Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of Aspergillus felis as Aspergillus viridinutans

Comparison of nucleic acid sequences is considered the new ‘gold standard’ for species identification in most fungal groups (Summerbell et al., 2005; Balajee et al., 2009). However, comparative sequence-based identification is still an evolving area of research, fuelled by the discovery of new fungal species and the constant addition of nucleotide sequences to a growing number of databases (Balajee et al., 2009).

We previously reported a case of two consecutive episodes of invasive aspergillosis (IA) in a patient with leukaemia, in which a first infection caused by Aspergillus calidoustus and Aspergillus nofumigatus was followed by a second infection caused by A. nofumigatus and Aspergillus viridinutans (Peláez et al., 2013). Fungal isolates were identified to the species level using a combination of traditional and molecular-based methods, and antifungal susceptibility testing revealed that these isolates were resistant to some azole drugs commonly used for the treatment of IA (Peláez et al., 2013).

More recently, Barrs et al. (2013) described Aspergillus felis as a novel heterothallic Aspergillus species belonging to the section Fumigati of that genus. To date, this new Aspergillus species has been isolated from domestic cats with invasive fungal rhinosinusitis, a dog with disseminated IA and some human patients with chronic invasive pulmonary aspergillosis (Barrs et al., 2013). Although the fungal-related disease in all these host species seemed to be refractory to aggressive antifungal therapeutic regimens (Barrs et al., 2013), the actual prevalence and clinical importance of A. felis infection remains to be elucidated.

Notably, A. felis seems to be closely related to A. viridinutans (Barrs et al., 2013) and, therefore, there is a risk for possible misidentification between these sibling species. In fact, on the basis of partial β-tubulin sequences, Barrs et al. (2013) suggested that one of the A. viridinutans isolates from our previously reported case of IA should be identified instead as A. felis, but a thorough phenotypic and genetic characterization of such an isolate was not attempted. In view of that observation, we considered it appropriate to reassess the taxonomic status of all the A. viridinutans isolates retrieved from our leukaemic patient with IA, as detailed below.

Isolates HGUGM 14162, HGUGM 14163 (=GM 02/39) and HGUGM 14220 were included in this study. These three isolates were grown on Sabouraud dextrose agar (SDA; Pronadisa), Czapek agar (Difco) and Czapek yeast autolysate agar (Difco) at 37 °C for morphological characterization. In all cases, fungal colonies grew rapidly (diameter of 6.2–8.7 cm in 7 days), but sporulated poorly. The scarce conidiophores were uniseriate with greenish stipes and subclavate, ‘nodding’ heads, a characteristic that is shared by A. felis and A. viridinutans (Barrs et al., 2013). Nevertheless, the studied isolates were able to grow to a diameter of 0.3–0.7 cm in 7 days on SDA plates incubated at 45 °C, a trait that can be used to rule out A. viridinutans (Barrs et al., 2013). None of the studied isolates showed growth on SDA after a 7-day incubation at 50 °C, also agreeing with the description of A. felis (Barrs et al., 2013).

To confirm the identification of our isolates as A. felis, we amplified and determined the sequences of the internal transcribed spacer (ITS) regions (comprising ITS-1, ITS-2 and the 5.8S rRNA gene), and parts of the β-tubulin (benA) and calmodulin (calM) genes, and the locus encoding the high-mobility group domain of the MAT1-2 gene. Partial benA sequences were taken from our previous study (Peláez et al., 2013), whilst ITS, calM and MAT1-2 sequences were obtained by following the same basic protocol as for benA but using primer pairs ITS-1 and ITS-4 (White et al., 1990), cmd5 and cmd6 (Hong et al., 2005) and MAT2_F6086 and MAT2_R6580 (Barrs et al., 2013), respectively. Similar PCRs using degenerate primers targeting the α-domain-encoding sequence from the MAT1-1 gene family (AFM1_F65655 and MAT1_R6215; Barrs et al., 2013) resulted in no amplification, thus confirming that the three studied isolates belonged to the MAT1-2 mating type. The nucleotide sequences obtained were then compared to those corresponding to the type strain of A. felis (CBS 130245T) through the use of BLAST analysis (http://www.ncbi.nlm.nih.gov/blast). In all cases, query sequences were ≥98.9% identical to A. felis CBS 130245T (Table 1). Sequence identity to the type strain of A. viridinutans (CBS 127.56T) was 99.1, 92.6 and 95.6% for the ITS region, benA and calM genes, respectively. GenBank search results using query MAT1-2 sequences did not show any significant alignment with A. viridinutans. Although consensus cut-off values for species definition on the basis of percentages of sequence identity are not yet available, these results strongly suggest that the studied isolates had indeed been misidentified as A. viridinutans in our previous case report.

