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CASE REPORT

Echinocandin Resistance in Two *Candida haemulonii* Isolates from Pediatric Patients

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We report 3 cases of patients with *Candida haemulonii* isolates that were obtained from hemocultures. In 2 of the 3 cases, isolates exhibited resistance to echinocandins and fluconazole. This is the first report of an echinocandin-resistant species of this fungus in pediatric patients.

CASE REPORTS

Case 1. A female Down syndrome patient aged 1 year and 7 months and diagnosed with cardiopathy (total atrioventricular septal defect) was hospitalized on 24 November 2009 for total surgical correction. During surgery, a double-lumen central venous catheter (CVC) was inserted in the right internal jugular vein. The patient exhibited good postsurgical recovery; the CVC was withdrawn and she was discharged from the intensive care unit (ICU) on 7 December 2009. Subsequently, she presented with chylothorax and was readmitted into the ICU 3 days later for drainage. Her fever was treated with cefepime and then vancomycin. On 14 December 2009, a new CVC was inserted, without any complications. A blood culture (BC) from the catheter was positive for coagulase-negative *Staphylococcus*. A new BC was obtained, and treatment with amphotericin B deoxycholate and meropenem was initiated. The BC showed growth of *Candida haemulonii*. Ultrasonography showed pleural effusion with septations; surgical debridement was performed to remove necrotic tissue from the lung apex. On 13 January 2010, a new episode of sepsis occurred, with the BC testing positive for *C. haemulonii*. Amphotericin B deoxycholate was replaced with liposomal amphotericin B and fluconazole. The patient exhibited good clinical recovery with this new antifungal treatment and completed 20 days of treatment. She was discharged on 12 February 2010 after the results of 2 BCs were negative.

Case 2. A 9-year-old female patient was hospitalized on 9 April 2010 with febrile neutropenia following chemotherapy for Ewing's sarcoma. She was treated with piperacillin-tazobactam according to the institutional protocol for febrile neutropenia. Three days later, she was underwent an odontological evaluation due to a suspected dental abscess, which confirmed the suspicion; she subsequently required drainage. On the next day, the antibiotic was replaced with imipenem. On the seventh and eighth day of admission, she had a fever of approximately 38°C. On the 10th day of admission, an echocardiogram showed an image on the interatrial septum, which suggested vegetation or a thrombus, and subsequently, vancomycin treatment was initiated. On the 19th day of admission, an echocardiography showed an increase in the size of the vegetation. Antibiotics were maintained through peripheral venous access (PVA). On the 22nd day, she presented with fever (38.5°C) and hyperemia at the PVA site together with pain at the insertion point of the catheter, and a new BC was collected. The culture showed the presence of *C. haemulonii*, probably due to phlebitis at the PVA site. Amphotericin B deoxycholate was administered for 14 days. The patient exhibited resolution of endocarditis after 28 days of antibiotics; the BC was negative for fungal infection after 14 days of antifungal treatment, and she was discharged subsequently.

Case 3. A 16-year-old female patient was diagnosed with acute myeloid leukemia on 18 May 2009. Since then, she had experienced various episodes of febrile neutropenia with multiple hospital admissions to treat several infectious presentations. Subsequently, she presented with fever due to phlebitis at the PVA site, and an ambulatory BC showed growth of *C. haemulonii*. The patient did not receive antifungal treatment or present with new febrile episodes; she was therefore characterized as exhibiting transient, auto-limited fungemia. The patient continued to follow her chemotherapy without further complications.

The clinical impact of fungal infections has increased substantially in recent years, particularly in immunocompromised patients. *Candida* spp. have emerged as one of the major opportunistic pathogens in such patients. These infections are difficult to diagnose and cause high levels of morbidity and mortality despite antifungal therapy (7). This paper describes and discusses findings in 3 pediatric patients with candidemia caused by *C. haemulonii* isolates, in two cases resistant to echinocandins.

