A new species, *Aspergillus persii*, as an agent of onychomycosis

MIRCA ZOTTI*, MARCO MACHETTI*, MADDALENA PEROTTI†, GIANFRANCO BARABINO‡ & AGOSTINO PERSI§

*Mycology Laboratory, Department for Territory and its Resources Study (DIP.TE.RIS), Polo Botanico “Hanbury”, University of Genoa, †Interdisciplinary Department for Surgery Sciences, Microbiology and Organ Transplantation (DISCMIT), Section of Microbiology, University of Genoa, ‡U.O. Department of Social Dermatology, San Martino Hospital, Genoa, and §Department of Endocrinology and Medical Sciences (DISEM), University of Genoa, Genoa, Italy

Onychomycosis due to non-dermatophytic fungi is a well-known and increasingly common infection. For the first time ever, we report two cases of onychomycosis caused by *Aspergillus persii*, a recently described new *Aspergillus* species. After ten and three years respectively, both patients showed relapsing nail infections, and mycological tests were still positive for *A. persii*. In vitro antifungal susceptibility tests demonstrated that both strains were only susceptible to itraconazole, voriconazole, posaconazole and terbinafine of the agents tested.

Keywords Onychomycosis, non dermatophyte, new *Aspergillus* species

Introduction

Onychomycoses are nail infections usually caused by dermatophytic fungi, but in recent years many authors have reported an increasing number of cases involving non-dermatophytes [1–4]. Due to this particular aetiology, only a reduced number of the antifungal drugs usually effective with dermatophytic fungi, has a spectrum that includes non-dermatophytic agents of onychomycosis.

*Aspergillus* is a well known cause of pulmonary and invasive infections, especially in immunocompromised hosts. While there is a lower number of reports regarding onychomycosis caused by *Aspergillus*, this might be the result of the inherent difficulties in distinguishing a true *Aspergillus* nail infection from the presence of the fungus as a contaminant. Moreover, the identification of non-dermatophytic fungi recovered from cases of onychomycosis, in particular *Aspergillus* spp., usually stops at the genus level [3–5]. The absences of species identification may lead to inappropriate treatments or to misleading evaluations of these pathogens’ clinical impact. When an unusual or a new fungus is involved, further difficulties may arise in the correct management of cases of onychomycosis.

Case report 1

In 1999, a 61-year-old female with a suspected onychomycosis of the left big toe was examined at the Dermatology Unit of S. Martino Hospital in Genoa. Despite a previous therapeutic onychectomy, the patient had suffered from a nail infection of the left big toe since the age of 40. The dermatological examination showed involvement of the nail lamina of her left big toe, suggestive for a distal lateral subungual onychomycosis. Discolouration was present on the free edge of the nail plate, as well as near the lateral nail fold, where the nail lamina was severely damaged. The invasion of the nail plate was associated with a subungual hyperkeratosis (Fig. 1).

Direct microscopic analysis of the nail revealed fungal filaments and inoculation of portions of the nail on modified Sabouraud medium (1.5% agar, 2% glucose, 1% neopeptone) yielded a yellow mould, with general morphological characters matching those of members of the genus *Aspergillus* P. Micheli ex Link, section *Circumdati*...
Onychomycosis due to *Aspergillus persii* (Fig. 2). The analysis of additional features, especially the small size of conidial heads, vesicles and club-like enlargements, together with the production of secondary metabolites, led to the isolate’s identification as *Aspergillus persii*, a new species described in detail in 2002 [6]. Subcultures of the isolate were deposited at the Mycotheque de l’Université Catholique de Louvain, Louvain-la-Neuve, Belgium (MUCL 41970), at the Centraalbureau voor Schimmelcultures, Utrecht, The Nederlands (CBS 112795), and at the Mycothea Universitatis Taurinensis, Turin, Italy (MUT 3318). The sequence of β-tubulin gene was deposited at GenBank (AY819988) by other authors [7].

The application of nail enamel containing 28% tioconazole was the first therapeutic approach, but it proved to be unsuccessful. Even the results of a second local therapeutic attempt, based on a nail varnish containing 5% amorolfin, were disappointing as well. Finally, we attempted a combination of local and oral therapies. The first step consisted of cleaning the infected portion of the nail lamina by means of a dental milling cutter. After full abrasion up to the nail bed, an ointment containing 1% amphotericin B was applied (since this product was not available in Italy, it was prepared as a galenic formulation). In addition, a daily oral dose of terbinafine (250 mg) was administered for three months. As a result of this combined approach, the patient slowly improved and achieved the clinical recovery in two years.

The patient remained stable for many years throughout follow-up, but probable relapse was noted in November 2008. Microscopic examination in January 2009 of the nail revealed a filamentous fungus and *Aspergillus persii* was again recovered in culture.

**Case report 2**

In June 2006, a 56-year-old male was examined due to a suspected onychomycosis of both the big toes at the same Dermatology Unit that was involved in Case 1. Although the patient did not have diabetes mellitus or any immunological disease disorder, he reported having infections of both big toe nails since 2005. Distal lateral type lesions affected the nail laminas of both toes and the nail plates were dystrophic to the same degree, maybe due to a direct action of the fungus or to minor nail traumas.

