Candidemia: species involved, virulence factors and antimycotic susceptibility

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SUMMARY

We investigate the characteristics of the *Candida* species involved in BSI episodes in our Institute, their phospholipase and protease activity and the susceptibility pattern towards the main antifungal agents currently available. From January 2009 to December 2010 we documented a total of 59 episodes of candidemia. The incidence of candidemia was 32% in General Surgery, 22% in the Intensive Care Unit (ICU), 13% in Oncology and 10% in Gastroenterology. *C. albicans* was the most common species (32 cases=48%), followed by *C. glabrata* (17 cases=26%) and *C. parapsilosis* (12 cases=18%), a significant production of phospholipase in all strains of *C. albicans* was detected. Among *Candida* non-*albicans* species, the production of this enzyme only occurred in 1/12 strains of *C. parapsilosis*. The expression of acid protease production was detected in 48% of *C. albicans* and no strains of *Candida* non-*albicans*.
All species of *Candida* were susceptible to amphotericin B. The rate of susceptibility to fluconazole was 100% for *C. albicans* and *C. parapsilosis*. Decreased susceptibility to fluconazole was mostly seen with *C. glabrata*, which was 76.5% susceptible in a dose-dependent manner. The echinocandins showed a good performance for *C. albicans,* and maintained a good MIC distribution in *C. glabrata*.

KEY WORDS: Candidemia, Virulence factors, Protease, Phospholipase, Antimycotic susceptibility.

Received February 02, 2012 *Accepted* April 24, 2012

INTRODUCTION

In the immunocompetent host, *Candida albicans* is a benign member of the human microbiota, and colonizes the human gastrointestinal, respiratory and reproductive tracts. However, when the host physiology is disrupted, this commensal-host interaction can degenerate and lead to an opportunistic infection (Mochon *et al.*, 2010). *Candida* bloodstream infections (BSI) are common causes of the nosocomial infections with the highest mortality rates (Tumbarello *et al.*, 2007), and currently represent the fourth most frequent pathogens involved in BSI in the United States (Ben-Ami *et al.*, 2008).

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In addition to predisposing factors relating to the host, *Candida* species are able to cause infection depending on intrinsic characteristics relating to the microorganism itself. Germ tube production, growth at 37°C, protease and phospholipase production and ability to adhere to buccal epithelial cells (BEC) are considered to be important factors in establishing the infection. Proteinase and phospholipase production can lead to dysfunction or even rupture of cell membranes, which facilitate adhesion of the microorganism to the host. In fact, successful colonization and infection by a microorganism depend upon their initial ability to adhere to host tissue (Costa *at al.*, 2010). Moreover, *C. albicans* forms a robust, architecturally complex biofilm on implanted synthetic material *in vivo,* most notably on intravascular catheters, but also on peritoneal dialysis catheters, ventricular peritoneal shunts and other implanted devices necessary for patient survival and quality of life. These biofilm infected catheters serve as reservoirs of infectious parti-

cles, releasing cells into the bloodstream where they gain access to distant sites, with the potential for metastatic infection of deep organs (Uppuluri *et al.*, 2010).

The estimated attributable outcomes of candidemia are represented by prolongation of hospital stay by 10 days, increased expenditure of around 40,000 dollars per case, and excess mortality of 14% to 49% (Gudlaugsson *et al.*, 2003; Zaoutis *et al*., 2005). Reported predictors of mortality include age, duration of hospitalization, severity-of-illness score, acute renal failure, intensive care unit (ICU) stay, retention of central venous catheter, mechanical ventilation, and *Candida* non-*albicans* species involved (Fraser *et al*., 1992; Nolla-Salas *et al.*, 1997; Zaoutis *et al*., 2005; Labelle *et al*., 2008). In addition, two studies have demonstrated that a delay in starting antifungal therapy is associated with increased mortality (Morrell *et al*., 2005; Garey *et al*., 2006), and inadequate antifungal therapy is an important predictor of adverse outcome (Ibrahim *et al*., 2000; Harbarth *et al*., 2002; Hajjeh *et al*., 2004).

