Invasive aspergillosis caused by cryptic Aspergillus species: a report of two consecutive episodes in a patient with leukaemia

Teresa Peláez,1,2,3,4 Sergio Álvarez-Pérez,1,2 Emilia Mellado,5 David Serrano,2,6 Maricela Valerio,1,2 José L. Blanco,7 Marta E. García,7 Patricia Muñoz,1,2,3,4 Manuel Cuenca-Estrella5 and Emilio Bouza1,2,3,4

1Servicio de Microbiología Clínica y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, Madrid, Spain
2Instituto de Investigación Sanitaria del Hospital Gregorio Marañón, Madrid, Spain
3Departamento de Medicina, Facultad de Medicina, Universidad Complutense, Madrid, Spain
4CIBER de Enfermedades Respiratorias (CIBERES CD06/06/0058), Palma de Mallorca, Spain
5Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
6Servicio de Hematología, Hospital General Universitario Gregorio Marañón, Madrid, Spain
7Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain

We report a case of two consecutive episodes of invasive aspergillosis caused by cryptic Aspergillus species in a patient with leukaemia. A first episode of pulmonary infection was caused by Aspergillus calidoustus and Aspergillus novofumigatus, and the second episode by A. novofumigatus and Aspergillus viridinutans. Fungal isolates were identified to species level using traditional and sequencing-based molecular methods.

Introduction

Invasive aspergillosis (IA) is an opportunistic fungal infection that has become a major cause for concern owing to its high morbidity and mortality rates in a variety of immunocompromised patients and the rapid expansion of populations at risk (Maertens et al., 2002). Development and outcome of IA are largely determined by the underlying disease, and IA can be particularly devastating in haematopoietic stem cell recipients with graft-versus-host disease (GVHD). Nevertheless, fungus-related factors, such as species identity and resistance of isolates to antifungal therapy, can also play a critical role in pathogenesis (Denning, 1998; Marr et al., 2002).

The versatile and ubiquitous fungus Aspergillus fumigatus has traditionally been considered the main aetiological agent of IA in humans (Denning, 1998; Marr et al., 2002; Muñoz et al., 2006). However, recent findings have demonstrated that other fungal organisms previously identified as A. fumigatus on the basis of standard mycological procedures actually belong to different – yet sometimes closely related – genospecies within the Aspergillus subgenus Fumigati, section Fumigati (Balajee et al., 2005a, b, 2006, 2007; Hong et al., 2005; Katz et al., 2005; Balajee & Marr, 2006; Yaguchi et al., 2007). Correct identification of these cryptic Aspergillus species is important, as they can differ in some clinically relevant characteristics, including virulence and antifungal resistance profile (Balajee et al., 2005a, 2006; Alcazar-Fuoli et al., 2008; Alhambra et al., 2008; Montenegro et al., 2009; Sugui et al., 2010; Van Der Linden et al., 2011).

Apart from A. fumigatus, several species within Aspergillus section Fumigati have been implicated in different human infections, namely, Aspergillus lentulus (Alhambra et al., 2008; Montenegro et al., 2009), Aspergillus viridinutans (Vinh et al., 2009a; Coelho et al., 2011), Neosartorya fischeri (Lonial et al., 1997), Neosartorya pseudoafricae (Padhye et al., 2007), and Neosartorya spinosa (Summerbell et al., 1992), Neosartorya hiratsukae (Guarro et al., 2002; Koutroutsos et al., 2010) and Neosartorya udagawae (Vinh et al., 2009b; Posteraro et al., 2011). Different Aspergillus species seem to be simultaneously involved in several such infections.
(Alhambra et al., 2008; Montenegro et al., 2009). Nevertheless, the pathogenic potential of most *A. fumigatus*-like species in single infections and/or co-infections remains virtually unknown.

In the present article, we report a double episode of IA in a patient with leukaemia, in which a first infection caused by *Aspergillus calidoustus* (*Aspergillus* section Usti) and *Aspergillus novofumigatus* (*section Fumigati*) was followed by a second infection caused by *A. novofumigatus* and *A. viridinutans* (*section Fumigati*). Isolates recovered from clinical samples showed resistance to someazole antifungals commonly used for the treatment of IA. Furthermore, to the best of our knowledge, this seems to be the first ever reported isolation of *A. novofumigatus* from a patient with IA.

**Case report**

A 48-year-old Caucasian woman was diagnosed with high-risk B-lineage acute lymphoid leukaemia in April 2005. She achieved complete remission after a standard 4-week cycle of chemotherapy followed by consolidation and maintenance therapy until January 2008. In November 2008, she experienced a recurrence. Rescue treatment with hyper-CVAD was prescribed, and she reached a second complete remission.

