

In vitro antifungal susceptibility of *Malassezia furfur* from bloodstream infections

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Fungaemia caused by *Malassezia* spp. in hospitalized patients requires prompt and appropriate therapy, but standard methods for the definition of the *in vitro* antifungal susceptibility have not been established yet. In this study, the *in vitro* susceptibility of *Malassezia furfur* from bloodstream infections (BSIs) to amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), posaconazole (POS) and voriconazole (VRC) was assessed using the broth microdilution (BMD) method of the Clinical and Laboratory Standards Institute (CLSI) with different media such as modified Sabouraud dextrose broth (SDB), RPMI and Christensen's urea broth (CUB). Optimal broth media that allow sufficient growth of *M. furfur*, and produce reliable and reproducible MICs using the CLSI BMD protocol were assessed. Thirty-six *M. furfur* isolates collected from BSIs of patients before and during AMB therapy, and receiving FLC prophylaxis, were tested. A good growth of *M. furfur* was observed in RPMI, CUB and SDB at 32 °C for 48 and 72 h. No statistically significant differences were detected between the MIC values registered after 48 and 72 h incubation. ITC, POS and VRC displayed lower MICs than FLC and AMB. These last two antifungal drugs showed higher and lower MICs, respectively, when the isolates were tested in SDB. SDB is the only medium in which it is possible to detect isolates with high FLC MICs in patients receiving FLC prophylaxis. A large number of isolates showed high AMB MIC values regardless of the media used. In conclusion, SDB might be suitable to determine triazole susceptibility. However, the media, the drug formulation or the breakpoints herein applied might not be useful for assessing the AMB susceptibility of *M. furfur* from BSIs.

Received 22 May 2014

Accepted 26 August 2014

INTRODUCTION

Malassezia furfur is a basidiomycetous yeast and part of normal human skin microbiota (Marcon & Powell, 1992; Ashbee, 2007; Gaitanis *et al.*, 2012; Findley *et al.*, 2013). The *Malassezia* genus comprises 14 lipophilic species, with *M. furfur*, *Malassezia sympodialis*, *Malassezia globosa* and *Malassezia restricta* being agents of dermatological disorders (i.e. pityriasis versicolor, seborrhoeic dermatitis, dandruff, atopic eczema and folliculitis) in immunocompetent patients (Ashbee, 2007; Tragiannidis *et al.*, 2010; Gaitanis *et al.*, 2012), and *M. furfur* and *Malassezia pachydermatis* causing systemic infections in severely immunocompromised hosts (Marcon & Powell, 1992; Chrystanthou

et al., 2001; Tragiannidis *et al.*, 2010; Oliveri *et al.*, 2011; Gaitanis *et al.*, 2012). A recent study demonstrated that *M. furfur* caused bloodstream infection (BSI) episodes in hospitalized neonates with a higher prevalence than that registered for *Candida* spp. (Iatta *et al.*, 2014). These findings highlighted the need for an appropriate diagnosis and for defining the most effective therapies.

Based on the renewed interest in rare invasive yeast infections, including those by *Malassezia* spp., clinical guidelines for the diagnosis and management of these infections have been recently published (Arendrup *et al.*, 2014). However, the *in vitro* antifungal susceptibility tests for *Malassezia* yeasts still require standardization (Arendrup *et al.*, 2014). The susceptibility of *Malassezia* spp. isolated from the skin of symptomatic and asymptomatic animals and humans has been assessed employing the modified Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) technique, using different media

Abbreviations: AMB, amphotericin B; BMD, broth microdilution; BSI, bloodstream infection; CA, categorical agreement; CLSI, Clinical and Laboratory Standards Institute; CVC, central venous catheter; EA, essential agreement; FLC, fluconazole; ITC, itraconazole; MIC_m, MIC mean value; POS, posaconazole; VRC, voriconazole.

(Jesus *et al.*, 2011; Cafarchia *et al.*, 2012a; Carrillo-Muñoz *et al.*, 2013; Yurayart *et al.*, 2013). Significant variations in MIC ranges have been recorded, depending on the medium used, eventually resulting in erroneous susceptibility classification (Cafarchia *et al.*, 2012a). Therefore, Christensen's urea broth (CUB) and Sabouraud dextrose broth (SDB) with 1% Tween 80 have been proposed as suitable culture media in CLSI BMD for testing *M. pachydermatis* susceptibility (Cafarchia *et al.*, 2012a). There is no data available on the correlation between the *in vitro* and *in vivo* susceptibility to antifungal agents. In addition, the *in vitro* susceptibility of *M. furfur* isolated from BSIs has been assessed for a single isolate (Velegaki *et al.*, 2004). Thus, this study aims to (i) assess the optimal medium for testing the susceptibility of *M. furfur* using the CLSI BMD protocol; and (ii) evaluate the *in vitro* susceptibility of *M. furfur* isolates from BSIs to amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), posaconazole (POS) and voriconazole (VRC).