Although *C. albicans* remains the most common fungal isolate recovered from blood, recent reports indicate a trend towards an increasing prevalence of infections caused by species of *Candida* other than *C. albicans* (Blumberg *et al*., 2001; Trick *et al*., 2002; Pappas *et al.*, 2003; Marr, 2004; Pappas *et al*., 2004; Nucci & Marr, 2005; Fridkin, 2005) which are associated with a high mortality rate, particularly in the case of bloodstream infection caused by *C. krusei*, which is innately resistant to fluconazole, and *C. glabrata,* which easily develops azole resistance (Morace & Borghi, 2010). Moreover, less common species may emerge as important opportunistic pathogens in the future, so it is important to describe the activities of both new and established antifungal agents as potential therapeutic options (Diekema *et al.,* 2009).

The aim of our present study was to investigate the characteristics of the *Candida* species involved in BSI episodes in our Institute to define the role of the biological characteristics of the species involved in pathogenicity, and the optimal therapeutic approach. We therefore studied the species involved, and determined the phopholipase and protease activity of *Candida* species isolated from blood infection and the susceptibility pattern towards the main antifungal agents currently available.

MATERIALS AND METHODS

From January 2009 to December 2010 we documented a total of 59 episodes of candidemia at the Humanitas Clinical Institute. A case of candidemia was defined as isolation of a *Candida* species from a blood culture. Only the first episode was reported in the case of patients with recurrent episodes of infection. After 5 days' incubation of blood culture bottles in the automated system (Bactec 9240, Becton Dickinson, Cocesville, USA) at 37°C, a total of 66 *Candida* strains were recovered. All isolates were identified by Gram staining, subcultures on CHROMagar™ Candida (Becton Dickinson, Cocesville, USA), and the API ID 20 C AUX testing kit (bioMérieux AS, Marcy-l'Etoile, France).

The antifungal susceptibility testing of all isolates was performed on Sensititre YeastOne panel (Trek Diagnostic Systems, East Grinstead, UK) containing Anidulafungin (AND), Micafungin (MF), Caspofungin (CAS), 5-flucytosine (FLU), Voriconazole (VOR), Itraconazole (IT), Fluconazole (FLU) and Amphotericin B (AB). The isolates were tested in accordance with the manufacturer's instructions, using an inoculum at the concentration of $1x10^6$ to $5x10^6$ cells per ml in RPMI 1640 broth. Plates containing twofold serial dilutions of antifungal agents across 12 dilutions were inoculated with the yeast suspension. They were then incubated at 35°C and read at 24 h. The MIC was determined as the lowest concentration of antifungal agent preventing development of a red color (the first blue well). MIC was evaluated according to CLSI and EUCAST criteria when available.

The MIC was interpreted blindly by two different laboratory operators. Proteinase production was detected by the method of Dagdeviren *et al.* (Dagdeviren *et al.*, 2005). The assay was performed in triplicate for each yeast isolate tested. Briefly, a yeast suspension was made in YEPD broth containing yeast extract, peptone and glucose. Ten µl of this suspension were put on a sterile paper disk placed on the surface of bovine serum albumin agar medium (pH 5.0). The plates were incubated at 30°C for 5 days, and were observed each day for increasing opacity around the disks caused by the growing fungi. Subsequently, clearing of the opacity corresponding to hydrolysis of precipitated albumin was recorded.

Proteinase activity (Pz) was determined as the ratio of the colony diameter to the diameter of the cleared proteolytic zone. Proteinase activity was evaluated as negative for no clearance and 1+ for mild activity (a 1-2 mm lysis zone around the disk) and a strong activity (a 3-5 mm lysis zone around the disk).

Phospholipase activity was estimated by the method of Tsang *et al.* (Tsang *et al.*, 2007). The assay was conducted in triplicate for each yeast isolate tested. About 5 µl of yeast suspension in sterile saline was placed on the surface of agar medium containing egg yolk (pH 4.3), and left to dry at room temperature. The plates were incubated at 37°C for 5 days. The diameter of the precipitation zone around the colony was considered as an indicator of phospholipase activity. This activity (Pz) was expressed as the ratio of the colony diameter to the colony diameter plus the precipitation zone (in mm). A Pz of 1.0 was evaluated as negative, 0.99-0.9 as weak, 0.89-0.8 as mild, 0.79-0.7 as relatively strong and <0.69 as a very strong positive.

