The European Confederation of Medical Mycology (ECMM) survey of candidaemia in Italy: \textit{in vitro} susceptibility of 375 \textit{Candida albicans} isolates and biofilm production

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Objectives: To investigate the \textit{in vitro} antifungal susceptibility pattern of 375 \textit{Candida albicans} bloodstream isolates recovered during the European Confederation of Medical Mycology survey of candidaemia performed in Lombardia, Italy and to test the ability to form biofilm.

Methods: \textit{In vitro} susceptibility to flucytosine, fluconazole, itraconazole, posaconazole, voriconazole and caspofungin was performed by broth microdilution following the NCCLS guidelines. Biofilm production was measured using the XTT reduction assay in 59 isolates selected as representative of different patterns of susceptibility to flucytosine and azoles.

Results: MICs (mg/L) at which 90% of the strains were inhibited were \(\leq 0.25\) for flucytosine, 0.25 for caspofungin, 4 for fluconazole and 0.06 for itraconazole, voriconazole and posaconazole. Flucytosine resistance was detected in five isolates and was associated with serotype B in 2/29 and serotype A in 3/346. Resistance to fluconazole was detected in 10 isolates; nine of these exhibited reduced susceptibility to the other azoles. Among the 10 patients with fluconazole-resistant \textit{C. albicans} bloodstream infection, only one, an AIDS patient, had been previously treated with fluconazole. Biofilm production was observed in 23 isolates (39%) and was significantly associated with serotype B. No relationship was detected with the pattern of antifungal susceptibility.

Conclusions: Resistance is uncommon in \textit{C. albicans} isolates recovered from blood cultures, while biofilm production is a relatively frequent event. Periodic surveillance is warranted to monitor the incidence of \textit{in vitro} antifungal resistance as well as of biofilm production.

Keywords: flucytosine, caspofungin, azoles, voriconazole, posaconazole

Introduction

Although the emergence of non-\textit{albicans} \textit{Candida} spp. as a cause of bloodstream infection (BSI) has been reported in all surveillance programmes,\(^1\) \textit{Candida albicans} remains the predominant species recovered. In the large prospective survey performed by the European Confederation of Medical Mycology (ECMM) \textit{C. albicans} was identified in 1178 out of 2089 reported episodes (56%),\(^2\) ranging from 43% in Spain\(^3\) to 67% in Sweden\(^4\) and was responsible for more than half of the cases in all the patient populations except in patients with haematological malignancies.\(^5\)

The introduction of fluconazole in the 1990s has improved the outcome of Candida BSIs. However, the formation of biofilms on inert or biological surfaces frequently associated with deep seated candidosis enhances resistance to antimicrobial agents and protection from host defences making these infections refractory to conventional therapy.\(^5\) Amphotericin B lipid formulations and echinocandins have been shown to have activity against \textit{Candida} biofilms as the inhibition of polysaccharide production could lead to lysis and dissolution of the extracellular matrix.\(^5\)

The aim of the present report is to investigate the antifungal susceptibility pattern of 375 \textit{C. albicans} isolates recovered from blood cultures during the ECMM survey of candidaemia (09/1997 to 12/1999) in Lombardia, Italy,\(^6\) and to test the ability to form biofilm.

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**Materials and methods**

**Isolates**

A total of 375 C. albicans strains isolated from blood (the first isolate from each episode) during the ECMM survey of candidaemia were studied. Thirty-five medical centres in Lombardia, Italy, participated in the survey from September 1997 to December 1999.

The strains received by the coordinating centre were subcultured on Chromagar Candida medium (CHROMagar Microbiology, Paris, France) to ensure viability and purity.

Yeast identification was checked by production of germ tubes in serum and chlamydompores in potato–carrot–ox gall agar. Serotype was identified by slide agglutination test with specific antisera (Candida Check; Iatron Laboratories, Tokyo, Japan).

Isolates were stored as suspensions in distilled water at room temperature until needed.

**Susceptibility testing**

The antifungals tested were fluconazole (Pfizer Central Research, Sandwich, UK), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ, USA), fluconitine (Sigma-Chemical, Milano, Italy) and caspofungin (Merck & Co., Whitehouse Station, NJ, USA). Susceptibility was determined by broth microdilution method performed following the recommendations of the NCCLS.7 Testing was performed in RPMI 1640 without sodium bicarbonate (Sigma-Chemical) and buffered to pH 7.0 with 0.165 M MOPS (Sigma-Chemical) and supplemented with 2% glucose and 0.03% L-glutamine (Sigma-Chemical). For strains exhibiting a significant trailing effect when tested against azoles, susceptibility testing was also performed with Casitone broth or RPMI broth with/without Alamar Blue. MICs for the quality control strain, C. parapsilosis ATCC 22019, were within the expected range.

