**In vitro** activity of two amphotericin B formulations against *Malassezia furfur* strains recovered from patients with bloodstream infections

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**Abstract**

Although guidelines for the treatment of *Malassezia furfur* fungemia are not yet defined, clinical data suggest that amphotericin B (AmB) is effective for treating systemic infections. In the absence of clinical breakpoints for *Malassezia* yeasts, epidemiological cut-off values (ECVs) are useful to discriminate between isolates with and without drug resistance. This study aimed to compare the distribution of minimal inhibitory concentration (MIC) and the ECVs for AmB of both deoxycholate (d-AmB) and liposomal (l-AmB) formulations of *M. furfur* isolates. The 84 *M. furfur* strains analyzed, which included 56 from blood, sterile sites and catheters, and 28 from skin, were isolated from patients with bloodstream infections. MICs were determined by the modified broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI). The l-AmB MIC and the ECVs were two-fold lower than those of d-AmB and a lower l-AmB mean MIC value was found for blood isolates than from skin. The ECVs for l-AmB and d-AmB were two-fold lower than those of d-AmB and a lower l-AmB mean MIC value was found for blood isolates than from skin. The ECVs for l-AmB and d-AmB were 8 mg/l and 32 mg/l, respectively. Three strains (3.6%) showed l-AmB MIC higher than ECV (MIC > 8 mg/l) of which two were isolated from the catheter tip of patients treated with micafugin, l-AmB and fluconazole, and one from skin. The results showed that the l-AmB might be employed for assessing the in vitro antifungal susceptibility of *M. furfur* by a modified CLSI protocol and that ECVs might be useful for detecting the emergence of resistance.

**Key words:** amphotericin B susceptibility, *Malassezia furfur*, CLSI, bloodstream infections, epidemiological cut-off.

**Introduction**

*Malassezia* spp. are lipid-dependent yeasts commonly isolated from human and animal skin, which may cause dermatologic and systemic diseases [1–3]. There are 14 currently recognized *Malassezia* spp., eight of which have been associated with human skin, with *M. globosa* and *M. restricta* the most frequently isolated [3], whereas *M. furfur* and *M. pachydermatis* are the most common in systemic
infections of immunocompromised hosts [1–3]. In particular, Malassezia fungemia is a catheter-related infection, which occurs in neonates with the infusion of total lipid parenteral nutrition or in children and adults with various forms of immunosuppression and underlying diseases [3–6]. The management of this infection requires the removal of the central venous catheter (CVC), the discontinuation of the parenteral lipid nutrition and the institution of systemic antifungal treatment [7]. Although guidelines for the treatment of Malassezia spp. systemic mycoses have not yet been assessed, clinical evidence indicates that amphotericin B (AmB) is effective in their treatment [3,7]. Lipid-based formulations of AmB (i.e., l-AmB) are currently used for the treatment of invasive fungal infections, as they are better-tolerated and less nephrotoxic when compared with deoxycholate AmB (d-AmB) [8]. Due to the limited information available concerning the in vitro antifungal susceptibility of M. furfur in bloodstream infections (BSI), their treatment is often challenging [7]. Indeed, the susceptibility testing of Malassezia spp. has not yet been standardized, either by the Clinical and Laboratory Standards Institute (CLSI) or by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [9]. This situation has resulted in the absence of clinical breakpoints (CBPs) and epidemiological cut-off values (ECVs), which are useful parameters for discriminating between isolates with and without drug resistance [10,11].

Recently, a modified CLSI protocol, using Sabouraud dextrose broth (SDB), was shown to be useful for testing the in vitro antifungal susceptibility of M. furfur and M. pachydermatis to triazole drugs but not to AmB deoxycholate [12,13]. Indeed, high AmB MIC values were found with isolates from BSI patients who were recovering from fungemia by using AmB deoxycholate [13]. Interestingly, it has been shown that l-AmB has excellent in vitro activity against Candida spp., and AmB resistance in Aspergillus spp. is better detected when AmBisome is used as the test drug in the CLSI BMD protocol [14,15]. No data regarding the antifungal activity of AmBisome against Malassezia yeasts is available despite its high in vivo efficacy [13]. Thus, this study aimed to compare the distribution of MICs and the ECVs for AmB in both deoxycholate and liposomal formulations in M. furfur isolates from patients with BSI.

