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 of the resistance 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 with a recurrent brain abscess was referred to the Hospital General Universitario Gregorio Marañón (HGUGM), Madrid, Spain, in July 2011. 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 a radical mastoidectomy. In November 2010, the patient had several postsurgical 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). Treatment with voriconazole at 200 mg (4 mg/kg) twice a day was thus initiated, and monitoring of serum drug levels for 8 weeks revealed good tolerance and apparent efficacy of the antifungal. However, 2 months later, the patient experienced a 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 cochlceostomy 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 again underwent a petrosectomy. Aspergillomas were removed from cerebral tissue, and biopsy specimens once more yielded *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 MRI showed that the lesions had disappeared.

**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.

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Address correspondence to Maricela Valerio, maricela.valerio@hotmail.com.

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**Antifungal susceptibility testing.** Antifungal susceptibility testing was performed by using the broth microdilution (BMD) method described in a 2008 EUCAST technical note (30) and the Etest in accordance with the manufacturer’s recommendations. The results of these assays are shown in Tables 1 and 2 and Fig. 1, respectively. The antifungal agents tested were

**CASE REPORT**

Resistance to Voriconazole Due to a G448S Substitution in *Aspergillus fumigatus* in a Patient with Cerebral Aspergillosis

Teresa Pelaez, Paloma Gijón, Eleonora Bunsow, Emilio Bouza, Waldo Sánchez-Yebra, Maricela Valerio, Beatriz Gama, Manuel Cuenca-Estrella, and Emilia Mellado

Clinical Microbiology and Infectious Diseases Department, Hospital General Universitario Gregorio Marañón and Department of Medicine, Faculty of Medicine, Universidad Complutense, Madrid, Spain; CIBER de Enfermedades Respiratorias (CIBERES CD06/06/0058), Palma de Mallorca, Spain; UGC Biotecnología, Unidad de Microbiología y Parasitología, Complejo Hospitalario Torrecárdenas, Almería, Spain; and Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

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), CM-237 (azole susceptible) and CR019 (multiazole resistant), were used as controls throughout this work.

**Molecular characterization of isolates.** Conidia from each of the aforementioned strains were inoculated into 3 ml of GYE 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 the identity of isolate TP1362 was confirmed as *A. fumigatus*, the full coding sequences of the cyp51B and cyp51A genes, including the cyp51A promoter, were amplified using the PCR conditions described by 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 among the cyp51B sequences of the three strains. However, the cyp51A gene 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 by using the broth microdilution (BMD) method described in a 2008 EUCAST technical note (30) and the Etest in accordance with the manufacturer’s recommendations. The results of these assays are shown in Tables 1 and 2 and Fig. 1, respectively. The antifungal agents tested were

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TABLE 1 EUCAST microdilution susceptibility test results of the three clinical strains analyzed

<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</td>
<td>4.0–8.0</td>
<td>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>

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 cutoff values (ECVs) recently published for A. fumigatus. Isolates with itraconazole and voriconazole MICs of ≤1 μg/ml were considered to belong 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 risk of death associated to cerebral aspergillosis usually exceeds 90%.

Resistance to triazoles in A. fumigatus was first described in the United Kingdom in the 1980s (8). Since then, reports of clinical isolates showing reduced susceptibility to different triazole antifungals have multiplied (8, 13, 22, 28, 32) and azole-resistant IA, whether a primary or a 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 (16, 22, 31, 32, 33). 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, 19, 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. The **TABLE 2** Etest susceptibility range values of the three clinical strains analyzed at 24 and 48 h

<table>
<thead>
<tr>
<th>Strain</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>0.12</td>
<td>0.75</td>
<td>0.12</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>

cyp51A gene sequencing revealed a G1413A mutation causing a G448S amino acid substitution. According to the azole MICs 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 isolate obtained later from the abscess drained by petrosectomy. We assume that the A. fumigatus strain acquired voriconazole resistance under voriconazole pressure, but we cannot rule out reinfection with another strain.

Azole resistance due to a G1413A mutation has been reported in the United Kingdom (13) and France (3). In both cases, the strains carrying the mutation were considered resistant to multiple azoles. It is notable that we found that the Etest showed a lack of correlation with BMD methods, 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 Etest. Surprisingly, this mutation was previously reported by Manavathu et al. (17, 18), and it was then defined as moderately resistant to itraconazole and posaconazole. The lack of agreement between the MICs for strains of different origins 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 appears 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 resistant to only voriconazole should necessarily change the 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 of the ERG11/cyp51-encoded cytochrome P450s of yeasts and filamentous fungi. The G448S amino acid substitution described here corresponds to the G464S substitution in Candida albicans ERG11 and the G484S substitution in Cryptococcus neoformans, both of which are involved in fluconazole resistance (25, 27). Several studies have demonstrated that this amino acid substitution detected in C. albicans 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 (15, 27). Although it is known that this mutation also alters susceptibility to voriconazole (and, to a lesser extent, to other azole drugs), the exact mechanism accounting for the resistance phenotype remains to be studied in more detail.

To our knowledge, this is the first report of an A. fumigatus clinical isolate carrying the specific G448S mutation in Spain. The observed in vitro resistance of the isolate to voriconazole led to treatment failure, and the patient was cured only after therapy with liposomal amphotericin B. Therefore, the data presented here confirm the role of this mutation in the development of decreased 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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