany) programmed as follows: 1 cycle of 3 min denaturation at 95 °C; 35 cycles of 40s denaturation at 94°C, primer annealing 45 s at 55 °C, primer extension 1 min at 72°C, Final 10 min elongation at 72 °C. DNA sequencing was performed by DiNAMYCODE s.r.l. (DiNAMYCODE, Turin, Italy). The sequence of β-tubulin gene was deposited at GenBank.

Results

Altogether, 44 NDF cases of onychomycosis were observed, 26 were caused by *Aspergillus*, belonging to 8 different species. Tab. 1 shows the 8 species identified and their occurrences.

Aspergillus species	Number of cases
Aspergillus sclerotiorum G.A. Huber	16
A. persii Corte & Zotti	3
A. tritici B.S. Mehrotra & M. Basu	2
A. flavus Link	1
A. nomius Kurtzman, B.W. Horn & Hesselt	1
A. melleus Yukawa	1
A. ochraceus K. Wilh.	1
A. terreus Thom	1

Table 1. List of microfungal species involved in onychomycosis. The second column reports the number of occurrences observed during the investigation period.

Discussion

NDF responsible for onychomycosis give rise to certain problems often associated with therapeutic difficulties. The identification of these fungi is longer and more complicated compared to the identification of dermatophytes. Moreover, nail infections due to non dermatophytic fungi often show a lower sensitivity to treatment and a clear attitude to recur [13].
Among NDF, the *Aspergillus* genus accounts for 7 to 100% of cases, according to different studies [1, 3, 14]. The genus seems to reveal an emerging role as agent of onychomycosis and the most frequently identified *Aspergillus* species related to nail infections are: *A. niger* Tiegh., *A. flavus*, *A. terreus*, *A. nidulans* (Eidam) G. Winter, *A. fumigatus* Fresen. and *A. versicolor* (Vuill.) Tirab. [2-4, 14, 15, 16]. Nail infections cases from *A. candidus* Link, *A. ochraceus*, *A. persii*, *A. sclerotiorum*, *A. sydowii* (Bainier & Sartory) Thom & Church, and *A. tamari* Kita have been occasionally observed [5-8, 14, 15].
It is worth noting that the absences of species identification may lead to inappropriate treatments or to misleading evaluations of these pathogens' clinical impact. As a matter of fact, whenever an unusual or a new fungus is involved, a number of difficulties may arise in the correct management of patients.
Although *Aspergillus* is a well known cause of pulmonary and invasive infections, especially in immunocompromised hosts, only a limited number of reports concerns onychomycosis caused by *Aspergillus*: this is probably due to the inherent difficulties in distinguishing a true *Aspergillus* nail infection from the presence of the fungus as a contaminant.
The deep analysis carried out during our activity allowed us to identify pathogenic agents at the level of species as shown in previous Tab. 1. The most interesting species are *A. persii*, *A. melleus* and *A. nomius*. The first one is a new *Aspergillus* species [8, 17] and was found in 3 cases. The other two species, were never described before in onychomycoses. Specifically, *A. nomius* was only observed in a case of mycotic keratitis [18]; *A. melleus* is never cited in the literature devoted to medical mycology: indeed, this species should be considered an emerging opportunistic fungus.
In the future, an important task is surely represented by the evaluation of the antifungal susceptibility for all the species encountered in order to improve the effectiveness of therapeutic actions.

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