# *In vitro* Synergistic Activity of Caspofungin Plus Polymyxin B Against Fluconazole-Resistant *Candida glabrata*

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## ABSTRACT

**Background:** *Candida* species account for most invasive fungal infections, and the emergence of fluconazole and caspofungin resistance is problematic. Overcoming resistance with synergism between 2 drugs may be useful. In a 2013 *in vitro* study, caspofungin plus colistin (polymyxin E) was found to act synergistically against fluconazole-resistant and susceptible *Candida albicans* isolates. The purpose of our study was to extend this finding by evaluating caspofungin plus polymyxin B for *in vitro* synergy against fluconazole-resistant *Candida glabrata* isolates.

**Materials and Methods:** A total of 7 fluconazole-resistant *C. glabrata* bloodstream infection isolates were obtained from 2010–2011. Of these, 2 isolates were also resistant to caspofungin. Minimum inhibitory concentrations (MICs) for caspofungin and polymyxin B were determined by Etest and broth microdilution. Clinical and Laboratory Standards Institute breakpoints were used for fluconazole and caspofungin MIC interpretations. No interpretive guidelines exist for testing polymyxin B against *C. glabrata*. Synergy testing with caspofungin (1 × MIC) and polymyxin B (½MIC) was performed using a modified bacterial Etest synergy method and time-kill assay.

**Results:** With the Etest synergy method, 4 out of 7 isolates showed *in vitro* synergy and 1 out of 7 showed additivity. The remaining isolates (both caspofungin resistant) showed indifference. Using the time-kill assay, 1 out of 7 isolates showed synergy, 1 showed additivity and the remaining 5 (including both caspofungin-resistant isolates) showed indifference.

**Conclusions:** Caspofungin susceptibility may be required for synergism between caspofungin and polymyxin B. Further synergy testing with caspofungin plus polymyxin B and additional fluconazole-resistant *C. glabrata* isolates should be performed. *In vitro* synergy/additivity may or may not correlate with *in vivo* benefit.


**Keying Indexing Terms:** *Candida glabrata*; Caspofungin; Fluconazole resistance; Polymyxin B; Synergy. **[Am J Med Sci 2016;351(3):265–270.]**

## INTRODUCTION

The number of *Candida* bloodstream infections (BSIs) has risen during the past few decades.[1] In the United States, *Candida* species are currently the fourth most common cause of healthcare-associated BSIs.[2,3] *Candida* BSIs have been shown to increase hospitalization stays and healthcare costs, with each episode costing approximately $40,000.[1] In addition, mortality rates for invasive *Candida* infections are the highest among all causes of nosocomial BSIs and can be as high as 45%.[3,4]

A total of 5 *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*) are known to cause up to 95% of all invasive *Candida* infections. Of these 5, the most commonly isolated species remains *C.* albicans, in the United States, there has been an increase in the proportion of *Candida* BSIs caused by *C. glabrata*.[5–8] Although the cause appears to be multifactorial, the increase in the number of *C. glabrata* infections is thought to be partially because of the increased use of fluconazole, which occurred after its approval in the 1990s, and the emergence of innate fluconazole-resistant *C. glabrata* that came along with it.[4,9]

Owing to the emergence of fluconazole resistance and the increased prevalence of *Candida* species with reduced fluconazole susceptibility, echinocandins are now being used more frequently for the treatment of invasive candidiasis.[9] Echinocandins (caspofungin, micafungin and anidulafungin) are the newest available antifungal agents and are considered the first-line treatment for *Candida* BSIs in critically ill patients, patients with a history of azole use, and in those infected with *C.* glabrata.[9,10] However, there have also been reports of echinocandin resistance among *Candida* species, especially in *C. glabrata*, and this appears to be on the rise.[3,5] A study found that the rate of echinocandin-resistant *C. glabrata* has increased from 4.9–12.3% between 2001 and 2010.[11] Even more alarming are the reports of *C. glabrata* isolates resistant to both fluconazole and echinocandins. A review of data from 2 large surveillance programs (the SENTRY Antimicrobial Surveillance

Program for years 2006-2010 and the Centers for Disease Control and Prevention population-based surveillance study for years 2008–2010) showed that 11% of the fluconazole-resistant *C. glabrata* BSI isolates collected during the course of 2 programs were also resistant to 1 or more of the echinocandins.[10]