In June 2009, the patient underwent a haploidentical allogeneic transplant of cord blood cells and CD34+ cells, depleted from lymphocytes, from a sibling donor. Neutrophil engraftment occurred (>500 cells mm⁻³ on day +12 after transplantation), and complete cord cell chimerism was achieved on day +54.

At day 30 after transplantation, the patient developed interstitial pneumonitis. The thoracic computed tomography (CT) image showed patchy ground glass infiltrates distributed throughout the lungs. Bronchoscopy and bronchoalveolar lavage (BAL) were performed. Histopathology revealed non-specific alveolar and interstitial damage. Screening of BAL and nasopharyngeal samples for respiratory viruses detected parainfluenza. The results of calcofluor white staining, PCR with *Aspergillus*-specific primers and mycological culture of BAL fluid were negative. Nevertheless, the *Aspergillus* antigen was detected in serum using enzyme immunoassay (Platelia Aspergillus, Bio-Rad; 0.97–1.24 ng ml⁻¹) and BAL (0.9 ng ml⁻¹). Treatment was started with oral ribavirin (1 g per day) and liposomal amphotericin B (3 mg kg⁻¹ per day) for 20 days, followed by voriconazole and caspofungin (4 mg kg⁻¹ every 12 h and 50 mg kg⁻¹ per day, respectively) for 16 days, although the patient’s condition did not improve.

A second bronchoscopy with BAL was performed on day 50 after transplantation, yielding results similar to those of the previous analyses: non-specific alveolar and interstitial damage, persistence of parainfluenza infection and negative results in mycological tests (culture, calcofluor white staining, *Aspergillus* antigenaemia and specific PCR). Consequently, a diagnosis of cryptogenic organizing pneumonia (COP) was proposed. Corticosteroids were started (methylprednisolone, 125 mg per day, for 5 days), and both the clinical and the radiological response were excellent.

Corticosteroids were progressively withdrawn. Three months after transplantation, the respiratory process reactivated, and corticosteroids were once again prescribed. Cyclosporin continued to be administered for prophylaxis of GVHD, with voriconazole (4 mg kg⁻¹ every 12 h), acyclovir and quinolones being concomitantly used for microbiological prophylaxis.

In the sixth month post-transplantation (December 2009), after a further reduction of the steroid therapy, the patient presented a new episode of COP, with dyspnoea on minimal exertion and cough. Thoracic CT showed multiple nodular infiltrates in both lungs, and bronchiectasis and patchy ground glass infiltrates, mostly in the upper and middle lobes of the right lung. Voriconazole was discontinued owing to elevated liver enzyme levels. Treatment was started with liposomal amphotericin B (3 mg kg⁻¹ per day), but it was discontinued and replaced with caspofungin (50 mg kg⁻¹ per day) due to decreased creatinine clearance.

In February 2010, the patient’s condition improved slightly, although immune recovery was still poor (CD3⁺ <10 cells mm⁻³, CD19⁺=360 cells mm⁻³, NK=109 cells mm⁻³, IgG=160 mg dl⁻¹ and IgM <7 mg dl⁻¹). *Aspergillus* antigen in serum reached 2.6 ng ml⁻¹. A third bronchoscopy and BAL were performed. *Aspergillus* antigen in BAL fluid was 8.2 ng ml⁻¹. Calcofluor white staining was positive, and mycological cultures yielded several colonies of *Aspergillus* identified as *A. calidoustus* and *A. novofumigatus* by partial sequencing of the β-tubulin (*benA*) gene and some additional physiological tests (see Methods). Antifungal susceptibility testing was performed using standard procedures (see Methods). The *A. calidoustus* and *A. novofumigatus* isolates showed high MIC values for itraconazole, posaconazole and voriconazole, while both isolates exhibited lower MIC values for amphotericin B and candins (Table 1). Histopathology of a pulmonary biopsy specimen revealed respiratory mucosa with minimal lung parenchyma and no granulomas. In view of these results, the patient was prescribed micafungin (100 mg per day, duration 20 days).