Nail samples from both toes were microscopically examined and inoculated on Sabouraud dextrose agar. While the microscopic analysis did not reveal the presence of fungi, *A. persii* was recovered in culture. Since positive cultures were not confirmed by direct microscopic analysis, a subsequent dermatological examination was planned to ascertain the identification of the etiologic agent. However, as the patient was abroad at that time, new examinations could only be performed prior to September 2006. The second direct microscopic examination revealed filamentous fungi and *A. persii* was again found in repeated cultures.

The sequence of the β-tubulin gene was obtained in order to confirm the identification. Total fungal genomic DNA was isolated by means of Qiagen DNeasy Plant Mini...
Aspergillus persii (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Amplification of β-tubulin gene was performed by using Bt2a and Bt2b primers [8]. Amplification 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 40 s denaturation at 94°C, primer annealing 45 s at 55°C, primer extension 1 min at 72°C; – Final 10 min elongation step at 72°C. DNA sequencing was performed by DiNAMICODE s.r.l. (DiNAMICODE, Turin, Italy). Sequence data, submitted to known sequence databases (NCBI/BLASTN algorithm), together with morphological data, confirmed the identification of the etiologic agent as Aspergillus persii.

Living cultures of this second A. persii strain were deposited at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS 124573), and at the Mycotheca Universitatis Taurinensis, Turin, Italy (MUT 4189). The sequence of β-tubulin gene was deposited at GenBank (GQ850380).

The patient was treated with both local and systemic therapy. Local therapy consisted of softening the nail plate with 50% urea and then applying 5% amorolfine enamel. Systemic therapy involved the daily administration of 400 mg of itraconazole for 1 week per month for 6 months (pulsed therapy). After this treatment the patient improved and finally reached clinical recovery.

Follow-up was uneventful until February 2009, when the big toes again showed nail lamina infections. A complete mycological examination was performed and A. persii was isolated again but the patient refused to start another therapeutic attempt.

Antifungal susceptibility testing

The in vitro antifungal drug susceptibility of isolates of A. persii from both cases was investigated by the following two methods:

**Microdilution method**

The broth microdilution method was performed according to CLSI document M38-A2 guidelines [9] using RPMI 1640 medium supplemented with 2% glucose. Inocula suspensions were prepared from 7-day-old cultures grown on potato dextrose agar and adjusted spectrophotometrically to optical densities from 0.09 to 0.17 (68–82% transmittance). The final inocula ranged from 0.5 × 10^4 to 5 × 10^4 CFU/ml, as demonstrated by quantitative colony counts. As recommended, colonies were counted as soon as possible after growth became visible.

The antifungals employed were amphotericin B, itraconazole, fluconazole, voriconazole, posaconazole, caspofungin and terbinafine. Drug concentration ranged from 0.125–256 μg/ml for fluconazole and from 0.008–16 μg/ml for itraconazole, voriconazole, posaconazole, caspofungin and amphotericin B and from 0.03–16 μg/ml for terbinafine.

The inoculated microdilution trays were incubated at 35°C and read after 48 h. The MIC endpoint for the azoles, amphotericin B and terbinafine was defined as the lowest concentration that produced complete inhibition of growth, whereas with fluconazole it was the level at which 50% inhibition was observed. The minimum effective concentration (MEC) endpoint for caspofungin was defined as the lowest concentration at which an abnormal growth was found.

Aspergillus flavus ATCC 204304 and A. fumigatus ATCC 204305 were employed as controls and results were within the recommended limits of the CLSI procedure.

**Sensititre YeastOne® method**

Sensititre YeastOne® panels (Trek Diagnostic Systems) was the second method for evaluation of antifungal susceptibility patterns. Inocula suspensions were prepared once again according to the M38-A2 method, and the suspensions were diluted 1:100 in YeastOne RPMI medium. The dried Sensititre YeastOne panels were rehydrated, 100 μl of the working suspension was added to each well, the plates covered with sealing strips, incubated at 35°C, and read after 48 and 72 h.

Colorimetric MIC were interpreted as the lowest concentration of antifungal solutions at which a change in colour from pink (growth) to blue (no growth) was observed.

**Drug susceptibility test results**

Drug susceptibility test results are summarized in Table 1. Both A. persii strains appeared susceptible in vitro to itraconazole, voriconazole, posaconazole, and terbinafine with only slight differences found between the two methods.

**Discussion**

Non-dermatophytic moulds are an increasing cause of onychomycosis. The incidence of these infections is probably influenced by environmental conditions as case numbers are increasing in tropical and humid climatic regions [1–5,10–12].