### Methods

**Malassezia furfur strains**

A total of 84 M. furfur isolates recovered from 14 patients with BSI were phenotypically (i.e., macroscopic and microscopic morphology) and physiologically identified, as previously reported [3,16]. They were then identified to the species level by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and sequencing of the internal transcribed spacer (ITS) of nuclear ribosomal DNA [3,17]. Isolates obtained from each BSI patient were collected from blood, urine, gastric aspirate and catheter tip (total of n = 56 strains; Group I), and from skin of the arm and chest (total of n = 28 strains; Group II). All the patients enrolled in the study were treated with l-AmB and recovered from BSI. Two of them were also treated with micafungin (10 mg/kg/d for 6 d), l-AmB (5 mg/kg/d for 20 d), followed by fluconazole (25 mg/kg/d for 20 d). A written informed consent from the parents or guardian of patients were obtained according to the current Italian legislation (Art. 81-D.Lgs.vo n.196/2003).

**In vitro susceptibility testing**

The antifungal susceptibility testing of M. furfur strains was performed by broth microdilution CLSI M27-A3 (BMD CLSI) protocol [18] using SDB (Liofilchem Diagnostic, Roseto degli Abruzzi, Italy) with 1% Tween 80 (Sigma Co, Milano, Italy). For the inoculum suspension, isolates were obtained according to the current Italian legislation (Art. 81-D.Lgs.vo n.196/2003). The antifungal susceptibility testing of M. furfur strains was performed by broth microdilution CLSI M27-A3 (BMD CLSI) protocol [18] using SDB (Liofilchem Diagnostic, Roseto degli Abruzzi, Italy) with 1% Tween 80 (Sigma Co, Milano, Italy). For the inoculum suspension, isolates were obtained according to the current Italian legislation (Art. 81-D.Lgs.vo n.196/2003).

### Definitions

A microorganism was defined as wild-type (WT) when it did not have any acquired resistance mechanisms to the drugs tested [10,11,19,20]. The typical MIC distribution for WT microorganisms covers three to five two-fold dilutions surrounding the modal MIC [10,11,19] by...
testing a single isolate/clinical specimen, for each infectious episode.

The epidemiological cut-off value (ECV) was obtained considering the MIC distribution, the modal MIC and the inherent variability of the test, usually within one doubling dilution. In general, the ECV should encompass at least 95% of the isolates in the WT distribution and should be calculated as two-fold dilution steps higher than the modal value \([10,19]\). Organisms with MIC results higher than the ECV were considered as resistant \([10,11,19]\). Data were also reported as MIC ranges, MIC mean value (mMIC), MIC at which 50% (MIC\(_{50}\)) and 90% (MIC\(_{90}\)) of the strains were inhibited.

**Statistical analysis**

Both on-scale and off-scale results were included in the analysis. The low and high off-scale MICs were converted as the lowest MIC or the highest MIC, respectively. MIC mean values of d-AmB and l-AmB in different groups were screened with paired Student t-Test. Data were statistically analysed using the R software (version 2.8.1, \text{http://www.r-project.org/}) and a \(P\)-value less than 0.05 was considered significant.

**Results**

In general, the MIC range found with l-AmB was broader than noted with d-AmB (Fig. 1). The MIC\(_{50}\), MIC\(_{90}\), modal MIC, and ECV for l-AmB were two-fold double dilutions lower than d-AmB (Fig. 1; Table 1). In particular, the ECVs for l-AmB and d-AmB were 8 mg/l and 32 mg/l, respectively, in isolates from Groups I and II (Table 1). The MIC values for each d-AmB and l-AmB, expressed as MIC\(_{50}\), modal MIC, and ECV, were identical within isolates from the two groups. A total of three strains had l-AmB MIC values of \(>8\) mg/l (i.e., MIC\(>\)ECV), with two being recovered from catheter tips (Group I) and one from a skin sample (Group II) (Table 1). The two Group I isolates were recovered from patients treated with micafugin, l-AmB and fluconazole. The l-AmB MIC\(_{50}\) varied according to the groups tested, in other words, MIC\(_{50}\) = 4 and 8 mg/l in Groups I and II, respectively (Table 1). A lower l-AmB mean MIC (mMIC) was associated with blood isolates (3.4 mg/l, Group I) than those obtained from skin (4.4 mg/l, Group II) \((P > 0.05)\). The l-AmB and d-AmB MICs for control strains, using the modified CLSI protocol, were within the expected ranges, in other words, 0.5 mg/l (incubation time of 24 h) or 1.0 mg/l (incubation time of 48 h) for \(C\). parapsilosis ATCC 22019 and 1.0 mg/l (incubation time of 24 h) or 2.0 mg/l (incubation time of 48 h) for \(C\). krusei ATCC 6258 \([18]\).

