Amphotericin B and Caspofungin Resistance in *Candida glabrata* Isolates Recovered from a Critically Ill Patient

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**Background** Consecutive *Candida glabrata* isolates recovered from a patient in an intensive care unit were resistant to amphotericin B (minimum inhibitory concentration, up to 32 μg/mL; determined by Etest [AB Biodisk]). Analyses at the national reference laboratory showed that some isolates were also resistant to azoles and caspofungin. In this study, 4 isolates were studied thoroughly using susceptibility assays and a mouse model and to determine clonality.

**Methods.** Different broth microdilution tests, Etests, and time-kill studies for antifungals were performed in different media. Three of the 4 isolates were examined in an in vivo experiment, in which mice were challenged intravenously with 1 of 3 isolates and treated daily with amphotericin B, caspofungin, or saline. For the clonality studies, arbitrarily primed polymerase chain reaction (PCR) was performed with the 4 isolates, 8 isolates obtained from nonrelated patients, and a reference strain.

**Results.** The murine model indicated that 1 isolate was resistant to amphotericin B, 1 had intermediate susceptibility, and 1 was fully susceptible. Two of the 3 isolates were resistant to caspofungin. Microdilution methods did not reliably differentiate between amphotericin B–susceptible and -resistant isolates. All assays identified caspofungin-susceptible and -resistant isolates. Arbitrarily primed PCR showed that the 4 isolates probably were of clonal origin.

**Conclusions.** We have documented the emergence of amphotericin B–resistant and caspofungin-resistant *C. glabrata* isolates during treatment of a critically ill liver transplant recipient. Only the Etest predicted amphotericin B resistance in the isolates. We recommend that important fungal strains recovered from patients who are receiving antifungal therapy should be tested for susceptibility to the antifungal drug used, because resistance can be present initially or may occur during treatment.

In vitro susceptibility testing of *Candida* species usually reveals an MIC of <2 μg/mL for amphotericin B [4–7]. However, MICs of up to 16 μg/mL have been reported for *Candida glabrata* when the Etest (AB Biodisk) is used [6]. For caspofungin, the MICs for *Candida* species are typically <4 μg/mL, and MICs for *C. glabrata* are typically somewhat greater than those for *Candida albicans* [8].

Although amphotericin B has been used for many years, resistance is rare, and to our knowledge, resistance to combined amphotericin B and caspofungin has not been reported. We report the recovery of *C. glabrata* isolates from a critically ill patient with in vitro and in vivo resistance to amphotericin B and caspofungin.

**CASE REPORT**

A 66-year-old woman was admitted to an intensive care unit at a tertiary care hospital. Before admission, the
Multidrug-Resistant C. glabrata Isolates

Figure 1. Antifungal treatments given to a patient in the intensive care unit (ICU) and Candida glabrata findings. Each mark indicates at least 1 isolation of C. glabrata. For C. glabrata findings that are marked on the line, isolates had an amphotericin B MIC (determined by Etest [AB Biodisk]) of ≤1 μg/mL; if the mark is above the line, at least 1 isolate had an MIC of >1 μg/mL. The isolates investigated in the text are marked with circles. The gray line refers to the right y axis and indicates the highest MIC of amphotericin B for C. glabrata isolates on the given day. Notable events occurred on day 12 (when the first isolate of C. glabrata with an MIC >1 μg/mL was recovered), day 47 (when the patient was subjected to isolation procedures), day 72 (when liver transplantation was performed), and day 144 (when the patient died). The C. glabrata strains investigated in this study were all isolated 40–61 days after admission.

The patient had undergone elective removal of the gallbladder because of gallstones at a local hospital. Complications occurred during the operation, and the liver artery, gall ducts, and spleen were harmed. The patient was transferred to the tertiary care hospital and underwent immediate surgery, with removal of the spleen and gallbladder, and the liver artery was sutured. The following weeks were complicated with numerous reoperations because of bleeding, organ dysfunction, the need for mechanical ventilation, and hemodialysis. A liver transplantation was performed 12 weeks after hospital admission because liver necrosis had occurred. The patient died of multiorgan failure and irreversible shock after 20 weeks in the intensive care unit.