In March 2010, the patient was admitted to hospital with fever and mild respiratory symptoms. A new thoracic and cranial CT revealed pansinusitis with air-fluid levels in both the maxillary and the sphenoid sinus. Thoracic CT images revealed areas of consolidation on the upper left lobe and right apex, as well as multiple bilateral infiltrates. The serum *Aspergillus* antigen level was 7.4 ng ml⁻¹. Calcofluor white staining of sputum isolates was again positive. Mycological cultures of sputum and BAL samples were positive for slow-growing *Aspergillus* species. Partial *benA* gene sequencing identified the isolates as *A.
Table 1. Antifungal susceptibility (MICs or MECs, in µg ml⁻¹) of the three different *Aspergillus* species isolated

<table>
<thead>
<tr>
<th>Species (n)*</th>
<th>AMB</th>
<th>AND</th>
<th>CAS</th>
<th>MYC</th>
<th>ITZ</th>
<th>POS</th>
<th>VOR</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. calidoustus</em> (1)</td>
<td>2</td>
<td>0.015</td>
<td>0.125</td>
<td>0.062</td>
<td>8</td>
<td>&gt;16</td>
<td>4</td>
<td>YES</td>
</tr>
<tr>
<td><em>A. novofumigatus</em> (7)</td>
<td>0.5</td>
<td>&lt;0.03</td>
<td>0.25</td>
<td>&lt;0.03</td>
<td>&gt;8</td>
<td>1</td>
<td>8</td>
<td>YES</td>
</tr>
<tr>
<td><em>A. viridinutans</em> (3)</td>
<td>1</td>
<td>&lt;0.03</td>
<td>1</td>
<td>&lt;0.03</td>
<td>1</td>
<td>0.5</td>
<td>4</td>
<td>YES</td>
</tr>
</tbody>
</table>

*Number of isolates tested.

**novofumigatus** and *A. viridinutans*. *A. calidoustus* was no longer recovered. The antifungal susceptibility profile of the isolates is shown in Table 1. Due to the patient’s critical condition, therapy with caspofungin and liposomal amphotericin B (50 mg kg⁻¹ per day and 3 mg kg⁻¹ per day, respectively) was reintroduced before the results of molecular identification and antifungal susceptibility were known. Three days later, the patient’s respiratory condition worsened as a result of progression of pulmonary infiltrates, and progressive desaturation was observed. The patient died of cardiac-respiratory arrest. No autopsy was granted.

The aforementioned clinical data suggested a picture of corticosteroid-sensitive post-transplantation COP. The need for prolonged immunosuppressive therapy and the poor immune reconstitution rendered the patient susceptible to subsequent episodes of invasive fungal infection, of which she eventually died.

**Methods**

**Mycolological procedures.** Mycolological culture of sputum and BAL samples was performed on Sabouraud-chloramphenicol medium (Oxoid). The incubation temperature was 37 °C. Fungal isolates were macro- and microscopically examined according to standard criteria (de Hoog et al., 2000). Isolates and their respective origins are listed in Table 2.

Additionally, to confirm the identification of the *A. calidoustus* isolate (HGU09-I4055), we assessed its growth characteristics at 37 °C on Czapek yeast extract agar (CZA; Difco) and its ability to produce indole. These assays were performed as described by Hageskal et al. (2011), but using Kovacs' instead of Ehrlich’s reagent to test for indole. These assays were performed as described by Hageskal et al. (2011), but using Kovacs' instead of Ehrlich’s reagent to test for indole.

**Molecular identification of fungal isolates.** DNA extraction from *Aspergillus* isolates was performed as described previously (Alvarez-Perez et al., 2010a, b). PCR mixtures for *benA* gene amplification contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 20 pmol each forward primer (5ʹ-AATTGTCTGCAGCTTTGCTG-3ʹ) and reverse primer (5ʹ-AGTTGTCGGGACGACAGATAG-3ʹ) (Isogen Life Science) (Balajee et al., 2005a, b), 200 µM each dNTP, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 2 µl DNA extract. The final volume was adjusted to 50 µl with MilliQ sterilized water (Millipore). Amplification was carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) and consisted of a denaturation step of 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 68 °C, and a final extension at 68 °C for 5 min. PCR amplicons were purified with the QIAquick PCR Purification kit (Qiagen Iberia), according to the manufacturer’s instructions. Both strands were sequenced (using the same primer sets as in PCR amplifications) with the ABI Prism Big Dye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems) and analysed on an ABI Prism 3730 sequencer (Applied Biosystems). The sequences obtained were compared with reference sequences from GenBank using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast).

**Antifungal susceptibility testing.** Antifungal susceptibility testing was performed using two broth microdilution methods: EUCAST (2008) and that of the Clinical and Laboratory Standards Institute (CLSI, 2008). The antifungal agents tested were amphotericin B (Sigma-Aldrich), itraconazole (Janssen Pharmaceutical), voriconazole (Pfizer), posaconazole (Merck), caspofungin (Merck), miconafungin (Astellas) and anidulafungin (Pfizer). Susceptibility tests were performed at least three times on different days with each strain. *Aspergillus flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains.