**Discussion**

The results of this in vitro study suggest that l-AmB was the more active formulation against \(M\). furfur. Indeed, a broader MIC distribution was noted with l-AmB than d-AmB, which might be due to substantial variations in drug concentrations as a result of the reduced solubility of

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- \text{Figure 1. Distribution of minimum inhibitory concentrations (MIC) of d-AmB and l-AmB of 84 Mamassezia furfur isolates. A value of >32 mg/l was assumed to be 64 mg/l.} 
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l-AmB formulations used in the in vitro tests as previously suggested [14,21,22]. However, the use of SDB with 1% Tween 80 might initiate a better release of amphotericin B from the liposomes and thereby prevent such a bias in drug bioavailability. In addition, the lipid presence in the cell wall and in the capsule in Malassezia yeasts [23–26] might facilitate the entry of the l-AmB through the outer layers, favoring the drug binding with the target in the fungal cell membrane (i.e., ergosterol). Accordingly, the lower l-AmB MIC values of isolates from blood in BSI patients, than in those from the skin, might be due to the parenteral nutrition received by BSI patients, which might increase the permeability of l-AmB. In addition, the lower l-AmB mMIC value (4.4 mg/l) than d-AmB (7.1 mg/l) in the skin isolates, might be the result of the fact that Malassezia yeasts usually colonize body skin sites high in fatty acids [27,28]. Interestingly, the finding of a lower l-AmB MIC than d-AmB in the M. furfur strain from the skin lesions of one patient, suggests that the solubilisation of l-AmB might be enhanced with skin lesions that could have directly modified the Malassezia cell wall composition [29,30]. The ECVs proposed were shown to be useful to detect from 96% to 100% l-AmB and d-AmB strains of M. furfur, respectively, within the susceptible population. However, the ECVs (i.e., l-AmB = 8 mg/l and d-AmB = 32 mg/l) were higher than those previously reported for any other organism [31], including M. furfur strains [32,33]. The methodology employed in these studies would seem to be appropriate as SDB was shown to be an optimal medium to evaluate the in vitro susceptibility of both M. furfur and M. pachydermatis [12,13,34] and acceptable results with QC organisms (i.e., Candida parapsilosis ATCC 22019 and Candida kru- sei ATCC 6258). Therefore, the low M. furfur susceptibility to AmB may be related to the variations in quantity or type of sterols in cell membranes, as well to the inhibition of oxidative action of AmB (i.e., high activity of fungal intracellular catalase and/or superoxide dismutase) as previously suggested [35,36]. In addition, considering that the patients recovered from fungemia after therapy with micafungin and l-AmB followed by fluconazole, the positive results might be due to the additional drugs which could explain the resistance to l-AmB in two strains isolated from catheter tips (Group I). Indeed an in vitro synergistic effect between echinocandin (i.e., anidulafungin) and amphotericin B or fluconazole, has been previously reported for Candida spp. [37]. Conversely, resistance mechanisms in a skin strain from a patient with a fungemia being resolved using only l-AmB (Group I) might suggest a different source of BSI.

In conclusion the study provides, for the first time, data on the susceptibility of two AmB formulations (deoxycholate and liposomal) in M. furfur strains from patients with BSI, showing that l-AmB was the most active for in vitro testing and confirming the excellent activity of l-AmB previously reported for Candida spp. and Aspergillus spp. [14,15]. The lipophilic nature of this yeast might support the higher antifungal activity of the l-AmB than the d-AmB. The ECVs proposed are higher than those previously reported for any other microorganism and might be related to the variations in quantity or type of sterols in cell membranes as well to the inhibition of oxidative action of AmB. Further studies are needed to corroborate this hypothesis. Since studies on M. furfur strains from bloodstream infections are scant and the number of isolates is limited, a multicenter laboratory study was not herein performed, but this limitation might be mitigated by the fact that MIC values were determined by three independent experiments and evaluated by three different operators, as previously reported [31]. Nonetheless, the susceptibility tests should be performed as multicentre studies in order to validate these data and to promptly develop therapeutic guidelines for Malassezia infections. In addition, the molecular mechanisms of drugs resistance of the strains that fall outside the ECVs should be addressed to identify the mechanisms for such a resistance.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References