RESULTS

During the 24-month study, we detected a total of 59 cases of candidemia for 66 isolates in our Institute. The age range of the patients was between 28 and 88 years, and 65% were male. The incidence of candidemia was 32% in General Surgery, 22% in the Intensive Care Unit (ICU), 13% in Oncology and 10% in Gastroenterology. 43% of the patients had had surgery, 30% had received antimicrobial agents such as vancomycin and ciprofloxacin, and neutropenia was present in 12% of cases.

The distribution of isolated *Candida* species is shown in Figure 1. *C. albicans* was the most com-

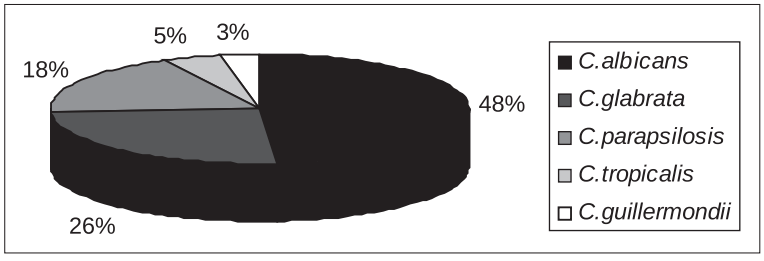

FIGURE 1 - *Distribution of Candida species. C. albicans species represents the 48% of isolates and C. non-albicans the 52%. Between C. non-albicans the most common species is C. glabrata.*

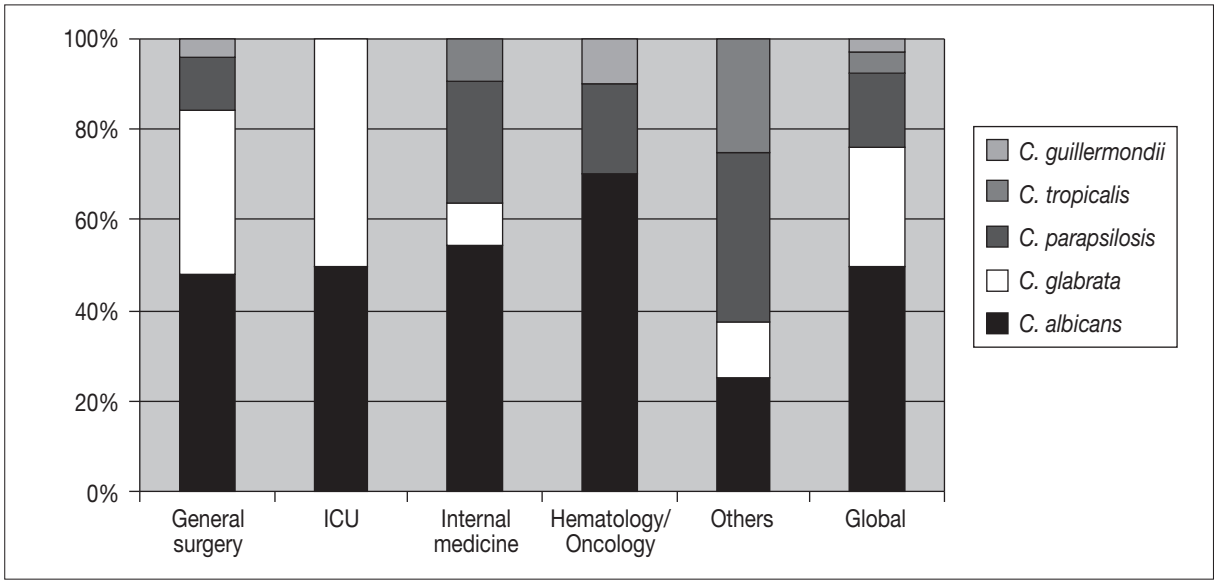

FIGURE 2 - *Distribution of Candida species according to hospital Unit. C. albicans dominated in Hemato-oncology while Candida non-albicans and especially C. glabrata occur frequently in General Surgery and ICU.*

