Transient Fungemia Caused by an Amphotericin B-Resistant Isolate of *Candida haemulonii*

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A bloodstream infection due to *Candida haemulonii* afflicting a patient with fever and a medical history of megaloblastic anemia is reported. The clinical isolate was misidentified by the API 20C and VITEK identification systems. The results of susceptibility tests showed that the MIC of amphotericin B for *C. haemulonii* was 4 µg/ml. Additional susceptibility testing procedures based on the use of antibiotic medium 3 and Iso-Sensitest broth were performed, and killing curves were determined. Two collection strains of *C. haemulonii* were employed as controls. The three isolates exhibited resistance to amphotericin B in vitro regardless of the antifungal susceptibility testing method employed. In addition, the MICs of fluconazole for the three isolates were high. Further studies are needed in order to ascertain whether this species exhibits innate or acquired resistance to amphotericin B and other antifungal agents.

Candida haemulonii (Van Uden & Kolipinski) S. A. Meyer & Yarrow (Yarrow and Meyer 1978) (syn. *Torulopsis haemulonii*) is a yeast species that has been reported in the scales of animals and in seawater and has been recently associated with an epidemic disease afflicting laboratory animals (*Ornithodoros moubata*) in the Czech Republic (5, 10, 11). This fungus is also involved in human infections such as onychia, ulcers of the feet or legs, and candidemia (10). Identification of *C. haemulonii* is difficult because it is phenotypically very similar to *Candida famata* (teleomorph, *Debaryomyces hansenii*) and *Candida guilliermondii* (teleomorph, *Pichia guilliermondii*), although it can be distinguished by its inability to assimilate cellobiose and its negative or weak assimilation of raffinose (8, 10). In addition, commercial yeast identification systems have failed to identify *C. haemulonii* isolates (16). Lehmann et al. studied 25 clinical isolates of *C. haemulonii* and described two genetically distinct groups within the species (group I and group II) on the basis of isoenzyme profiles, DNA reassociations, and physiological characteristics (9, 10, 16). No differences in clinical associations between the groups were described, but it was determined that *C. haemulonii* constitutes a species complex.

The susceptibility pattern of *C. haemulonii* is unknown, but an anecdotal report has suggested that it could be resistant to amphotericin B (AMB) and other antifungal agents (4). Here, we describe a case of transient fungemia due to a *C. haemulonii* strain which exhibited resistance to AMB and fluconazole in vitro. Killing curves were determined and additional susceptibility tests were performed to assess the activity of AMB against the isolate.

Case report. An 83-year-old male patient presenting with fever was admitted to Hospital Fernández in Buenos Aires,

Argentina. The patient had a medical history of megaloblastic anemia, which was being treated. Blood cultures were performed, his intravenous lines were changed, and the fever disappeared. The blood cultures were positive for yeast species after 24 h of incubation. The case was considered an instance of catheter-related candidemia. Long-term catheterization and treatment with broad-spectrum antibiotics were the patient's associated risk factors for candidemia. The patient had not received antifungal therapy previously.

Identification of the isolate. The clinical strain was recovered from blood cultures with Sabouraud glucose agar. Aerobic growth on cornmeal agar yielded off-white, smooth colonies. The cells were elongate and cylindrical and appeared singly and in chains. Pseudohyphae were not present. The API 20C (bioMérieux SA, Marcy l'Etoile, France) and VITEK (bioMérieux SA) clinical yeast identification systems were used for identification of the isolate. The API 20C system yielded the code 6102170, which is not included in the API 20C database, andthe VITEK system identified the strain as *Pichia ohmeri* (86% probability of identity). The isolate was then sent to the Mycology Reference Laboratory of Argentina (INEI, ANLIS, Dr. Carlos G. Malbrán, Buenos Aires) and was identified as *C. haemulonii* by morphological and physiological tests (17). The isolate was also sent to the Mycology Reference Laboratory of the National Center for Microbiology in Spain, where the strain was labeled CNM-CL-3458 (Spanish Centro Nacional de Microbiología yeast culture collection), and the identification of the isolate was reconfirmed by routine methods (17). The fermentation and assimilation profiles as well as the morphological characteristics of the isolate are shown in Table 1.