METHODS

***M. furfur* strains.** A total of 36 *M. furfur* isolated from the BSIs of neonates and paediatric patients were identified phenotypically (i.e. macroscopic and microscopic morphology) and physiologically, as reported elsewhere (Guého *et al.*, 1996; Cafarchia *et al.*, 2011; Iatta *et al.*, 2014). Validation of the strains to species level was achieved by matrix-assisted laser desorption ionization-time of flight MS and sequencing of the internal transcribed spacer of nuclear rDNA (Kolecka *et al.*, 2014).

Upon collection, isolates were divided into three groups: group I consisting of 16 isolates [13 from blood samples through central venous catheters (CVCs) and 3 from CVC tips] from 5 BSI patients before receiving AMB therapy; group II comprising 16 isolates (12 from blood samples through CVCs and 4 from CVC tips) from 8 patients (5 from group I and 3 from group III) receiving AMB therapy for at least 3 days (mean duration 14 days); and group III comprising 4 isolates from blood samples through CVCs from 3 BSI patients receiving FLC prophylaxis [3 mg kg^{-1} (72 h^{-1})], for at least 1 month, and before receiving AMB therapy. All patients received total lipid parenteral nutrition via CVC. Catheters were removed from 7 of 8 patients after yeast isolation from the blood cultures. All patients recovered from BSI in about 16 days [mean values are described by Iatta *et al.* (2014)]. All *M. furfur* strains were deposited in the fungal collection at the Department of Veterinary Medicine of the University of Bari (Italy) with the following code numbers: CD1006–CD1008, CD1016–CD1019, CD1022–CD1028, CD1030–CD1033, CD1036–CD1039, CD1041, CD1044–CD1050, CD1054, CD1055, CD1061, CD1072, CD1124 and CD1136.

In vitro growth of *Malassezia* yeasts using different media.

Growth of *Malassezia* strains in various media broths (see below) was evaluated after 48 and 72 h of incubation. SDB (Liofilchem Diagnostici) was supplemented with 1% Tween 80 (Sigma), RPMI 1640 broth was buffered with MOPS and supplemented with 2% glucose, 0.4% ox bile, 1% Tween 40, 0.2% glycerol (v/v) and 0.2% oleic acid (RPMI), and CUB was supplemented with 0.1% Tween 80 and 0.5% Tween 40 (Rincón *et al.*, 2006; Cafarchia *et al.*, 2012a). For the inoculum suspension, isolates were obtained using colonies grown for 5 days in Dixon agar, suspended in 5 ml sterile distilled water and thoroughly vortexed to achieve a smooth suspension. Turbidity was read at a wavelength of $565 \pm 15 \text{ nm}$ and adjusted spectrophotometrically (Biosan DEN 1) to an optical density of 2.4 McFarland,

corresponding to $1\text{--}5 \times 10^6 \text{ c.f.u. ml}^{-1}$, as inferred by quantitative plate counts of c.f.u. in Dixon agar. Subsequently, two dilutions (1:100 and 1:20) of the inoculum were performed using each medium and a total of 100 μl of the final dilution was transferred into a 96-well microtitre plate containing 100 μl each medium. Triplicates of each plate were incubated for 48 and 72 h at 32°C together with a negative control (medium only), and growth of *Malassezia* was observed, measured spectrophotometrically and expressed as the mean of the optical density values at 595 nm minus the mean of optical density of the negative control.

***In vitro* susceptibility testing.** The susceptibility of *M. furfur* strains in CUB, SDB and RPMI (Velegaki *et al.*, 2004; Rincón *et al.*, 2006; Cafarchia *et al.*, 2012a) to antifungal compounds was assessed using the CLSI BMD M27-A3 protocol (CLSI, 2008). The stock inoculum suspensions were prepared as described above.

The following antifungal drugs were supplied by the manufacturers as pure standard compounds: AMB and ITC (Sigma-Aldrich), FLC and VRC (Pfizer Pharmaceuticals), and POS (Schering-Plough). FLC was dissolved in sterile water, whereas the remaining drugs were solubilized in DMSO (Sigma-Aldrich). The concentration of each antifungal drug ranged from 0.008 to 16 mg l^{-1} , except for FLC (i.e. from 0.06 to 128 mg l^{-1}). Visual reading of plates was performed after 48 and 72 h of incubation at 32°C .

For azoles, the MIC end point was defined as the lowest concentration that produced a prominent decrease in turbidity ($\geq 50\%$) relative to that of the drug-free control. The MIC for AMB was defined as the lowest concentration at which no visible growth could be detected. Quality control strains (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 from the American Type Culture Collection, Manassas, VA, USA) were included to assess the accuracy of the drug dilutions and the reproducibility of the results (CLSI, 2008). Two different tests for each isolate were performed. Data obtained were reported as MIC ranges, modal MIC, MIC mean value (MIC_m), and MIC at which 50% (MIC₅₀) and 90% (MIC₉₀) of the isolates were inhibited.