Samples from all catheters and of body fluids were obtained for microbiological evaluation as a part of routine surveillance and because ongoing infections were suspected. With the exception of an episode of bacteremia due to Enterococcus faecium, no other major bacterial infections were diagnosed. The patient was colonized with various fungi; she was initially colonized with Candida tropicalis in the airways, with 2 subsequent episodes of fungemia on hospital days 8 and 43, respectively, but this fungus was not isolated thereafter. The patient was colonized with C. glabrata, which was found in numerous samples obtained from shortly after hospital admission. The first case of C. glabrata fungemia occurred on hospital day 81; thereafter, C. glabrata was found in almost every blood culture until the patient died on hospital day 144. All of the findings regarding C. glabrata and antifungal therapy are shown in figure 1. Amphotericin B (50 mg 3 times per day) was administered only orally as part of the decontamination mixture (which also included cefuroxim and tobramycin) on days 6–46, and voriconazole (250 mg twice per day) was initially given intravenously for 12 days (starting on hospital day 42) and was given perorally thereafter until day 109. Caspofungin was given from hospital day 9 to day 144 (70 mg on the first day, followed by 50 mg iv daily). When Etests repeatedly revealed MICs of amphotericin B for C. glabrata of ≥2 μg/mL (often 4–8 μg/mL, and sometimes even 24–32 μg/mL), isolation procedures were performed for the rest of the patient’s stay—even though the reference laboratory (The Mycological Unit, Statens Serum Institut, Copenhagen, Denmark) found that all MICs of amphotericin B were ≤4 μg/mL. Fourteen of the numerous C. glabrata isolates recovered from this patient were examined at the reference laboratory during the patient’s stay in the intensive care unit. Four isolates were found to be resistant to caspofungin (MIC, ≥2 μg/mL), of which 1 isolate was also resistant to all azoles.

MATERIALS AND METHODS

C. glabrata isolates. Four C. glabrata isolates (designated isolates A, B, C, and D) were chosen from a small collection of 19 stored C. glabrata isolates recovered from the patient during hospital days 40–82 and on hospital day 134. Isolates A, C, and
The arbitrarily primed PCR (AP-PCR) study included 8 isolates. The isolates were recovered 40–61 days after admission (figure 1). The concentration of amphotericin B for these isolates were 1.5–32 μg/mL, and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

D were recovered from gall drainages, and isolate B was recovered from a urine sample. Etests determined that the MICs of amphotericin B for these isolates were 1.5–32 μg/mL, and the isolates were recovered 40–61 days after admission (figure 1). The arbitrarily primed PCR (AP-PCR) study included 8 C. glabrata isolates recovered from blood cultures from other patients who had invasive candidiasis during the same period and 1 reference strain (C. glabrata ATCC 90030).

**Antifungal agents.** The amphotericin B and caspofungin powder used in the broth microdilution studies were purchased from Sigma-Aldrich Chemie or kindly provided by MSD, respectively. For the time-kill studies and the animal model, amphotericin B and caspofungin were purchased as the infusion powders Fungizone (Bristol-Myers Squibb) and Cancidas (MSD).

**Susceptibility testing.** Broth microdilution methods for amphotericin B, caspofungin, fluconazole, voriconazole, and itraconazole were performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) M27-A2 (former NCCLS) [9] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) E.dis 7.1 [10] documents. (Azole data are not shown; isolates A, B, and D usually displayed dose-dependent susceptibility, whereas isolate C was considered resistant to all azoles.) Breakpoints of ≤1 μg/mL and ≤2 μg/mL were used to interpret the amphotericin B and caspofungin findings, respectively. The broth microdilution tests were performed with RPMI 1640 and antibiotic medium 3 (Becton Dickinson). The range of the antifungal concentrations was 0.06–8 μg/mL. Etests (AB Biodisk) were performed in accordance with the manufacturer’s recommendations.

**Table 1. MICs and stationary concentrations obtained by various in vitro methods.**

<table>
<thead>
<tr>
<th>Agent, isolate</th>
<th>Etest</th>
<th>Broth microdilution method</th>
<th>Stationary concentration from time-kill study, μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RPMI 1640 medium</td>
<td>AM3 medium</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td></td>
<td>CLSI method</td>
<td>EUCAST method</td>
</tr>
<tr>
<td>Isolate A</td>
<td>32 to &gt;32</td>
<td>2</td>
<td>1–2</td>
</tr>
<tr>
<td>Isolate B</td>
<td>8–12</td>
<td>1–2</td>
<td>1</td>
</tr>
<tr>
<td>Isolate C</td>
<td>1.5–2</td>
<td>0.5</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>Isolate D</td>
<td>6–8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Caspofungin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate A</td>
<td>0.19</td>
<td>0.5</td>
<td>0.125–0.25</td>
</tr>
<tr>
<td>Isolate B</td>
<td>&gt;32</td>
<td>8</td>
<td>4–8</td>
</tr>
<tr>
<td>Isolate C</td>
<td>0.38</td>
<td>1</td>
<td>0.25–1</td>
</tr>
<tr>
<td>Isolate D</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>8 to &gt;8</td>
</tr>
</tbody>
</table>