Microbiological data. Hemocultures were processed according to the standard protocol of the American Society for Microbiology (19) and inoculated at 37°C using a BD Bactec 9120 blood culture system (Becton, Dickinson, Franklin Lakes, NJ). Colonies on Sabouraud dextrose agar had a white, smooth, glabrous yeast-like appearance. Microscopic morphology showed numerous ovoid-to-globose-shaped budding yeast-like cells. No pseudohyphae were produced. A germ tube test was negative, hydrolysis of urea was negative, growth on cycloheximide medium was positive, and growth at 37°C was positive (13). Phenotypic identifica-

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TABLE 1 Results from the antifungigram of the 3 isolates of *Candida haemulonii*

Antifungal	MIC (μg/ml) and susceptibility type[a]								
	Case 1			Case 2			Case 3		
	S	SDD	R	S	SDD	R	S	SDD	R
Fluconazole		32.0			16.0			16.0	
Amphotericin	1.0			1.0			1.0		
Micafungin	0.5					4.0			4.0

[a] S, sensitivity; SDD, dose-dependent sensitivity; R, resistance.

tion of the isolates was performed using chromogenic agar (CHROMagar, Paris, France) and an API 20C AUX system (bioMérieux, Marcy l'Etoile, France), and isolates were identified as *Kodamaea* (*Pichia*) *ohmeri* in all 3 samples. The API ID code was approximately 90% for all 3 strains. The Vitek yeast biochemical card (YBC) (bioMérieux, Durham, NC) system was also used for identification; this yielded inconclusive results for the first isolate and identified the other 2 isolates as *C. haemulonii.*

Molecular data. The isolates were identified using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Samples were analyzed in triplicate, using the Putignani method (12). Scores were satisfactory (above 2) and identification was compatible with *C. haemulonii* for all 3 samples. PCR followed by DNA sequencing was used as the gold standard for identification. The internal transcribed spacer (ITS) and D1/D2 regions of the ribosomal gene were amplified. For amplification of the ITS region by PCR, the external primers V9G and LS266, which specifically amplify this region in fungi, were used, and primers NL1 and NL4 were used to amplify the D1/D2 region (5, 18). For sequencing of the amplified products, ITS1 and ITS4 primers (21) were used for the ITS region, and NL1 and NL4 primers were used for the D1/D2 region. Sequencing was performed using the dideoxy chain-terminating method (16), using an automatic sequencer with BigDye Terminator (Applied Biosystems, Foster City, CA). The consensus sequences of the ITS and D1/D2 regions were aligned using the Sequencher 4.1.4 program and analyzed by comparison with DNA databases, using the BLAST tool to identify the species. The gene bank used was the NCBI database: the 3 samples were identified molecularly as *C. haemulonii.* The parameters determined by BLAST analysis were 96% identity and an E value of 6×10^{-175} for the ITS region and 100% identity and an E value of 0.0 for the D1/D2 region. These results are considered highly reliable for the identification of species.

Analysis of the microbiological results indicates that the phenotype tests were inadequate to identify *C. haemulonii* but that the molecular tests (PCR and sequencing, as well as MALDI-TOF) showed 100% agreement. Similar results have been reported by Stevenson et al. (17).

Antifungigram. Susceptibility assays to the antifungal agents amphotericin B (Sigma-Aldrich Quimica, Madrid, Spain), fluconazole (Pfizer, Madrid, Spain), and micafungin (Mycamine; Astellas Pharma Inc., Toyama, Japan) were performed, using the broth microdilution method, in accordance with protocol M27-A3 of the Clinical and Laboratory Standards Institute (1, 2). The results (Table 1) showed that the MIC was 1.0 μg/ml for amphotericin B for all 3 samples; for fluconazole, the MICs were 16 μg/ml in 2 samples and 32 μg/ml in the other sample. For micafungin, an MIC of 0.5 μg/ml was found in 1 sample and an MIC of 4.0 μg/ml in the other 2 samples. These results indicate that the samples presented a dose-dependent response to fluconazole, were resistant to micafungin, and were sensitive (borderline) to amphotericin B, according to the CLSI 2008 M27-A3 protocol. However, although the strains with MICs of >2 μg/ml for echinocandins were not considered susceptible, the cutoff for resistance has not yet been established due to a lack of clinical isolates available from patients with therapeutic failure (11).

Species-specific clinical breakpoints (BPs) have been developed for fluconazole (8) and micafungin (9) and do not cover rare species such as *C. haemulonii*. The BPs used in this research were determined by the Clinical and Laboratory Standards Institute (1, 2).