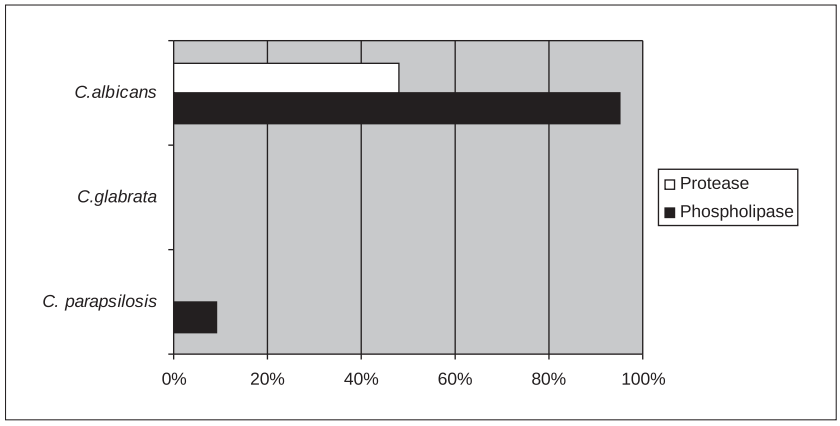

FIGURE 3 - *Protease and phospholipase activity of Candida strains. C. albicans strains had phospholipase activity, and 48% exhibited protease activity. In Candida non-albicans strains, proteinase and phospholipase activity was not detected in C. glabrata, and in C. parapsilosis only one strain secretes protease.*