As described in different reports, the most common species involved belong to the genera Scopulariopsis, Aspergillus, Acremonium and Fusarium [1,4,12,13]. From a clinical point of view, there is no general agreement about differences in the manifestations of onychomycosis caused by dermatophytes and nondermatophytes [1,4,14]. However, the Aspergillus strains usually show a clear keratinophilic
Onychomycosis due to Aspergillus persii

Suffered from a reinfection or from a new infection in the same nail plate. Other Aspergillus species described as agents of onychomycosis are A. terreus, A. flavus and A. sclerotiorum, belonging to the sections Terrei, Flavi and Circumdati [10,12,13,16–18] respectively. The members of the section Circumdati, which includes A. sclerotiorum and A. persii, typically exhibit predominant biseriate conidial heads with yellow (or buff or ochraceus) colored conidia [19]. In particular, A. persii colonies on Sabouraud medium are pastel to light yellow in colour, with conidial heads loosely columnar to radiate, vesicles 10–25 μm in diameter, conidia globose and smooth 2.5–3 μm in diameter [6].

Activity, which causes partial or total dystrophy of the affected nail [11]. According to the criteria provided by Gupta et al. in 2001, positive cultures from repeated nail examinations are required to confirm the diagnosis of nondermatophytic onychomycosis [15].

Both our patients showed, to a variable degree, dystrophy of the affected nail plates and A. persii was repeatedly isolated in pure culture. Therefore we believe that the data clearly indicates that this fungus was responsible for the nail infections observed in our two patients. This conclusion was further supported by the recovery of the same fungus from relapse nail infections in the patients after three and ten years. Since the fungus is not a common pathogen, it is difficult to understand if our patients suffered from a reinfection or from a new infection in the same nail plate.

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Table 1 Aspergillus persii drug susceptibility test results obtained with broth microdilution method (CLSI document M38-A2) and with Sensititre YeastOne® method.

<table>
<thead>
<tr>
<th>MIC (μl/ml)</th>
<th>M38A2</th>
<th>YeastOne</th>
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<tbody>
<tr>
<td>AmB</td>
<td>FZ</td>
<td>IZ</td>
</tr>
<tr>
<td>Case 1</td>
<td>8/8</td>
<td>&gt;256/256</td>
</tr>
<tr>
<td>Case 2</td>
<td>16/8</td>
<td>&gt;256/256</td>
</tr>
</tbody>
</table>

*MIC (μl/ml) M38A2/MIC (μl/ml) YeastOne.
AmB, amphotericin B; FZ, fluconazole, IZ, itraconazole; VZ, voriconazole; POS, posaconazole; CAS, caspofungin; TERB, terbinafine.

Fig. 3 Conidial heads and septate club-like enlargements of Aspergillus persii from culture on MEA 25°C medium. (A and B) Conidial heads. Magnification ×10 and ×100, respectively. (C) Conidial head and septate club-like enlargements. Magnification ×100. (D) Detail of the septate club-like enlargements. Magnification ×100. Black arrows in Fig. 3C and D indicate the septate club-like enlargements.
Therefore, on a strictly morphological basis, *A. terreus* and *A. flavus* are easily distinguishable from *A. persii*. Indeed, when cultured on Sabouraud medium, colonies of *A. terreus* are in the brownish orange to camel colour range with compact columnar conidial heads and very small and smooth conidia (2–2.5 μm in diameter). In contrast, *A. flavus* colonies are olive yellow to olive green, with smooth to finely roughened conidia (3–6 μm in diameter).

In contrast, *A. persii* is far more difficult to distinguish from *A. sclerotiorum* on the basis of morphologic characters as the key features of *A. persii* are only evident on certain substrates when grown at specific temperatures. Unlike *A. sclerotiorum*, *A. persii* scarcely grows on Czapek dox agar (Cz) not even after ten days as opposed to its better development on Czapek Yeast Agar (CYA) at 37°C, with abundant conidiogenesis similar to those obtained on CYA at 25°C. Moreover, *A. persii* does not show exudates on CYA at 25°C and the colonies reverse is coloured in red brown or orange brown on Cz, CYA and Malt Extract Agar (MEA).

Finally, the presence of septate club-like enlargements on hyphae [6] seems to be the most discriminating morphological character and was found with both strains. The occurrence of these enlargements on all culture media suggests they are typical of the species and not a mere morphological alteration of an opportunistic fungus (Fig. 3).

It is possible to distinguish *A. persii* from *A. sclerotiorum* by means of DNA analysis, as reported by Frisvad et al. [7] and Peterson [20]. The use of molecular analysis meets the polyphasic approach (molecular, morphological, physiological and ecological data) recommended by Samson et al. [21] in order to describe a new *Aspergillus* species.

In conclusion, our case reports are the first clinical descriptions of onychomycosis caused by *A. persii*. The fungi had previously been isolated and described for the first time just from the first clinical case [6,16].

Since the identification of the etiologic agents of onychomycosis is generally only to the generic level, it is quite possible that other cases ascribed to yellow-colonies *Aspergillus* spp., might have actually been caused by *A. persii* [3–5]. As regards the antifungal susceptibility, the isolates were susceptible in *vitro* to itraconazole, voriconazole, posaconazole and terbinafine.

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**References**