Case report

Resistance to voriconazole due to a G448S substitution in *Aspergillus fumigatus* in a patient with cerebral aspergillosis.

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Running title: Voriconazole resistance in cerebral aspergillosis

Key words: Cerebral Aspergillosis; *Aspergillus fumigatus*; voriconazole resistance; G448S Cyp51A amino acid substitution.
ABSTRACT
A voriconazole-resistant isolate of *Aspergillus fumigatus* was recovered from an immunocompetent patient receiving long-term antifungal therapy for cerebral aspergillosis. A G448S amino acid substitution in the azole target (Cyp51A) was identified as the cause accounting for the resistant phenotype. This article describes the first isolation of a voriconazole-resistant *A. fumigatus* isolate from an immunocompetent patient in Spain.

CASE REPORT
A 45-year-old woman was referred to Hospital General Universitario Gregorio Marañón [HGUGM], Madrid, Spain) in July 2011 with recurrent brain abscess. She was immunocompetent, with no comorbid conditions, and had presented with chronic otitis media with cholesteatoma. Two years before (in 2009) the patient had undergone open tympanoplasty, which was unsuccessful and she required radical mastoidectomy. In November 2010, the patient presented several post-surgical complications, namely, persistent otorrhea, gradual facial palsy, and an abscess in the right temporal lobe that was successfully removed. Culturing of the resected tissue yielded a fungal isolate identified as *Aspergillus fumigatus* by standard mycological procedures and molecular methods (see below). A treatment with voriconazole 200 mg bid (4 mg/kg) was thus initiated, and monitoring of serum levels for eight weeks revealed good tolerance and apparent efficacy of the antifungal. However, two months later, the patient experienced continuous and intense earache. Magnetic resonance imaging (MRI) revealed a new abscess at the cerebellopontine angle and signs of petrosal bone osteomyelitis. The abscess was drained and a petrosectomy was performed. Resection was extensive, with cochleostomy and removal of granulation tissue and the whole facial nerve, whose second and third portions were completely infiltrated by the fungus. *A. fumigatus* was again isolated from mucus and bone biopsy specimens. Treatment with voriconazole was continued, but the patient’s condition deteriorated. Eventually, voriconazole was replaced with liposomal amphotericin B.
Upon admission to the HGUGM (in July 2011) the patient underwent again petrosectomy. Aspergillomas were removed from cerebral tissue, and biopsy specimens yielded once more *A. fumigatus*. Two months later, the patient was still receiving liposomal amphotericin B (3 mg/kg/day) with no further complications. She had no apparent symptoms and the lesions had disappeared on MRI.

Mycological procedures

Culturing of clinical samples was performed on Sabouraud-chloramphenicol medium (Oxoid, Madrid, Spain). The incubation temperature was 37°C. Fungal isolates were macro- and microscopically examined according to standard procedures.

The *A. fumigatus* isolate recovered from biopsy samples taken at the HGUGM after the second petrosectomy (see above), which was named TP1362, was used in subsequent detailed phenotypic and molecular studies. Two *A. fumigatus* strains from a previous study (21) were used as a control throughout this work [CM-237 (azole-susceptible), and CR019 (multiazole-resistant)].

Molecular characterization of isolates

Conidia from each of the aforementioned strains were inoculated into 3 ml of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and grown overnight at 37°C, after which mycelium mats were harvested and DNA was extracted as previously described (9). Species-specific identification was performed by partial PCR amplification and subsequent sequencing of the β-tubulin gene, as described elsewhere (1). After confirming the identity of isolate TP1362 as *A. fumigatus*, the full coding sequences of the *cyp51B* and *cyp51A* genes, including the *cyp51A* promoter, were amplified using the PCR conditions described in Mellado et al. (21). To rule out the possibility that any sequence changes identified were due to PCR-induced errors, each isolate was independently analyzed twice. No differences were found between the *cyp51B* sequences of the three strains. However, the *cyp51A* gen from isolate TP1362 carried a g1413a mutation that was responsible for a G448S amino acid
substitution. No further changes were found in the sequence of the remainder of the cyp51A gene or in the promoter region.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed using the broth microdilution method described in the EUCAST Technical Note (2008) (30) and the E-test, following the manufacturer’s recommendations. The results of these assays are shown in Table 1, and Table 2 and Figure 1, respectively. The antifungal agents tested were: itraconazole (Janssen Pharmaceutical S.A., Madrid, Spain), voriconazole (Pfizer, S.A., Madrid, Spain), posaconazole (Schering Plough, Madrid, Spain), and liposomal amphotericin B (Sigma, Madrid, Spain). Susceptibility profiles were determined at least three times for each isolate on different days. Aspergillus flavus ATCC 204304 and A. fumigatus ATCC 204305 were used as quality control strains. In vitro susceptibility and resistance were defined according to the epidemiological cut-off values (ECVs) recently published for A. fumigatus. For itraconazole and voriconazole isolates with MIC values ≤1 µg/mL were considered as belonging to wild-type populations; for posaconazole this value was ≤0.25 µg/mL (10, 26).

DISCUSSION

Invasive aspergillosis (IA) is an important cause of morbidity and mortality and A. fumigatus is among the most prevalent airborne fungal pathogens worldwide (12, 23). Cerebral aspergillosis is one of the most severe clinical forms of IA (29), usually presenting a poor prognosis and resulting in a fatal outcome. In fact, the mortality risk associated to cerebral aspergillosis usually exceeds 90%.