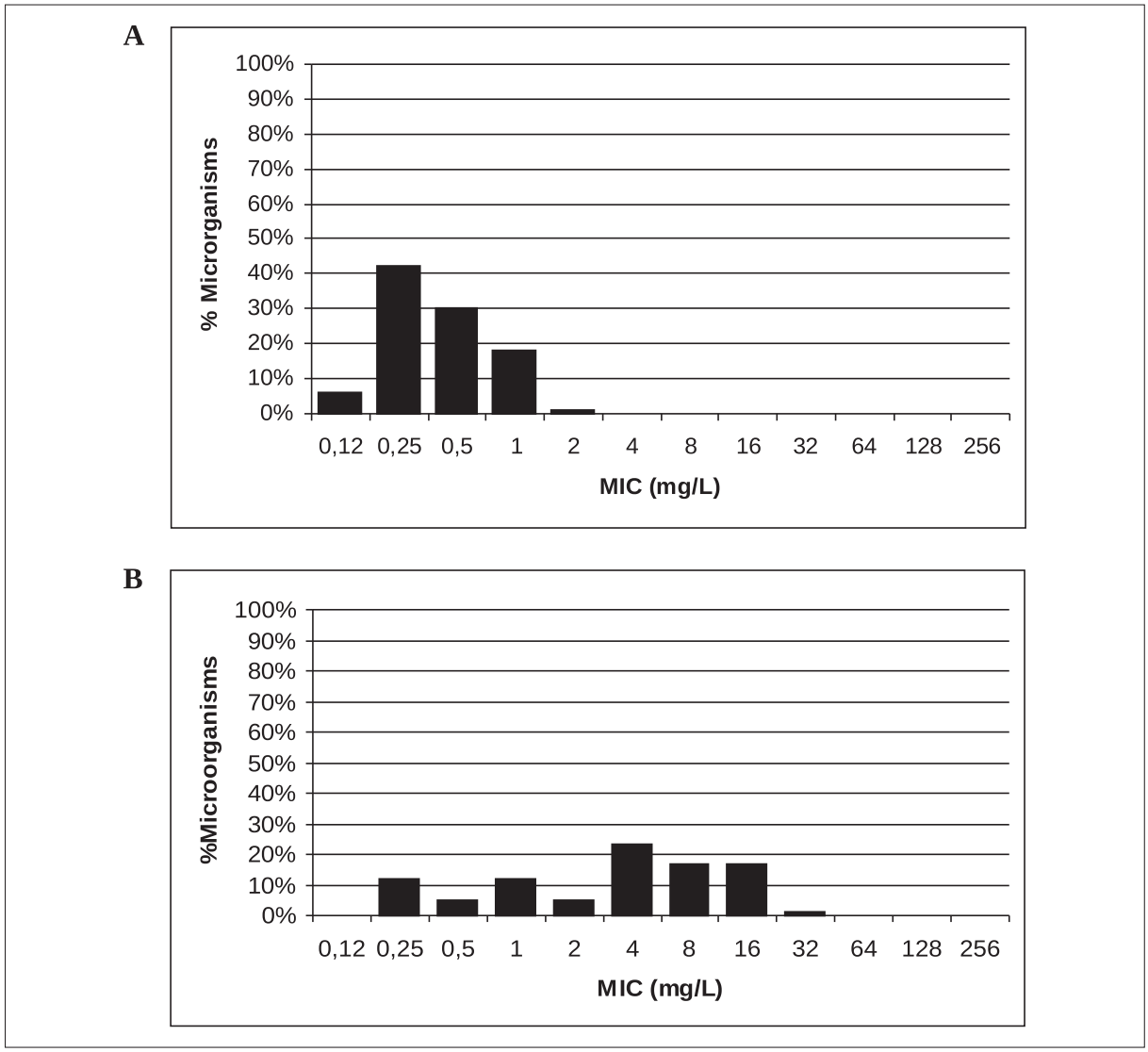

FIGURE 4 - *Fluconazole MIC distribution in C. albicans (A) and C. glabrata (B).*

mon species (32 cases =48%), followed by *C. glabrata* (17 cases =26%), *C. parapsilosis* (12 cases=18%), *C. tropicalis* (3 cases =5%) and *C.guillermondii* (2 cases =3%).

The distribution of *C. albicans* and *C.* non-*albicans* strains differed according to the type of patient population, as shown in Figure 2. In General Surgery, *C. albicans* was isolated in 48% of cases, *C. glabrata* in 36% and *C. parapsilosis* in 12%; in ICU *C. albicans* accounted for 50% of cases and *C. glabrata* for the other 50%; in Internal Medicine *C. albicans* was isolated in 55% of cases and *C. parapsilosis* in 27%, and in Hemato-Oncology *C. albicans* was isolated in 70% of cases and *C. parapsilosis* in 20%.

The present study has detected a significant production of phospholipase in all strains of *C. albicans*. Among *Candida* non-*albicans* species, the production of this enzyme only occurred in 1/12 strains of *C. parapsilosis*. As regards the production of acid protease, the expression of this activity was detected in 48% of *C. albicans* and no strains of *Candida* non-*albicans*. However, no significant relationship was detected between production of these enzymes and antifungal susceptibility (Figure 3).

