Amphotericin B in vitro resistance is associated with fatal Aspergillus flavus infection

INÈS HADRICH*, FATTOUNA MAKNI*, SOUROUR NEJI*, FATMA CHEIKHROUHOU*, HATEM BELLAJJ†, MOEZ ELLOUMI‡, ALI AYADI* & STÉPHANE RANQUE‡

*Laboratoire de Biologie Moléculaire Parasitaire et Fongique, Faculté de Médecine, Sfax, Tunisia, †Service d’Hématologie, CHU Hedi-Chaker, Sfax, Tunisia, and ‡Laboratoire de Parasitologie-Mycologie, Aix-Marseille Univ., AP-HM Timone, Marseille, France

Whether in vitro antifungal susceptibility findings correlate with the outcome of patients with invasive aspergillosis (IA) remains debated. This study aimed to test whether IA patients’ outcomes were associated with in vitro susceptibility results. To do so, we determined the in vitro susceptibility to amphotericin B (AMB) of 37 Aspergillus flavus isolates from 14 patients with haematological malignancies diagnosed with proven or probable IA, of which 13 were treated with AMB deoxycholate. Minimal inhibitory concentrations (MICs) were determined by Etest with the isolates classified as in vitro sensitive (AMB-S) or resistant (AMB-R) if their MICs were <2 or ≥2 mg/l, respectively. The association of the patients’ death with primary disease, administered antifungal treatment, and infection with AMB-R A. flavus was tested using generalized estimating equations logistic regression. We assessed AMB-R in 31/37 (84%) isolates. In the patients treated with AMB, the survival rate was 2/3 (67%) and 2/9 (22%) for those infected with AMB-S or AMB-R A. flavus, respectively. Both infection with AMB-R A. flavus (P = 0.014) strain and acute myelocytic leukaemia as the underlying primary disease (P = 0.036) were independent predictors of death. Our findings indicate that in vitro resistance predicts a poor outcome in patients with A. flavus invasive disease treated with AMB. Recent advances in non-culture-based microbiological methods should not discourage efforts to obtain in vitro antifungal susceptibility results, which are critical for the choice of antifungal therapy in patients with IA.

Keywords Aspergillus flavus, Etest, amphotericin B, outcome, haematological malignancies

Introduction

Whether in vitro antifungal susceptibility findings correlate with the outcome of patients with invasive aspergillosis (IA) remains fiercely debated [1]. Two conflicting situations are observed. An infecting fungus may be resistant in vitro, and the patient may respond to antifungal therapy. Similarly, an infecting fungus may be susceptible in vitro, but therapy may fail to result in clinical cure. The factors known to influence the outcome of IA are drug pharmacokinetics, site of infection, host factors, and the infecting fungus characteristics, including its susceptibility to the drug. In this setting, in vitro antifungal minimal inhibitory concentration (MIC) determination cannot reliably predict in vivo clinical outcome in a patient. In practice, while it may not always be possible to identify which patients will respond to therapy, in vitro susceptibility tests may be used to identify those who will have a decreased probability of responding. Lassl-Flörl [2] recently reviewed the conflicting data, including those of experimental models of in vivo correlation of antifungal susceptibility testing results in Aspergillus spp. Three clinical studies found a tendency towards a correlation of amphotericin B (AMB) MIC with IA outcome. Only the first one [3] was statistically significant, the

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Correspondence: Stéphane Ranque, Aix-Marseille Université, Parasitologie-Mycologie, Marseille, France. E-mail: stephane.ranque@ap-hm.fr

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two others [4,5] were not. Our study’s objective was to test whether the patients’ clinical outcomes was associated with in vitro susceptibility to AMB of the infecting Aspergillus.