**NOTE.** All tests were performed at least in duplicate, except for time-kill studies with AM3 medium. Etest was manufactured by AB Biodisk. CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

**Time-kill studies.** Time-kill studies were performed using AM3 and RPMI. Overnight cultures were diluted to a concentration of 2 × 10⁴–1 × 10⁵ cfu/mL for the starting inoculum. The concentrations of the antifungal agents used were 0.125–8 × MIC. To determine the concentration of fungi in colony-forming units per milliliter (at 0, 3, 6, and 24 h), 10-μL spots were plated after subsequent 10-fold dilutions. The plates were examined after 48 h at 35°C. The stationary concentration (SC), defined as the lowest concentration for which the initial cfu count was higher than the cfu count at 24 h, was determined.

**Animal model.** Isolates A, C, and D were examined in a mouse model with immunocompetent mice [11]. The model was approved and controlled by the Animal Experiments Directorate. A total of 54 outbred, female Naval Medical Research Institute mice (30 g) were used. Mice, in 3 groups of 18, were challenged intravenously in a tail vein with a 200-μL inoculum of 10⁷ cfu/mL of either isolate A, C, or D. Six mice from each group were treated intraperitoneally once daily on days 2, 3, and 4 with amphotericin B (6 mg/kg per day), caspofungin (5 mg/kg per day), or saline, all in total volumes of 0.5 mL per day. Kidneys were removed aseptically after the sacrifice, placed in 1.5 mL of sterile saline, and stored at −80°C before homogenization [11]. Ten-fold dilutions of the homogenate were performed, after which the number of colony-forming units was determined by plating 20-μL spots on Sabouraud agar. Plates were read after incubation at 35°C for 48 h [11]. The Kruskal-Wallis test for differences between groups was performed, and P values of <0.05 were considered to be statistically significant.

**AP-PCR.** DNA of isolates A-D, the 8 other C. glabrata
Multidrug-Resistant \textit{C. glabrata} Isolates

\textbf{RESULTS}

\textbf{Amphotericin B susceptibility test findings.} Etests yielded MICs ranging from 1.5 to \textgreater 32 \textmu g/mL (table 1). Broth microdilution methods resulted in markedly lower MIC values (0.25–2 \textmu g/mL). When AM3 was used as medium, isolate C was slightly more distinguished from the other isolates, but only when the EUCAST method was used.

\textbf{Caspofungin susceptibility test findings.} Etests and all microdilution methods separated the isolates into 2 groups. Isolates A and C were fully susceptible to caspofungin, whereas isolates B and D were resistant to caspofungin (table 1). MICs of caspofungin were markedly reduced when AM3 was used as medium instead of RPMI 1640.

\textbf{Time-kill studies.} The stationary concentration values for amphotericin B obtained from the time-kill studies could not distinguish the isolates (table 1 and figure 2). The stationary concentration values for caspofungin, however, convincingly showed that isolates A and C were susceptible and isolates B and D were resistant. In addition, the time-kill studies showed that the fungicidal effect of both amphotericin B and caspofungin is concentration dependent.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Time-kill curve examples, with mean values from 2 experiments, as well as minimum and maximum values. Isolate C and amphotericin B \textit{(A)} and isolate A and amphotericin B \textit{(C)} had stationary concentrations of 2 \textmu g/mL, despite the large difference in MICs found using Etest (AB Biodisk; \textasciitilde 2 vs. \textasciitilde 32 \textmu g/mL). Isolate C and caspofungin \textit{(B)} and isolate D and caspofungin \textit{(D)} had clearly different results, with isolate C being fully susceptible to caspofungin and isolate D being considered resistant to caspofungin. \textit{C. glabrata}, Candida glabrata.}
\end{figure}
The animal model. Infections were established in the mice for all 3 C. glabrata isolates, since the log cfu/mL for all 3 control groups was significantly different from 0 (\( P < .003 \)). The number of colony-forming units for the mice challenged with isolate A and treated with amphotericin B was not significantly different from that for the control group (\( P > .05 \)) but was different from 0 (\( P < .001 \)) (figure 3). However, caspofungin treatment of isolate A was successful (\( P < .003 \) for difference with controls). Thus, isolate A was proven to be resistant to amphotericin B and susceptible to caspofungin. Isolate C was clearly susceptible to both amphotericin B and caspofungin (\( P < .001 \) for differences with controls). The number of colony-forming units for the mice challenged with isolate D and treated with amphotericin B was found to be neither significantly different from 0 (\( P > .05 \)) nor significantly different from that of the controls (\( P > .05 \)). This indicates that treatment was inadequate, and isolate D was considered to be intermediately susceptible to amphotericin B and resistant to caspofungin (\( P > .05 \) for difference with controls; \( P = .002 \) for difference from zero).