Additionally, the partial benA and calM sequences of our clinical isolates and those of reference strains of A. felis, A. viridinutans and other closely related species were imported into MEGA5 software (Tamura et al., 2011). CLUSTAL W alignments and maximum-parsimony trees were subsequently created using the same parameters as described by Barrs et al. (2013). The results of these phylogenetic analyses, which are shown in Fig. S1 (available in the online Supplementary Material), confirmed the high-level relatedness of the studied clinical isolates and the type strain of A. felis.
Finally, the antifungal susceptibility data available for our isolates were reanalysed. As with most A. felis strains identified to date, the studied isolates showed high MICs to voriconazole (VOR; see Table 1 in Peláez et al., 2013). They also had high MICs to caspofungin, but not to amphotericin B, itraconazole (ITZ), posaconazole (POS), andilafungin and micafungin (Peláez et al., 2013). Nevertheless, A. felis isolates displaying cross-resistance to ITZ/VOR or ITZ/VOR/POS have also been detected (Barrs et al., 2013).

The taxonomy of the A. novofumigatus isolates obtained from the same case of IA was not brought into question, as GenBank searches of partial benA sequences suggested that these had not been misidentified (≥99.6% sequence identity to A. novofumigatus CBS 117520T, but <96.4% identity to the type strains of other species). Similarly, the single A. calidoustus isolate recovered during the first episode of IA in the leukaemic patient showed 99.3% sequence identity to A. calidoustus CBS 121601T for the benA gene, and was clearly different from Aspergillus ustus and other sibling species on the basis of nucleotide sequence data and the analysis of some key phenotypic traits (Peláez et al., 2013).

In conclusion, the multilocus sequence analysis scheme and complementary phenotypic tests used in this study confirmed that the isolates recovered from our previously reported case of IA in a leukaemic patient actually belong to the species A. felis and not to A. viridinutans. Future work should aim to assess the prevalence of A. felis as an agent of IA in different medical institutions and patient populations.

Furthermore, as data from clinical reports of fungal infections could prove invaluable for determining their exact aetiology and best management practices, we encourage other authors to reassess the identity of relevant isolates from their previously published cases in the face of the current concepts and methodologies in fungal taxonomy.

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Abbreviations: IA, invasive aspergillosis; ITS, internal transcribed spacer; ITZ, itraconazole; POS, posaconazole; VOR, voriconazole.

Table 1. Relevant pheno- and genotypic characteristics of the three A. felis isolates from our previous report of IA in a leukaemic patient

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Isolate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HGUGM 14162 (=CECT 20860)</td>
</tr>
<tr>
<td>Origin</td>
<td>Sputum</td>
</tr>
<tr>
<td>Nucleotide sequence identity to A. felis CBS 130245T</td>
<td>99.2% (KF652062)</td>
</tr>
<tr>
<td>ITS</td>
<td></td>
</tr>
<tr>
<td>benA</td>
<td>99.0% (HQ127259)</td>
</tr>
<tr>
<td>calM</td>
<td>99.4% (KF652065)</td>
</tr>
<tr>
<td>MAT1-2</td>
<td>98.9% (KF652068)</td>
</tr>
<tr>
<td>Growth on SDA at:</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>+</td>
</tr>
<tr>
<td>45°C</td>
<td>w</td>
</tr>
<tr>
<td>50°C</td>
<td>–</td>
</tr>
</tbody>
</table>

*The isolates have been deposited in the Spanish Type Culture Collection (GenBank accession nos are given in parentheses).
The GenBank/EMBL/DDBJ accession numbers for the ITS, benA, calM and MAT1-2 sequences of Aspergillus felis are KF652062–KF652064; HQ127256, HQ127257 and HQ127259; KF652065–KF652067; and KF652068–KF652070, respectively.

One supplementary figure is available with the online version of this paper.