Patients and methods

Study participants

The cohort study included immunocompromised patients at high risk of IA hospitalized in the haematology unit of the Sfax Teaching Hospital, Tunisia, who were receiving treatment for haematological malignancies, as previously described [6–8]. IA was diagnosed as per the international definitions of invasive fungal disease published by the Mycoses Study Group of the European Organization for Research and Treatment of Cancer/Mycoses Study Group [9]. Diagnosis of IA was proven for one case and probable for 13 cases. Underlying haematological diseases were myeloblastic leukaemia (AML; n = 10) acute lymphoblastic (ALL; n = 2) or medullar aplasia (MA; n = 2). There was no antifungal prophylaxis policy during the study period. Treatment with IV AMB deoxycholate monotherapy was given as prophylaxis in 13 patients, starting with 0.1 mg/kg, after a test dose of 1 mg, and escalating up to 1.5 mg/kg at the time of IA diagnosis. AMB lipid formulations were not available in this hospital. One patient was treated with oral 400 mg/12 h VO monotherapy. The patients’ characteristics (including their underlying diseases and status, whether there was neutropenia at diagnosis of IA, resolution of neutropenia) and antifungal MICs and microsatellite types of the Aspergillus flavus isolates, are detailed in Table 1. The files of the patients who died were reviewed by experts (IH, AHB, and ME) to ascertain the cause of death and its attributability to IA was scored on a 0–10 scale.

Aspergillus isolates

All A. flavus isolates were recovered from various respiratory tract sites in 14 patients with IA who were hospitalized in the onco-hematology service of Hedi Chaker Hospital (Sfax, Tunisia). All strains were cultured on Sabouraud dextrose agar (SDA, AES, France) and identified by conventional phenotypic characteristics and ITS rRNA regions DNA sequence analysis.

Antifungal MIC determination

Etest strips containing a continuous concentration gradient (0.002–32 μg/ml) of itraconazole (IT), voriconazole (VO), posaconazole (PO), or AMB were obtained from AES (France). RPMI 1640 plates were incubated at 30°C and the MIC was determined after 48 h incubation in accordance with the manufacturer’s instructions. In vitro AMB resistance (AMB-R) was defined by a MIC ≥ 2 μg/ml and isolates with lower MICs were considered sensitive (AMB-S) [2].

Molecular typing

The A. flavus isolates were typed using 12 microsatellite markers, as previously described [8].

Statistical analysis

Population structure in AMB-R or AMB-S A. flavus isolates was investigated using factorial analysis and Wright’s F-statistics [10]. The genetic differentiation between both populations was measured using the pairwise fixation index, Fst, and the log-likelihood ratio G test [11], computed with the Fstat V2.9.3 software [11].

The association of patients’ outcomes (death vs. survival) with AMB-R/S A. flavus isolates; acute primary disease, AML vs. ALL or MA; recovery from neutropenia; and antifungal treatment, AMB vs. voriconazole (VO) ± AMB, were tested by logistic regression. Generalized estimating equations (GEE) assuming an exchangeable structure for the correlation matrix were used to account for the non-independence of isolates from the same patient. All variables with a P value <0.20 in the univariate analysis were introduced in multivariate analysis. Results were expressed as odds ratio with 95% confidence interval (CI) and associated P value. All tests were two-sided with a final significance level of P <0.05. The statistical analyses were performed using the SAS statistical software ver. 9.1 (SAS Institute).