Conventional antifungal susceptibility testing. The susceptibility testing strictly followed the NCCLS recommendations for microdilution procedures (13) but included the modifications recommended by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial

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TABLE 1. Morphological, physiological, and biochemical characteristics of the *C. haemulonii* clinical isolate

Characteristic	Result(s)[a] and/or description	Characteristic	Result(s)[a] and/or description
Carbon source fermentation after 14 days of growth		Erythritol	−
Glucose	+	Ribitol	−
Trehalose	−	Xylitol	−
Galactose	−	L-Arabinitol	−
Lactose	−	D-Glucitol	+
Maltose	−	D-Mannitol	+
Raffinose	W, D	Galactitol	−
Sucrose	+	Gluconate	+
		D-Glucuronate	−
Assimilation, after 14 days of growth, of:		DL-Lactate	−
Glucose	+	Inositol	−
Galactose	−	Citrate	−
L-Sorbose	−	Methanol	−
D-Glucosamine	+	Ethanol	−
Xylose	−	Nitrate	−
L-Arabinose	−	Nitrite	−
D-Arabinose	−	Ethylamine	−
L-Rhamnose	+	Cadaverine	+
Sucrose	+	Creatine	−
Cellobiose	−	Creatinine	−
Salicin	−	Cycloheximide (0.01%)	+
Melibiose	−		
Lactose	−	Growth	
Raffinose	+, D	25°C	+
Melezitose	−	30°C	+
Starch	−	35°C	+
Glycerol	−	37°C	+
		Morphology	White to cream-colored colonies, vegetative reproduction by budding, no filaments, no pseudohyphae, no sexual reproduction

[a] W, weak reaction; D, delayed reaction.

Susceptibility Testing. These modifications included the use of RPMI 1640 medium supplemented with 2% glucose as the assay medium (RPMI–2% glucose), an inoculum size of 10^5 CFU/ml, flat-bottomed trays, spectrophotometric readings, and the definition of the concentration end point for azole agents and flucytosine as that at which 50% of the isolate was inhibited (2).

The antifungal agents used in the study were AMB (Sigma-Aldrich Quimica, SA, Madrid, Spain), flucytosine (Sigma-Aldrich Quimica), fluconazole (Pfizer SA, Madrid, Spain), and itraconazole (Janssen Farmaceutica, Madrid, Spain). AMB, fluconazole, and itraconazole were dissolved in 100% dimethyl sulfoxide (Sigma-Aldrich Quimica). Flucytosine was dissolved in sterile distilled water. RPMI 1640 medium (Sigma-Aldrich Quimica) without sodium bicarbonate, with L-glutamine, buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich Quimica), and supplemented with 18 g of glucose/liter to reach a final concentration of 2% was employed as the assay medium (2). Sterile plastic microtitration plates containing flat-bottomed wells were utilized. The plates contained twofold serial dilutions of the antifungal drugs with 100 μl of assay medium per well. Two wells containing drug-free medium were used as the sterility and growth controls. Each well of the trays was inoculated with 100 μl of the final inoculum $(0.5 \times 10^5$ to 2.5×10^5 CFU/ml).

Additional susceptibility testing to detect AMB resistance. Susceptibility tests were also performed with both antibiotic medium 3 (AM3) (Becton-Dickinson, Madrid, Spain) and Iso-Sensitest broth (Oxoid Unipath, Madrid, Spain) as assay media. It has been suggested that the utilization of these media

instead of RPMI 1640 medium improves the detection of resistance to AMB. The susceptibility testing methodology strictly followed the recommendations of previous reports (1, 14).

End point determination. The MICs were determined spectrophotometrically at 24 and 48 h. The MICs were obtained by measuring the absorbance at 530 nm with an MRX II reader (Dynatech, Cultek, Madrid, Spain). For AMB, the MIC end point was defined as the lowest drug concentration at which the growth of the isolate was reduced by 90% or more compared with that of the control. For flucytosine and the azole drugs, the MIC end point was defined as the lowest drug concentration at which the growth of the isolate was reduced by 50% or more compared with that of the control.

Control strains. Two other clinical isolates of *C. haemulonii* were used as control strains in each set of susceptibility tests. CNM-CL-178 was isolated from human nails and was obtained from the yeast culture collection of the Spanish National Center for Microbiology. CNM-CL-4098 was kindly provided by P. E. Verweij of University Hospital, Nijmegen, The Netherlands; there is no clinical data available for this isolate. Both isolates were identified by routine procedures (17). Likewise, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains for the experiments.