Only five isolates (1.3%) showed resistance to fluconazole (MIC ≥ 32 mg/L) confirming the low rate of resistance reported in the literature.9 Resistance was associated with serotype B in 2/9 (22%) and with serotype A in 3/346 (0.9%).

Resistance to fluconazole (MIC ≥ 64 mg/L) was detected in 10 isolates (2.7%), all identified as serotype A. This is in agreement with the negligible proportion of resistance among C. albicans bloodstream isolates reported elsewhere.1,9,11 Among the 10 patients with fluconazole-resistant C. albicans BSI only one, an AIDS patient, had been treated with fluconazole during the 2 weeks preceding candidaemia. The isolate from this patient also exhibited resistance to fluconazole.

Isolates were highly susceptible to the other azoles. MIC90s of itraconazole, voriconazole and posaconazole were 0.06 mg/L. However, as previously noted,1,10 the fluconazole-resistant isolates tended to be less susceptible to the other azoles and nine of these exhibited reduced susceptibility to itraconazole, voriconazole and posaconazole.

Caspofungin MICs ranged from 0.03 to 1 mg/L, and 86% and 98% of isolates were inhibited by 0.12 and 0.25 mg/L of inoculated in flat-bottom 96-well microtitre plates. Biofilm production was measured after 24 h by using a 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT; Sigma-Chemical) reduction assay. XTT assay absorbance was read spectrophotometrically (Multiskan MS; Labsystems, Needham Heights, MA, USA) at 490 nm. The percentage transmittance (%T), calculated from absorbance, was inversely proportional to the cellular density of the biofilm. Biofilm production was scored as 6+ (%T ≤ 5), 5+ (%T 6–10), 4+ (%T 11–20), 3+ (%T 21–40), 2+ (%T 41–60) or 1+ (%T > 60). Isolates showing scores of 6+ or 5+ were considered good producers of biofilm.

**Results and discussion**

Table 1 summarizes the in vitro susceptibilities of the 375 bloodstream C. albicans isolates to flucytosine, fluconazole, itraconazole, voriconazole, posaconazole and caspofungin. MICs (mg/L) at which 50% (MIC50) and 90% (MIC90) of the strains were inhibited and the range of MICs are reported. Ninety-nine isolates (26.4%) exhibited a significant trailing effect when tested against azoles. These isolates, however, tested susceptible in repeated tests performed using Casitone broth or RPMI broth with/without Alamar Blue. MICs for the quality control strain, C. parapsilosis ATCC 22019, were within the expected range.

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<th>Table 1. MIC50, MIC90 and range of MICs (mg/L) for 375 C. albicans bloodstream isolates and MIC for the quality control strain</th>
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aAccording to NCCLS breakpoints; ≥2 has been adopted as presumptive breakpoint for voriconazole, posaconazole and caspofungin.

bSusceptible-dose-dependent according to NCCLS.

cC. parapsilosis ATCC 22019.
In vitro antifungal susceptibility of C. albicans and biofilm production

Figure 1. Biofilm production according to different patterns of fluconazole susceptibility.

Caspofungin, respectively. Eighteen isolates showed the well described paradoxical effect of a regrowth at highest concentrations (4 and 8 mg/L) after growth inhibition in the wells with lower echinocandin levels (0.25–0.5 to 2). This phenomenon was more common reading after 48 h of incubation. The paradoxical effect was more frequent in serotype B than in serotype A strains (9 out of 29 versus 9 out of 346, \( P \leq 0.001 \)).

Biofilm production was observed in 23 isolates (39%) out of the 59 tested strains and was significantly associated with serotype B (19/28 versus 4/31 in serotype A, \( P < 0.001 \)). No relationship between biofilm production (scores of 6+ and 5+) and a specific pattern of susceptibility to fluconazole was detected (Figure 1). This lack of correlation could be explained by the use of planktonic populations in the antifungal susceptibility testing performed according to NCCLS guidelines.

In conclusion, the low proportion of antifungal resistance detected in this survey, consistent with data reported in both European and American surveillance programmes, appears reassuring and supports the indication to perform antifungal susceptibility testing only in case of failure of response to initial therapy or of breakthrough candidaemia in patients receiving fluconazole prophylaxis. However, the relatively high rate of biofilm production among the tested isolates is noteworthy, as biofilm lifestyle has been demonstrated to increase levels of resistance to the most commonly used antifungal agents.

Therefore, periodic surveillance is warranted to monitor the incidence of in vitro antifungal resistance as well as of biofilm production.

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References