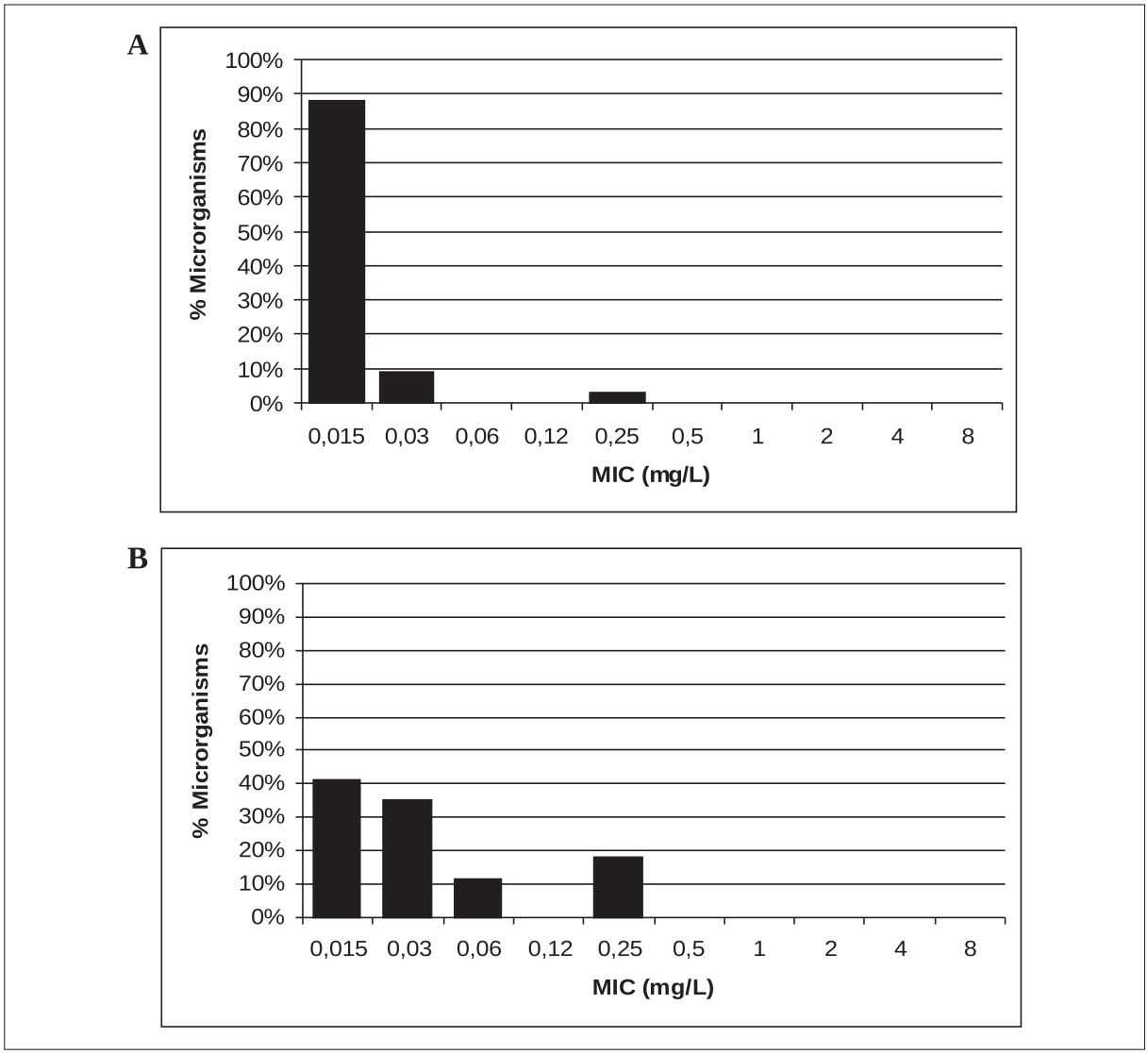

FIGURE 5 - *Anidulafungin MIC distribution in C. albicans (A) and C. glabrata (B).*

All species of *Candida* were susceptible to AB. The rate of susceptibility to fluconazole was 100% and 100% for *C. albicans* and *C. parapsilosis* according to the CLSI and EUCAST breakpoints respectively. Decreased susceptibility to fluconazole was mostly seen with *C. glabrata*, which was 76.5% susceptible in a dose-dependent manner (SDD) for CLSI, while for EUCAST there is insufficient evidence to establish the interpretation breakpoint.

Figure 4 shows the results of the *in vitro* activity of fluconazole in *C. albicans* and *C. glabrata*. For most *Candida* species tested, the MIC values were low for all three echinocandins and below the susceptibility breakpoint. In particular, the rate of susceptibility to Anidulafugin was 100% and 97% for *C. albicans* and 100% and 88.2% for *C. glabrata* according to the CLSI and EUCAST breakpoints respectively. Figure 5 shows the results of the *in vitro* activity of Anidulafungin for *C. albi-*