Time-kill curves (TKC). Killing-curve experiments were performed following a standardized procedure (7, 15). Duplicate tests were performed on two separate days. The killing rate tests included RPMI–2% glucose-based and AM3-based procedures. Fungi were subcultured on RPMI–2% glucose agar and AM3 agar and were incubated overnight at 35°C. *Candida*
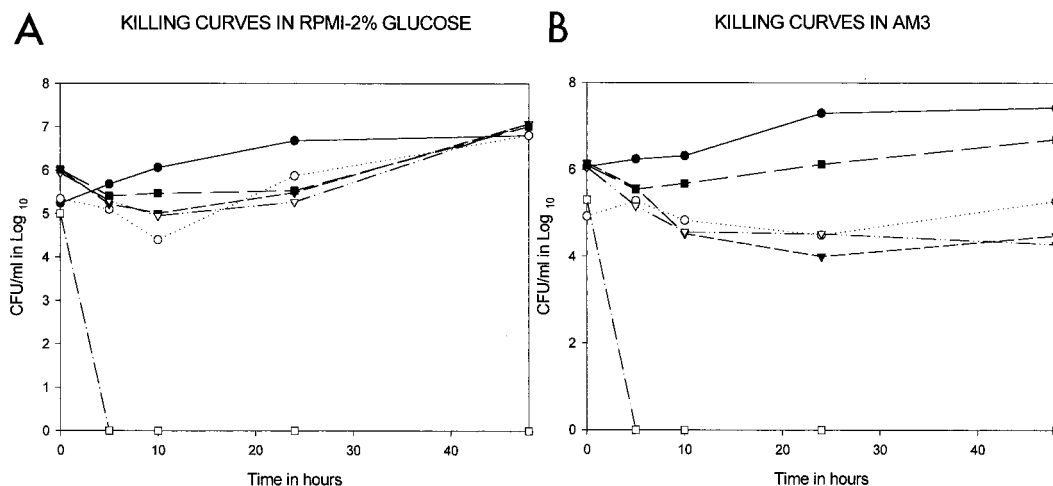
FIG. 1. Killing curves for *C. haemulonii* CNM-CL-3458 tested with various concentrations of AMB in RPMI–2% glucose (A) and AM3 (B). Symbols: ●, control; ○, AMB at 8 μg/ml; ▼, AMB at 4 μg/ml; ▽, AMB at 2 μg/ml; ■, AMB at 1 μg/ml. Also shown are the killing curves for *C. albicans* ATCC 64550 at an AMB concentration of 1 μg/ml (□).

albicans ATCC 64550 was used as the quality control strain. Suspensions were prepared by picking five distinct colonies with diameters of ≥1 mm and suspending them in 5 ml of distilled sterile water. The final inoculum suspension contained between 1×10^7 and 5×10^7 CFU/ml (McFarland standard no. 2).

The assay media for the killing-curve experiments, RPMI–2% glucose broth and AM3 broth, were prepared as described above. AMB was the antifungal agent tested. Stock solutions of reagent-grade powder of AMB (Sigma Chemical Co., St. Louis, Mo.) were prepared in dimethyl sulfoxide (Sigma Chemical Co.) at a concentration of 8 mg/ml. Twofold serial dilutions of AMB were prepared in sterile plastic tubes containing 9 ml of RPMI–2% glucose or AM3 broth. The final concentrations of AMB ranged from 1 to 8 μg/ml. Two tubes containing drug-free medium were used as the sterility and growth controls. The tubes were inoculated with 1 ml of the final inoculum suspension and were incubated at 35°C for 48 h. Samples (0.5 ml) from each tube were extracted after 0, 4, 12, 24, and 48 h of incubation and serially diluted 1:10 four times in the appropriate medium. Thirty-microliter samples of these suspensions were dispensed onto yeast malt agar plates and distributed evenly with sterile, bent glass rods. The plates were incubated for 72 h at 35°C, and the colony counts were determined. Figure 1 displays the colony counts determined at periodic intervals, with the killing rates represented by a plot of the number of survivors present after exposure to AMB. The end point was defined as a killing rate of ≥99.9% of the colony count at time zero.

Results and discussion. The clinical isolate of *C. haemulonii* could not be identified by the API 20C system and was misidentified by the VITEK system. Lehmann et al. (10) reported the existence of two groups within the *C. haemulonii* species and associated some API 20C codes with *C. haemulonii*. One of those codes (code 6102170) is the code that was obtained for the clinical isolate causing the transient fungemia. Curiously, this code is not included in the API 20C database. The results of the identification reference procedures are exhibited in Table 1. The clinical strain demonstrated negative assimilation reactions on galactose, sorbose, melezitose, arabinose, and galactitol and was classified as a group 1 isolate (10). The clinical isolate, like the first isolate described in medical literature, did not produce pseudohyphae or hyphae. However, it has been reported that some strains of this species have the ability to develop limited hyphal growth (8).