Results

The characteristics of the 14 patients with A. flavus IA are detailed in Table 1. Their mean age was 29.4 (±11.9) years; the male to female ratio was 11/3; 11 were treated with AMB monotherapy, with two receiving AMB followed by VO, and one with VO monotherapy. IA was proven for one and probable for 13 patients. The case fatality rate was 71.4% (95% CI [41.9–91.6]). For each of the 10 patients who died, the experts judged whether the death was attributable (score ≥ 8/10) to IA rather than to the underlying haematological condition. The three patients who did not recover from neutropenia died (Table 1), but this did not reach statistical significance (Fisher Exact test, P = 0.307). The in vitro susceptibility testing results of the 37 A. flavus strains are detailed in Table 1. Similar in vitro antifungal activity was found with all azoles in that the...
### Table 1: Patients’ characteristics and antifungal MICs and microsatellite types of *Aspergillus fumigatus* isolates.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/Sex</th>
<th>Primary disease*</th>
<th>IA EORTC 2008 criteria</th>
<th>Antifungal treatment†</th>
<th>Outcome</th>
<th>PMN at IA diagnosis</th>
<th>PMN at end of follow-up</th>
<th>AMB MICs§</th>
<th>Days from isolation to AMB treatment</th>
<th>Clinical sites</th>
<th>Microsatellite markers alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 21</td>
<td>ALL</td>
<td>Proven</td>
<td>AMB + VO²</td>
<td>Survival</td>
<td>300</td>
<td>1400</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>2</td>
<td>M, 42</td>
<td>AML</td>
<td>Probable</td>
<td>AMB</td>
<td>Death</td>
<td>100</td>
<td>1000</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>3</td>
<td>F, 25</td>
<td>AML</td>
<td>Probable</td>
<td>AMB</td>
<td>Death</td>
<td>100</td>
<td>1200</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>4</td>
<td>M, 18</td>
<td>AML</td>
<td>Probable</td>
<td>AMB</td>
<td>Death</td>
<td>200</td>
<td>800</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>5</td>
<td>M, 17</td>
<td>AML</td>
<td>Probable</td>
<td>AMB</td>
<td>Death</td>
<td>200</td>
<td>1000</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>6</td>
<td>M, 19</td>
<td>AML</td>
<td>Probable</td>
<td>AMB</td>
<td>Death</td>
<td>200</td>
<td>200</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>7</td>
<td>M, 40</td>
<td>MA</td>
<td>Probable</td>
<td>AMB</td>
<td>Survival</td>
<td>300</td>
<td>1400</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>8</td>
<td>M, 24</td>
<td>AML</td>
<td>Probable</td>
<td>AMB</td>
<td>Death</td>
<td>200</td>
<td>1200</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>9</td>
<td>F, 25</td>
<td>ALL</td>
<td></td>
<td>AMB</td>
<td>Death</td>
<td>300</td>
<td>800</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>10</td>
<td>F, 42</td>
<td>AML</td>
<td></td>
<td>AMB</td>
<td>Death</td>
<td>100</td>
<td>200</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>11</td>
<td>M, 50</td>
<td>AML</td>
<td></td>
<td>VO</td>
<td>Survival</td>
<td>200</td>
<td>800</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>12</td>
<td>M, 47</td>
<td>MA</td>
<td></td>
<td>AMB + VO</td>
<td>Death</td>
<td>200</td>
<td>300</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>13</td>
<td>M, 21</td>
<td>AML</td>
<td></td>
<td>AMB</td>
<td>Survival</td>
<td>300</td>
<td>1000</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
<tr>
<td>14</td>
<td>M, 20</td>
<td>AML</td>
<td></td>
<td>AMB</td>
<td>Death</td>
<td>200</td>
<td>1200</td>
<td>AMB</td>
<td>5</td>
<td>AFLA 1 2 3 4 5 6</td>
<td>AFLA 1 2 3 4 5 6 AFPM 1 2 3 4 5</td>
</tr>
</tbody>
</table>

*ALL, acute lymphocytic leukemia; AML, acute myelocytic leukemia; MA, medullary aplasia.

†AMB, Amphotericin B deoxycholate; VO, voriconazole.

‡BAL, Bronchoalveolar lavage fluid.

§PMN, Polymorphonuclear leukocytes counts (× 10⁹/l).

#AMB: amphotericin B (minimal inhibitory concentration in mg/l).

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AMB, Amphotericin B deoxycholate; VO, voriconazole.

BAL, Bronchoalveolar lavage fluid.

PMN, Polymorphonuclear leukocytes counts (× 10⁹/l).

AMB: amphotericin B (minimal inhibitory concentration in mg/l).
MICs were below 1 mg/l for VO and posaconazole in 100% of the isolates, and in 91.8% of the isolates for itraconazole. The proportion of AMB-R *A. flavus* isolates was 31/37 (83.7%).