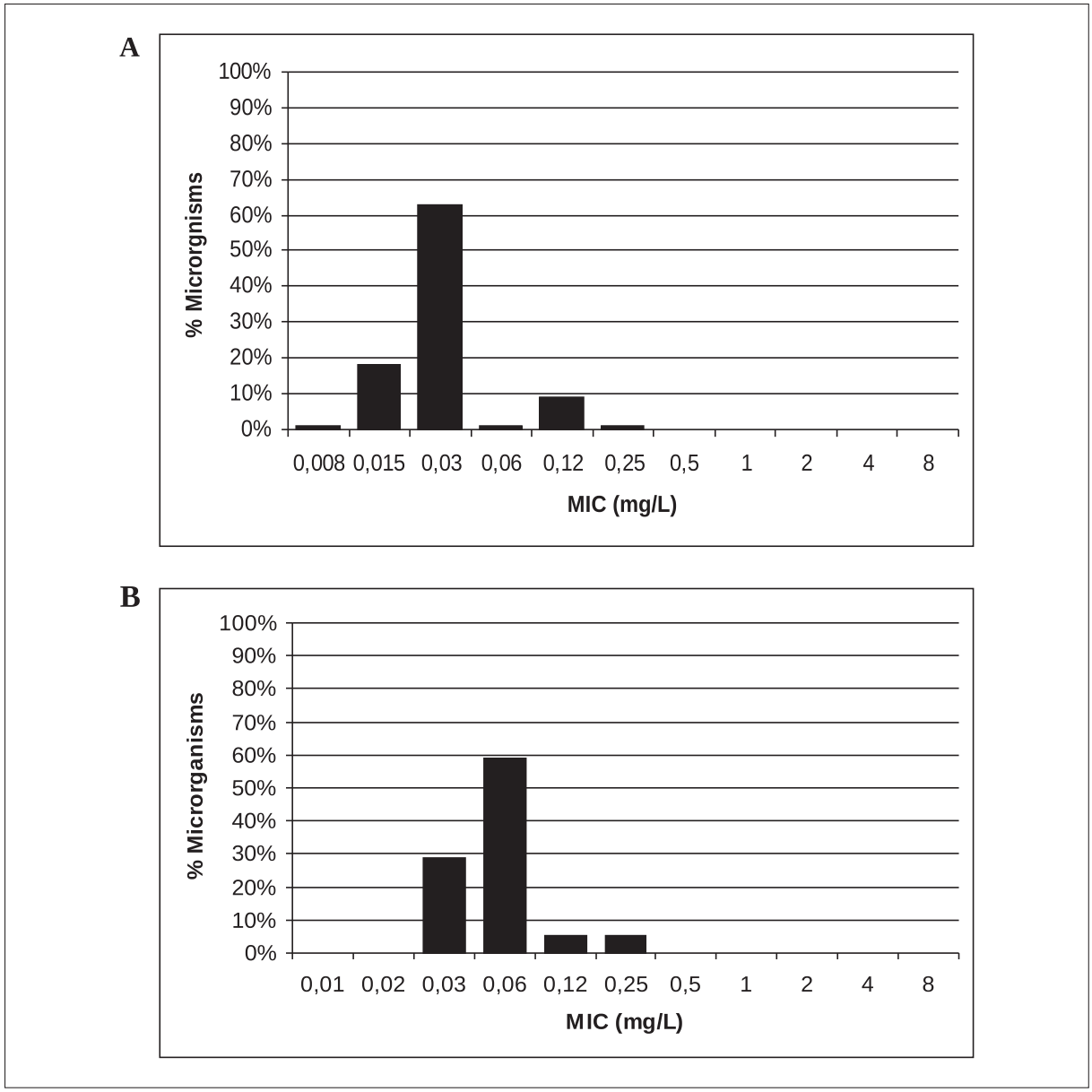

FIGURE 6 - *Caspofungin MIC distribution in C. albicans (A) and C. glabrata (B).*

cans and *C. glabrata*. Figure 6 shows the results of the *in vitro* activity of Caspofungin for *C. albicans* and *C. glabrata*.

DISCUSSION

Some recent studies have reported a shift in the etiology of candidemia. In particular, the epidemiology of the species responsible for BSI has been changing from *C. albicans* to *C.* non-*albicans* species (Bassetti *et al*., 2011; Mikulska *et al*., 2001; Pereira *et al*., 2010). Our data demonstrate that there is an equivalent distribution of isolates between *C. albicans* species (48%) and *C.* non-*albicans* species (52%), and these data must be taken into consideration for empirical antimycotic therapy in our Institution. This trend is described in the literature, and in particular seems to be related to geographical distribution, with a north-south drift, showing a prevalence of *C. albicans* in northern countries, moving towards a *C.* non-*albicans* prevalence in southern countries, described by Cisterna *et al.* (Cisterna *et al*., 2010) in Spain, Stylianakis *et al.* in Greece (Stylianakis *et al.,* 2010) and Bassetti *et al.* in Italy (Bassetti *et al.,* 2011). Among the *C.* non-*albicans* strains in southern countries, the most common species is *C. parapsilosis* (Mikulska *et al*., 2001; Pereira *et al*., 2010; Cisterna *et al*., 2010; Stylianakis *et al.,* 2010), while in our Institute, the most common species among the *C.* non-*albicans* strains is *C. glabrata,* as described by Das *et al.* (Das *et al.,* 2008) in a tertiary referral hospital in the United Kingdom. The epidemiology of *Candida* infections must be studied at local level rather than on a worldwide scale (Mikulska *et al*., 2001), and we are hopeful that every Institute will promote epidemiological surveillance to describe the local epidemiology, especially the *Candida* non-*albicans* species most likely to influence the empirical therapeutic options.