AP-PCR amplifications. Three primers generated distinct and reproducible bands in the gel electrophoresis patterns for all 13 isolates. As expected, differences in the band patterns were observed with the different primers. Bands were sometimes observed in the lane with the control sample without DNA template (figure 4, lane 15). This may be due to bacterial contamination of the taq polymerase [15], and if bands of the same size were found in other lanes, they were not included in the binary analysis. Two results could be drawn from the cluster analysis: the isolates from this patient could not be distinguished from one another, and the isolates could be distinguished from the isolates recovered from the nonrelated patients (figure 4). This indicates that isolates A–D were of clonal origin.

DISCUSSION
A series of consecutive C. glabrata isolates with resistance to caspofungin and amphotericin B were recovered from a critically ill patient in an intensive care unit. The animal model
Figure 4. Top, Gel electrophoresis findings for arbitrarily primed PCR products with primer AP50-1. Lane 1, size marker; lanes 2–5, isolates A–D, respectively; lane 6, isolate AH; lane 7, isolate BH; lane 8, isolate FH; lane 9, isolate HH; lane 10, isolate RH200; lane 11, isolate RH201; lane 12, isolate RH202; lane 13, isolate RH250; lane 14, Candida glabrata ATCC 90030; lane 15, control without DNA; lane 16, size marker. Isolates RH200, RH201, RH202, and RH250 were recovered from the intensive care unit in the same hospital where the index patient was treated; the isolates recovered from other hospitals were AH, BH, FH, and HH. Bottom, Dendrogram based on Jaccard’s coefficient and the unweighted pair group method with arithmetic mean clustering method.

proved that some of these C. glabrata isolates were less susceptible and probably had therapeutic resistance to treatment with amphotericin B, whereas others were fully susceptible to amphotericin B. The Etest method was superior to broth microdilution methods in distinguishing these isolates, and only the Etest identified the intermediately susceptible isolate. This finding is in agreement with the findings of others [6, 16]. A susceptibility breakpoint for amphotericin B has yet to be established, but ≤1 μg/mL has been suggested [4, 7, 17]. On the basis of this breakpoint, all 4 isolates recovered from our patient would be categorized as resistant to amphotericin B according to the Etest, although the mouse model revealed that at least the isolate with an MIC of 1.5 μg/mL was treatable. The use of AM3 as medium for susceptibility testing for amphotericin B has been suggested [18]; however, in our hands, this method only improved the discriminatory power slightly.

No breakpoint has been established for caspofungin. In this study, a breakpoint of ≤2 μg/mL was used to interpret the data. All methods identified the caspofungin-susceptible and -resistant isolates. The mouse model clearly showed that resistance to caspofungin was of major importance. These results are in agreement with the work of Hernandez et al. [19] on caspo-
fungin-resistant C. albicans, providing additional evidence that good correlations between MIC values and outcome are seen when highly resistant isolates are included [19, 20]. The time-kill studies showed that the fungicidal effect of both amphotericin B and caspofungin is concentration dependent, supporting previous findings [1–3]. Surprisingly, resistance to amphotericin B found in the animal model could not be seen with the same strength in the time-kill curves, although reduced susceptibility was identified (figure 2 and table 1).

Molecular investigations of the relationship between the isolates suggested a clonal origin of the study isolates and that these isolates were different from C. glabrata isolates recovered from other patients in the same and other hospitals. Amphotericin B and/or caspofungin resistance has not since been detected in this hospital; thus, hygiene precautions, such isolation of the patient, apparently prevented cross-infection to other patients.

In conclusion, we have documented the emergence of amphotericin B– and caspofungin-resistant C. glabrata isolates during treatment of a critically ill patient. This report demonstrates the importance of antifungal susceptibility testing in patients who are receiving or who have previously been exposed to antifungal treatment. We recommend that all important fungin-resistant Candida strains recovered from patients treated with antifungal therapy should be tested for susceptibility, because resistance can be present initially or may even occur during treatment. Furthermore, the study illustrates the value of various susceptibility testing methods, as well as the limits of these different methods (e.g., the Etest was superior to microdilution methodologies for amphotericin B susceptibility testing).

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References