The emerging resistance associated with existing antifungals has become a serious health threat, and finding new ways to combat this changing *Candida* population is becoming increasingly more important. Researchers have looked toward synergism among different antimicrobials as a possible solution. Polymyxins are a group of antibiotics that work by binding to the bacterial cell membrane, which alters its permeability and ultimately causes cell death, but the exact mechanism of their weak antifungal activity is unknown.[12-16] In a 2013 *in vitro* study, caspofungin plus colistin (polymyxin E) was found to act synergistically against fluconazole-resistant and susceptible *C. albicans* isolates. The same study also found synergy *in vivo*, using mice models, between caspofungin and colistin against fluconazole-susceptible *C. albicans* isolates.[13] Unlike this *in vitro* study, our present study evaluated the combination of caspofungin plus polymyxin B against fluconazole-resistant *C. glabrata*. In 2014, a study from our laboratory was published in which *in vitro* synergy was found between polymyxin B and fluconazole against fluconazole-resistant and fluconazole-susceptible *C. glabrata* isolates.[12] For the present study, these findings were evaluated further by testing caspofungin plus polymyxin B for synergy against fluconazole-resistant *C. glabrata* BSI isolates, using a modified Etest synergy method and time-kill assay.

## MATERIALS AND METHODS

### Microorganisms, Media and Antimicrobial Agents

In total, 7 fluconazole-resistant *C. glabrata* BSI isolates were collected in 2010–2011 from individual patients at Ochsner Medical Center, New Orleans, LA. Approval for the study was granted by the Institutional Review Board of the Ochsner Clinic Foundation. Isolates were identified using the API 20C yeast identification system (bioMérieux, Durham, NC). Plated media used included Sabouraud dextrose agar (Becton-Dickinson Microbiology Systems, Sparks, MD), used for the initial subculture of yeast isolates and spiral plating during the time-kill assay, and RPMI 1640 agar with morpholinepropanesulfonic acid and 2% glucose (Remel, Lenexa, KS), used for Etest minimum inhibitory concentration (MIC) determination and synergy testing. Caspofungin and polymyxin B Etest strips (bioMérieux, Durham, NC) were also used for Etest MIC determination and synergy testing. For broth microdilution MIC determination and time-kill assay, standard laboratory powders of caspofungin and polymyxin B (Sigma-Aldrich, St. Louis, MO) were used. Quality control testing was performed using *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.[17,18]

### Antimicrobial Susceptibility Testing

#### *Etest MICs*

MICs for caspofungin and polymyxin B were determined in triplicate using the Etest method, following manufacturer's guidelines for *Candida* species. Mean values were used for interpretation. Fluconazole MICs were determined in a previous study. Clinical and Laboratory Standards Institute (CLSI) 2012 breakpoints used for MIC interpretation for Etest and broth microdilution were caspofungin, $\leq$0.12 μg/mL susceptible, 0.25 μg/mL intermediate and $\geq$0.5 μg/mL resistant; fluconazole, $\leq$32 μg/mL susceptible-dose dependent, $\geq$64 μg/mL resistant.[17] No interpretive guidelines are available for testing polymyxin B against *C. glabrata*. RPMI plates were incubated at 35°C in a loosely folded plastic bag to maintain moisture, and MICs were read at 24 hours. Caspofungin MICs were read at 80% inhibition of visual growth (when trailing end points or other growth/inhibition phenomena occurred), and polymyxin B MICs were read at the lowest concentration to show complete inhibition of visual growth. Etest MIC values between 2-fold dilutions were rounded up to the next 2-fold value for interpretive categorization.

#### *Broth Microdilution MICs*

MICs for caspofungin and polymyxin B were also determined by broth microdilution, following the 2008 CLSI guidelines.[19] Fluconazole MICs were determined in a previous study. Microtiter trays were inoculated and incubated at 35°C. MICs were read at 24 hours. Caspofungin MICs were read at 50% inhibition of visual growth, and polymyxin B MICs were read at the lowest concentration to show complete inhibition of visual growth.

### Synergy Testing

#### *Etest Method*

*In vitro* synergy was evaluated with caspofungin (1 × MIC) plus polymyxin B (½MIC) in triplicate using a modified bacterial Etest MIC:MIC synergy method (Figure).[12] RPMI agar plates were inoculated twice with a suspension adjusted to the turbidity of a 0.5 McFarland standard of each isolate. Caspofungin and polymyxin B Etest strips were placed on different areas of each agar plate and allowed to incubate at room temperature for 1 hour. The agar was marked adjacent to the previously determined MIC value on each strip (caspofungin, the MIC; polymyxin B, ½MIC). The strips were removed, and a new caspofungin strip was placed on the area of the original polymyxin B strip, so that the caspofungin MIC corresponded with the mark for ½MIC polymyxin B. A new polymyxin B strip was placed in reciprocate fashion. The plates were incubated at 35°C in loosely folded plastic bags to maintain moisture,