In our Institute, candidemia was predominant in General Surgery and not in ICU or Hemato-oncology, as described in Italy by others (Viscoli *et al*., 1998; Bassetti *et al.*, 2009) probably because our Institute has a strong surgical bias. As described by other authors (Bassetti *et al*., 2011), we found a low rate of *C. krusei* and *C. tropicalis*. *C. albicans* dominated in Hemato-oncology, in contrast with the recent papers (Bassetti *et al.*, 2009; Tortorano *et al*., 2006) and in Internal Medicine, while *Candida* non-*albicans* and especially *C. glabrata* occur frequently in General Surgery and ICU. In particular in our Hemato-oncology unit, the prophylaxis regimen is based on echinocandin, not fluconazole.

As regards the proteinase and phospholipase secretions implicated as potential virulence factors, especially for catheter-related candidemia in intensive care unit (ICU) patients with indwelling devices, as in other studies (Mohan Das & Ballai, 2008) all our *C. albicans* strains had phospholipase activity, and 48% exhibited protease activity. In *Candida* non-*albicans* strains, proteinase and phospholipase activity was not detected in *C. glabrata,* and in *C. parapsilosis* only one strain secretes protease.

These data agree with a previous study (Senevirtatne *et al*., 2011) involving bloodstream isolates of *Candid*a from Hong Kong and Finland, where *C. albicans* showed higher proteinase activity and *C. glabrata* showed no proteinase activity, and with a study in Turkey (Gultekin *et al*., 2011) where the proteinase activity of *C. albicans* was higher than that of *C.* non-*albicans* species. In fact, the *C. albicans* strains involved in bloodstream infections are characterized in all the studies by excretion of virulence factors.

No antifungal resistance was found in our study, and as in other studies (Ostrosky-Zeichner *et al.*, 2003; Cisterna *et al*., 2010), none of our *Candida* bloodstream isolates had an MIC >2 mg/mL for amphotericin B. Fluconazole maintained its sensitivity (100% with CLSI and EUCAST) to *C. albicans,* as described by other authors (IshiKawa *et al*., 2010), and as expected, *C. glabrata* was fluconazole-resistant or SDD.

The echinocandins (caspofungin, micafungin and anidulafungin), which are attractive treatment options for invasive fungal infections, showed a good performance for *C. albicans,* and maintained a good MIC distribution in *C. glabrata*. With the epidemiology of our Institute regarding *Candida* non-*albicans* strains, empirical treatment with echinocandins will be desirable. Regarding *C. glabrata* with reduced susceptibility to echinocandins upon treatment, the results of the Japanese study (Oxman *et al.*, 2010) with four *C. glabrata* strains with a high MIC for micafungin must be taken into account.

In conclusion, there is an equivalent distribution

of isolates between *C. albicans* species and *C.* non-*albicans* species in our Institute. The most common species among the *C.* non-*albicans* strains is *C. glabrata,* candidemia was predominant in General Surgery and not in ICU or Hemato-oncology. All our *C. albicans* strains had phospholipase activity and 48% exhibited protease activity while proteinase and phospholipase activity was not detected in *C. non albicans*. No antifungal resistance was found in our study and none of our *Candida* bloodstream isolates had a MIC of >2 mg/mL for amphotericin B. Fluconazole maintained its sensitivity to *C. albicans, C. glabrata* was fluconazole-resistant or SDD.
The echinocandins showed a good performance for *C. albicans,* and maintained a good MIC distribution in *C. glabrata*.

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