**Discussion**

In the past, it was generally accepted, sometimes without significant mycological or clinical evidence, that most cases of IA were caused by *Aspergillus* isolates susceptible to azoles. However, acquired resistance to azoles has been described for *A. fumigatus* (Snelders et al., 2008; Verweij et al., 2009; Howard & Arendrup, 2011), and reports of aspergillosis caused by non-*fumigatus* *Aspergillus* species from different sections showing reduced susceptibility to azoles have multiplied in recent years (Pavie et al., 2005; Panackal et al., 2006; Varga et al., 2008; Vinh et al., 2009a, b; Coelho et al., 2011; Posteraro et al., 2011). Therefore, established dogma on the aetiology and management of IA should be revised. Data from clinical reports could prove invaluable for such a task.

In this article, we present what we believe to be the first description of two consecutive episodes of IA caused by different cryptic species of *Aspergillus*. The first of these episodes was caused by two species belonging to different sections, namely *A. calidoustus* (section *Ustil*) and *A. novofumigatus* (section *Fumigati*), while the second episode was caused by two *Aspergillus* section *Fumigati* members (*A. novofumigatus* and *A. viridinutans*). The relevance of this case is also highlighted by the fact that all three *Aspergillus* species implicated showed decreased susceptibility to the azoles commonly used for the treatment of IA.

*A. calidoustus* is as an emerging mould pathogen (Hageskal et al., 2011). This species is usually confounded with *A. ustus*, and isolates typically show resistance to multiple antifungal drugs (Varga et al., 2008; Alastruey-Izquierdo et al., 2010). *A. viridinutans* was originally isolated in 1954 from rabbit dung (McLennan et al., 1954), but it was later reported in retrospective analyses of culture collections.
(Katz et al., 2005; Yaguchi et al., 2007; Alcazar-Fuoli et al., 2008) and implicated in clinical cases of chronic IA (Vinh et al., 2009a; Coelho et al., 2011). The more recently described *A. novofumigatus* (Hong et al., 2005) has not been implicated in clinical infections (Balajee & Klaassen, 2009). In fact, to our knowledge, the present work is the first to report the isolation of this *A. fumigatus*-like species from clinical samples.

In conclusion, *A. novofumigatus*, *A. viridinutans* and other *A. fumigatus*-like species can cause refractory infections in immunocompromised patients. Multicentre studies assessing the incidence of these ‘usual’ species as causal agents of IA, as well as their antifungal resistance profiles and other clinically relevant traits, should be a priority for investigation.

### Acknowledgements

We thank Thomas O’Boyle for editing and proofreading the article, and Beatriz Gama for invaluable technical support. We also appreciate the helpful comments of two anonymous reviewers, which greatly improved the quality of this article. S.A.-P. is supported by a Sara Borrell contract from the Instituto de Salud Carlos III, and co-financed by the European Development Regional Fund ‘A way to build Europe’ and the Spanish Network for Research in Infectious Diseases (REIPI RD06/0008).

### References


Table 2. *Aspergillus* isolates recovered and GenBank accession numbers of partial *benA* sequences

<table>
<thead>
<tr>
<th>Episode</th>
<th>Date</th>
<th>Fungal species</th>
<th>Isolate</th>
<th>Origin</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>11 February 2010</td>
<td><em>A. calidoustus</em></td>
<td>HGUGM-14055</td>
<td>Sputum</td>
<td>JX888460</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. novofumigatus</em></td>
<td>HGUGM-14028</td>
<td>Sputum</td>
<td>HQ127280</td>
</tr>
<tr>
<td>Second</td>
<td>5 March 2010</td>
<td><em>A. novofumigatus</em></td>
<td>HGUGM-14158</td>
<td>Sputum</td>
<td>HQ127264</td>
</tr>
<tr>
<td></td>
<td>8 March 2010</td>
<td><em>A. novofumigatus</em></td>
<td>HGUGM-14159</td>
<td>Sputum</td>
<td>HQ127265</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. novofumigatus</em></td>
<td>HGUGM-14164</td>
<td>Sputum</td>
<td>HQ127263</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. novofumigatus</em></td>
<td>HGUGM-14165</td>
<td>Sputum</td>
<td>HQ127273</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. viridinutans</em></td>
<td>HGUGM-14219</td>
<td>BAL</td>
<td>HQ127274</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. viridinutans</em></td>
<td>HGUGM-14162</td>
<td>Sputum</td>
<td>HQ127259</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. viridinutans</em></td>
<td>HGUGM-14163</td>
<td>Sputum</td>
<td>HQ127257</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. viridinutans</em></td>
<td>HGUGM-14220</td>
<td>BAL</td>
<td>HQ127256</td>
</tr>
<tr>
<td></td>
<td>13 March 2010</td>
<td><em>A. novofumigatus</em></td>
<td>HGUGM-14222</td>
<td>Sputum</td>
<td>HQ127271</td>
</tr>
</tbody>
</table>