Among the 12 patients treated with AMB, three (Table 1, patients 6, 7 & 13) were infected with AMB-S and nine with AMB-R *A. flavus* strains. Two (66.7%, 95%CI [9.43–99.16]) of the patients infected with AMB-S and two (22.2%, 95%CI [2.81–60.01]) of those infected with AMB-R isolates survived. In the multivariate GEE analysis (Table 2), both infection with AMB-R *A. flavus* (*P* = 0.0140) and AML as the patient’s primary disease (*P* = 0.0359) were independently associated with an increased risk of death.

**Microsatellite analysis**

Microsatellite polymorphism analysis showed that the *A. flavus* isolates from these patients with IA in this haematology unit were very genetically heterogeneous. The mean gene diversity over the microsatellite loci was 0.559 and 0.960 for AMB-R and AMB-S populations, respectively. Nei’s unbiased expected heterozygosity was 0.596 overall. The pairwise Fst value was 0.082 (*P* > 0.07), indicating the absence of population structure associated with AMB-R/S phenotype, as illustrated in the factorial analysis graph (Fig. 1). The Log-likelihood G Test (*P* = 0.664) confirmed the absence of significant genetic differentiation within AMB-R or AMB-S *A. flavus* populations.

**Discussion**

This study’s major finding is the significant association of in vitro susceptibility results with the outcome IA in our study population. AMB-R was an independent predictor of a fatal outcome. We would like to discuss the limitations of our study. First, only three of the analyzed patients were infected by an AMB-S strain. This is in keeping with the known relatively high proportion of *A. flavus* isolates with high AMB MICs [12,13]. But despite the relatively low number of patients analyzed, this study had enough power to detect a statistically significant effect of in vitro AMB-R on the patients’ death in a multivariate analysis. Second, many factors associated with death in patients with severe haematological malignancies were not taken into account in the statistical analysis, either because they were unmeasured or because they occurred in only some patients. We thus used the type of haematological disease as a proxy for a measure of underlying disease severity. Nevertheless, this proxy’s statistically significant association with the patients’ outcome in the multivariate analysis indicates that it is probably closely correlated to the underlying disease’s severity. Third, another limitation is that AMB treatment

**Table 2** Univariate and multivariate GEE logistic regression results of the factors associated with survival in hematological patients with *Aspergillus flavus* invasive aspergillosis.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% Confidence interval</td>
</tr>
<tr>
<td>Age</td>
<td>1.0209</td>
<td>0.9152–1.1388</td>
</tr>
<tr>
<td>Year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antifungal treatment*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB vs. VO ≥ AMB</td>
<td>0.2692</td>
<td>0.0207–3.4987</td>
</tr>
<tr>
<td>AMB in vitro susceptibility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMB-R (MIC ≥ 2 mg/l) vs. AMB-S (MIC &lt; 2 mg/l)</td>
<td>0.0296</td>
<td>0.0015–0.5749</td>
</tr>
<tr>
<td>Primary disease**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML vs. ALL or MA</td>
<td>0.0600</td>
<td>0.0044–0.8156</td>
</tr>
</tbody>
</table>

*AMB, Amphotericin B deoxycholate; VO, voriconazole; **AML, acute myelocytic leukemia; ALL, acute lymphocytic leukemia; MA, medullar aplasia.
was started in some patients before and after fungus isolation and as a result, the efficient treatment duration differed from patient to patient. This heterogeneity might negatively impact on the power to detect an association between MIC and treatment.

In our patients 100% of the isolates were *A. flavus* of which 84.7% were AMB-R. As previously detailed [8], the absence of significant population structure, clearly illustrated by the factorial analysis correspondence graph (Fig. 1), indicates that AMB-S/R phenotype might either be acquired or lost too rapidly to be predicted by more stable genomic markers. Although most of the data regarding AMB resistance mechanisms comes from studies conducted in yeasts, Chamilos and Kontoyiannis [14] extensively reviewed the mechanisms of AMB resistance in *Aspergillus* spp. Briefly, these mechanisms include (i) depletion in the membrane ergosterol (the drugs’ target), through mutations in the ergosterol synthetic pathway and/or prior exposure to azoles, and (ii) increased production of reducing enzymes (e.g., catalases) that confer resistance to oxidative stress, as AMB also acts as an oxidizing agent.