Resistance to triazoles in A. fumigatus was first described in the UK in the 1980s (8). Since then, reports of clinical isolates showing reduced susceptibility to different triazole antifungals have multiplied (8, 13, 22, 32), and azole-resistant IA, whether primary or breakthrough infection, is well documented (2, 4-7, 13, 22). Development of resistance in azole-treated patients and an environmental route of resistance have also been reported (22, 31, 32). The main mechanisms accounting for triazole resistance in A. fumigatus are point
mutations in the cyp51A gene encoding 14-α sterol demethylase, which is the
target of azole drugs (9, 13, 14, 20, 21). Specific mutations in cyp51A may
result in resistance to one, two, or all three triazoles (9, 13, 14, 21, 24).
However, only a few mutations have been clearly associated with in vitro and in
vivo resistance to voriconazole (31).

In this article, we report a case of cerebral aspergillosis caused by a
voriconazole-resistant isolate of A. fumigatus in an immunocompetent patient
treated with that antifungal. cyp51A gene sequencing revealed a g1413a
mutation causing a G448S amino acid substitution. According to the azole MIC
values obtained, and considering the ECVs (10, 26), this A. fumigatus isolate
should be considered resistant to voriconazole but not to itraconazole or
posaconazole. Unfortunately, we did not have the previous isolate obtained
from the abscess in the right temporal lobe to compare it with the later isolate
obtained in the abscess drained by petrosectomy. We assume that the
A. fumigatus acquired the voriconazole resistance under voriconazole pressure,
but, we can not rule out a re-infection by another strain.

Azole-resistance due to g1413a mutation has been reported in the UK (13) and
France (3). In both cases, the strains carrying the mutation were considered
resistant to multiple azoles. Noticeably, we found that the E-test showed a lack
of correlation with broth microdilution methods (BMD), especially in the case of
itraconazole, as has been described elsewhere (11). It should be noted that
ECVs determined using BMD may not be applicable to the results of E-test.
Surprisingly, this mutation was previously reported by Manavathu et al. (18, 19),
and it was then defined as moderately resistant to itraconazole and
posaconazole. The lack of agreement between the MIC values in strains of
different origin carrying the same single mutation (G448S) could be due to
differences in the methods used for antifungal susceptibility testing.
Alternatively, the A. fumigatus strains described by Bellete et al. (3) and Howard
et al. (13) might rely on more than one mechanism that would render them
resistant to multiple azoles. So far, resistance to itraconazole appeared to be
common to all azole-resistant A. fumigatus strains and thus this trait is
commonly used as a marker in screening for azole resistance. The emergence
of strains only resistant to voriconazole should necessarily change this latter approach and voriconazole will have to be included in antifungal susceptibility testing methods.

Residue G448 forms part of the conserved heme-binding domain and it is conserved in all cytochrome P450 ERG11/Cyp51 of yeasts and filamentous fungi. The G448S amino acid substitution described here corresponds to the G464S in \textit{Candida albicans} \textit{ERG11} and G484S in \textit{Cryptococcus neoformans}, both of which are involved in fluconazole resistance (25, 28). Several studies have demonstrated that this amino acid substitution detected in \textit{C. albicans} \textit{ERG11} confers a change in the orientation of the P450 heme-binding domain, leading to decreased azole binding and decreased catalytic activity of the enzyme (28, 15). Although it is known that this mutation also alters the susceptibility to voriconazole (and, to a lesser extent, to other azole drugs), the exact mechanism accounting for the resistant phenotype remain to be studied in more detail.

To our knowledge, this is the first report of an \textit{A. fumigatus} clinical isolate carrying the G448S specific mutation in Spain. The observed \textit{in vitro} resistance of the isolate to voriconazole led to treatment failure, and the patient was only cured after therapy with liposomal amphotericin B. Therefore, the data presented here confirm the role of this mutation in the development of decreased \textit{in vivo} susceptibility to voriconazole, as well as the importance of antifungal resistance as a factor determining the clinical outcome of IA, not only in immunocompromised patients, but also in immunocompetent hosts.

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POTENTIAL CONFLICTS OF INTEREST

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References


Figure Legend.

Figure 1. E-test susceptibility testing to voriconazole (VOR), itraconazole (ITC) and posaconazole (POS) for an-azole susceptible strain of *A. fumigatus* (CM-237), a multiresistant strain (CR019), and the clinical isolate recovered from our patient (TP1362).
Table 1. EUCAST microdilution susceptibility testing of the three clinical strains analyzed. Ranges of MIC values are in mg/L

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amphotericin B</th>
<th>Itraconazole</th>
<th>Voriconazole</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM-237</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
<td>0.03 - 0.06</td>
</tr>
<tr>
<td>TP1362</td>
<td>0.25</td>
<td>0.50 - 0.50</td>
<td>4.0 - 8.0</td>
<td>0.125 - 0.125</td>
</tr>
<tr>
<td>CR019</td>
<td>0.25</td>
<td>&gt;8.0</td>
<td>4.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 2. E-test susceptibility testing range values of the three clinical strains analyzed at 24 and 48 hours. MIC values are in mg/L

<table>
<thead>
<tr>
<th>Strains</th>
<th>Itraconazole</th>
<th>Voriconazole</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
<td>48h</td>
<td>24h</td>
</tr>
<tr>
<td>CM-237</td>
<td>0.125</td>
<td>0.75</td>
<td>0.125</td>
</tr>
<tr>
<td>TP1362</td>
<td>0.75 - 1.5</td>
<td>2.0 - 4.0</td>
<td>0.50</td>
</tr>
<tr>
<td>CR019</td>
<td>&gt; 32</td>
<td>&gt; 32</td>
<td>1.5</td>
</tr>
</tbody>
</table>