In invasive fungemia, *Candida albicans* is the most common isolated species, although other non-*C. albicans* species, such as *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, and *Candida glabrata*, have a global distribution and are clinically important (7, 10). New *Candida* species have appeared and been recognized as potential pathogens, being described as emerging fungi. Some of these are closely related to other less rare fungi: *Candida orthopsilosis*, *Candida metapsilosis*, *K. ohmeri*, and *C. haemulonii* are all closely related to *C. parapsilosis* (6, 20). *C. haemulonii* is also related to *Candida famata* (teleomorph *Debaryomyces hansenii*) and *Candida guilliermondii* (teleomorph *Pichia guilliermondii*), which hampers its identification (3). Most *Candida* species respond well to treatment with azoles, amphotericin, and echinocandins; however, some species are resistant to these treatments (10). Laboratory identification of *C. haemulonii* is important because there is evidence of decreased susceptibility of this species to the antifungals typically used in clinics (15).

Clinical presentation of infection by *C. haemulonii* is characterized by fever, without specificities related to other nosocomial infections. The interesting feature of these 3 cases of *C. haemulonii* infection was the fact that they did not involve very young children, despite having been isolated in a pediatric hospital. Other predisposing factors frequently reported for this type of candidemia are the insertion of a CVC, malignancy, and mechanical ventilation (MV) (3, 4, 14). The patient in case 1 was hospitalized in an ICU and therefore had various predisposing factors such as MV, prolonged use of a CVC, and prolonged use of antimicrobials. In cases 2 and 3, both patients had oncohematologic diseases and candidemia was clearly associated with phlebitis, with the PVA site being the entry point.

C. haemulonii is quite resistant to antifungals and appears to be associated with environmental contamination. Accurate identification of this species in clinical samples is of major importance; however, traditional methods of *Candida* identification are not efficient at identifying these rare species (4). Although the API system is frequently used in the identification of fungi, Lee et al. have shown that 3 strains of *Candida* were not adequately identified by this method, with 2 later being identified as *C. haemulonii* and 1 as *C. parapsilosis* (6). Similarly, Rodero et al. showed in 2002 that isolates of *C. haemulonii* could be erroneously identified as *K. ohmeri* (86% probability of identification) using the same API 20C system and the Vitek identification system (14). Published results have shown that the Vitek 2 system is more consistent than mo-

lecular methods in identifying *C. haemulonii* but also that it cannot differentiate *C. haemulonii* from closely related species such as *Candida pseudohaemulonii* (4).

In the cases reported here, the identification of *C. haemulonii* by the API 20C system was incorrect, with all strains being identified as *K. ohmeri*. Upon performance of PCR and sequencing, these strains were identified as *C. haemulonii*; MALDI-TOF mass spectrometry, an easily accomplished method that is increasingly being used to identify species, also correctly identified these strains.

This case description also highlights the resistance of *C. haemulonii* isolates to antifungals. Previous studies reported that *C. haemulonii* was resistant to fluconazole and amphotericin B but susceptible to echinocandins (3, 4, 14). In our report, the isolates obtained from the 3 patients showed identical patterns of susceptibility, with borderline sensitivity to amphotericin B, dose-dependent sensitivity to fluconazole, and resistance to the echinocandins tested (micafungin). Once the genome of *C. haemulonii* is sequenced, molecular studies will be able to identify mutations that are responsible for this resistance.

Echinocandin treatment was not used in any of the clinical cases. In case 1, the patient showed improvement only after fluconazole was combined with amphotericin B treatment. The therapeutic failure of amphotericin B and therapeutic response associated with fluconazole could be explained by the fact that the isolate presented a dose-dependent sensitivity to fluconazole (used in this case at the maximum dose of 12 mg/kg/day) and borderline sensitivity to amphotericin B. In cases 2 and 3, it is likely that the patients had transient candidemia provoked by inflammation (barrier loss) at the PVA site, which is considered an access point to the bloodstream for fungi, because the withdrawal of the catheter leads to an improvement in phlebitis and a negative hemoculture. However, in the second case, the patient received amphotericin B for 14 days, and her improvement may have been related to this therapy, even if the MIC for this antifungal was a borderline concentration.

These cases illustrate the importance of correctly identifying *Candida* species and the difficulty in identifying *C. haemulonii* using phenotypic methods. PCR methods, sequencing, and MALDI-TOF mass spectrometry presented similar results, confirming the identification of this fungus. Susceptibility antifungal tests are fundamental to the identification of emerging fungi, and these are the first cases of *C. haemulonii* isolates with resistance to echinocandins in a Brazilian pediatric hospital.

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