After more than three decades of study, antibacterial testing can serve to predict the response to therapy as the ‘90-60 rule’, i.e., infections due to susceptible isolates respond to therapy ∼90% of the time, whereas infections due to resistant isolates respond ∼60% of the time [15]. Antifungal susceptibility testing has been shown to have a similar predictive utility and it is now used as a routine adjunct in the treatment of yeasts infections [16]. In contrast, *in vitro* testing of filamentous fungi remains limited in the routine clinical laboratory. Favorable responses to AMB therapy occurred in 66% of IA cases caused by AMB-S and in 22% of AMB-R *A. flavus* isolates. Despite the higher fatality rate in IA, the difference in survival rates is in keeping with antibacterial testing predictions. Note that the case fatality rate in the patients infected with an AMB-S isolate was similar to those reported in clinical trials in patients with haematological malignancies treated with AMB for IA [17].

In Lass-Flörl et al. [3] CLSI antifungal susceptibility tests revealed that all *A. terreus* strains were AMB-R, and 41% of *A. flavus* and 28% of *A. fumigatus* were also resistant (MIC ≥ 2 mg/l), and that this was associated with the AMB therapy failure. In our study, 84% of *A. flavus* isolates were AMB-R. This difference might be due to geographical disparities but also to the use of the Etest antifungal susceptibility test. The Etest determines the MIC and the susceptibility to AMB more precisely than do the micro-broth dilution methods [18] and MIC obtained with Etest correlates with response to therapy in patients with candidemia [16] whereas those obtained with micro-broth dilution methods do not [19].

The Transplant-Associated Infection Surveillance Network study [20] found that higher voriconazole MICs of *Aspergillus* spp. correlated with increased mortality at 6 weeks in transplant recipients with IA but it did not reach statistical significance, maybe because VO therapy was predominantly used in patients primarily infected with azole susceptible *A. fumigatus*. Azole resistance is now increasingly reported in *A. fumigatus* [21–23] but the microbiological criterion for IA diagnosis is more often galactomannan antigen detection than culture [24]. Thus, every endeavour should be made to obtain fungal cultures, which is mandatory to perform comprehensive susceptibility testing. Indeed, susceptibility testing results are, among various other factors, critical for the choice of antifungal therapy in patients with IA.

Our study was characterized by: the combination of a local epidemiological setting where a single species, *A. flavus*, was involved in all diagnosed IA cases; the use of Etest, an efficient *in vitro* MIC determination method for AMB; and the almost exclusive use of AM deoxycholate treatment, because voriconazole or other recently licensed antifungals were not available at the beginning of the study. In contrast to Baddley et al. [20] but in line with Lass-Flörl et al. [3], this remarkably homogeneous setting was particularly helpful in detecting a statistically significant association between AMB MICs and patient outcome. Our findings are representative of the local situation in Tunisia at the time of the study and cannot be extrapolated to distinct settings. Yet, they likely predict poorer countries’ situation in the near future. Because haematological treatments improve faster than both mycological diagnosis and accessibility to recommended antifungal treatments, we would like to stress the need for both enhancing mycological diagnosis capacities and making recommended first-line antifungal treatments for IA accessible in developing countries.

This study’s findings highlight the relatively frequent AMB resistance in *A. flavus* clinical isolates and bring new evidence that *in vitro* resistance is an independent predictor of a poor outcome in patients with invasive disease treated with AMB. While both *A. fumigatus* azole resistance and more resistant species are emerging, recent advances in non-culture-based microbiological methods [24] should not discourage efforts to obtain MIC results that, among the various other factors, are critical in choosing antifungal therapy for patients with IA. Now, in line with Rex and Pfaller’s perspective on yeast infections a decade ago [15], antifungal susceptibility testing has come of age in invasive aspergillosis.

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