The results of the susceptibility and TKC tests are shown in Table 2 and Fig. 1, respectively. Remarkably, susceptibility testing with RPMI 1640 medium revealed a high AMB MIC for the clinical isolate of *C. haemulonii* (CNM-CL-3458). As described previously (1, 14), this medium yields a range of AMB MICs that spans only 3 or 4 twofold serial dilutions, and this short range precludes the making of a reliable distinction between AMB-susceptible and AMB-resistant isolates. So AM3- and Iso-Sensitest agar-based procedures were also performed. Table 2 shows the MICs of AMB obtained with RPMI–2% glucose, AM3, and Iso-Sensitest agar as the assay media. The MICs for the clinical isolate of *C. haemulonii* after 24 h of incubation were 4 μg/ml when testing was done with RPMI 1640 medium, 2 μg/ml with AM3, and 2 μg/ml with Iso-Sensitest agar. As previous studies have suggested, isolates for which the MICs of AMB are ≥0.25 μg/ml with AM3 as the assay medium and ≥0.12 μg/ml with Iso-Sensitest broth as the assay medium should be classified as resistant in vitro (1, 14).

Two *C. haemulonii* collection strains were included as control strains. The AMB MICs for these isolates were high, with the exception of that for CNM-CL-178 at 24 h after incubation, which was lower (≤ 0.5 μg/ml) regardless of the assay medium employed. In addition, the MICs of fluconazole for all the isolates were high, as were those of flucytosine and itraconazole for CNM-CL-4098. In order to evaluate the fungicidal activity of AMB against *C. haemulonii* isolates, TKC tests were performed. These assays confirmed the resistance of these strains. Figure 1 shows that the clinical isolate of *C. haemulonii* was not killed at AMB concentrations of 8 μg/ml. The discrepancies between the MICs (2 to 4 μg/ml) and these killing-curve results can be explained in terms of methodological differ-

TABLE 2. Results of antifungal susceptibility testing of *C. haemulonii* clinical and collection isolates

| Antifungal agent | Incubation time (h) | MIC (µg/ml) on indicated medium for: | | | | | | | | |
| | | CNM-CL-3458[a] | | | CNM-CL-178[b] | | | CNM-CL-4098[b] | | |
		RPMI[c]	AM3	Iso-Sensitest agar	RPMI	AM3	Iso-Sensitest agar	RPMI	AM3	Iso-Sensitest agar
AMB	24	4.0	2.0	2.0	0.5	0.5	0.25	2.0	1.0	1.0
	48	4.0	8.0	2.0	1.0	1.0	0.50	4.0	2.0	1.0
Flucytosine	24	0.12	ND	ND	0.25	ND	ND	128.0	ND	ND
	48	0.12	ND	ND	0.25	ND	ND	128.0	ND	ND
Fluconazole	24	32	ND	ND	8.0	ND	ND	64.0	ND	ND
	48	64	ND	ND	32.0	ND	ND	64.0	ND	ND
Itraconazole	24	0.12	ND	ND	0.12	ND	ND	1.0	ND	ND
	48	0.25	ND	ND	0.25	ND	ND	1.0	ND	ND

[a] Clinical isolate.
[b] Collection isolate.
[c] RPMI, RPMI–2% glucose.
[d] ND, not done.

ences. In contrast to the determination of the MIC, which provides only inhibitory data, the killing-curve technique measures microbicidal activity and offers a dynamic picture of antimicrobial action. In addition, the killing-curve procedure is a macrodilution method with an inoculum size and end point that are different from those of the microdilution method used for MIC determination. For the collection isolates, CNM-CL-178 and CNM-CL-4098, a fungicidal effect was obtained at 4 and 8 µg/ml, respectively. The *C. albicans* quality control strain was killed at concentrations of 1 µg/ml.

There have been a few reports associating *C. haemulonii* with clinical specimens. However, over the last decade, several outbreaks of candidiasis in hospitals have involved infections due to this species (4, 5, 10, 16). In addition, *C. haemulonii* has been recognized as one of the most frequently occurring human pathogenic *Candida* species by experts designing DNA-based systems for the rapid identification of yeast species (3, 12).

Resistance to AMB is rare among *Candida* spp., although high MICs of AMB have been described for some clinical isolates of *C. tropicalis*, *C. rugosa*, *C. lusitaniae*, *C. guilliermondii*, and *C. parapsilosis* (1, 6, 14). There are no data available for *C. haemulonii* antifungal susceptibility patterns. The AMB MICs for the clinical and collection isolates included in this study were high regardless of the assay medium employed (RPMI 1640 medium, AM3, or Iso-Sensitest agar), and the results obtained by the killing-curve tests demonstrated the tolerance of these strains to AMB. In addition, the fluconazole MICs for the three isolates were high. It is unknown whether *C. haemulonii* is inherently or secondarily resistant to AMB. However, the clinical strain was isolated from a patient with no history of antifungal therapy. This case illustrates the significance of the proper identification of yeast species and the need for medical doctors to know the antifungal susceptibility profiles of organisms, particularly those isolates causing bloodstream infections.

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