Results interpretation. Tentative breakpoints have been established for azole compounds, allowing isolates of *Candida* spp. tested according to CLSI guidelines to be classified as susceptible (S), susceptible dose-dependent (SDD) or resistant (R) (CLSI, 2008). However, since breakpoints have not yet been established for *M. furfur*, the *Candida* spp. criteria were used in this study for the interpretation of results (CLSI, 2008): FLC, S $\leq 8 \text{ mg l}^{-1}$, SDD $16\text{--}32 \text{ mg l}^{-1}$, R $\geq 64 \text{ mg l}^{-1}$; ITC, S $\leq 0.125 \text{ mg l}^{-1}$, SDD $0.25\text{--}0.5 \text{ mg l}^{-1}$, R $\geq 1 \text{ mg l}^{-1}$; VRC, S $\leq 1 \text{ mg l}^{-1}$, SDD 2 mg l^{-1} , R $\geq 4 \text{ mg l}^{-1}$. Since AMB and POS breakpoints have not yet been established, the breakpoints for VRC were used for POS, whereas AMB isolates were considered susceptible when MIC was $\leq 1 \text{ mg l}^{-1}$ (Diekema *et al.*, 2009). MIC results with no more than twofold dilutions between the methods (i.e. cultures in different media) were defined as being in essential agreement (EA), whereas results falling within the same interpretive category were considered in categorical agreement (CA) (Iatta *et al.*, 2011).

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_m in different groups and media transformed in $\log_e(x+1)$ were screened with ANOVA, followed by Tukey test for post-hoc comparison. The Fisher's exact test was employed to compare the prevalence of *M. furfur* strains S, SDD and R to the antifungal drugs tested in different groups and media. Data were statistically analysed using SPSS for Windows, version 13.0. A *P* value less than 0.05 was considered significant.

RESULTS

All quality control MIC values determined in each medium were within the ranges established by the CLSI (2008). A good growth of *M. furfur* was observed in RPMI, CUB and SDB (48 h optical density $\pm$ SD: 0.099 ± 0.016 , 0.079 ± 0.016 and 0.093 ± 0.023 , respectively) at 32 °C for 48 and 72 h (data not shown). In particular, growth in CUB led to a statistically significant ($P < 0.05$) lower optical density than that observed for strains cultured in other media.

The antifungal susceptibility of the 36 *M. furfur* isolates cultured in CUB, SDB and RPMI media and determined by CLSI BMD method are reported in Tables 1 and 2, MICs after 48 h incubation were onefold to twofold lower dilutions than those after 72 h incubation and were not statistically significantly different (data not shown). The modal MIC, MIC₅₀, MIC₉₀ and MIC_m varied depending upon the media employed (Tables 1 and 2). ITC, VRC and POS displayed lower MICs values than FLC and AMB regardless of the medium employed and the group of isolates tested (Tables 1 and 2). FLC showed higher modal MIC (i.e. 128 mg l⁻¹) and MIC_m (i.e. 75 mg l⁻¹ in group I, 100.5 mg l⁻¹ in group II and 96 mg l⁻¹ in group III) when strains were cultured in CUB or SDB and SDB, respectively, compared to the other media (Tables 1 and 2). AMB showed lower MICs values in isolates cultured in CUB and SDB than those in RPMI (Tables 1 and 2).

The interpretive classification of the 36 *M. furfur* isolates cultured in CUB, SDB and RPMI media are reported in

Tables 1 and 3. The highest EA and CA was observed for all the drugs except for VRC when strains were cultured in RPMI and SDB (Table 1). The highest number of resistant *Malassezia* strains was registered for AMB and FLC regardless of the media employed (Tables 1 and 3).

A total of three isolates (i.e. two from group I and one from group II) were AMB susceptible when cultured in CUB, whereas two strains (i.e. one from group I and one from group II) were AMB susceptible when cultured in SDB (Tables 1 and 3). All *M. furfur* from BSI patients receiving FLC prophylactic treatment (group III) were FLC-resistant only when tested in SDB (Table 3). A higher number of triazole-resistant strains were registered in group II than in group I (for ITC 25 vs 12.5 %; for POS 12.5 vs 0 %; for VRC 31.25 vs 0 %, respectively) using SDB medium (Table 3).

DISCUSSION

The results of the present study provide evidence on the susceptibility of *M. furfur* isolates from BSIs and suggest the optimal medium for testing the susceptibility of this species. In particular, a good growth of *M. furfur* incubated for 48 and 72 h was observed in CUB, SDB and RPMI 1640 ($P > 0.05$); thus, suggesting that the three media might be employed for testing the susceptibility of this yeast species to antifungal agents using the CLSI BMD protocol. In addition, since no statistically significant differences were detected between the MIC values registered after 48 and

Table 1. AMB, FLC, ITC, POS and VRC MIC range and modal value (mg l⁻¹) data of 36 *M. furfur* isolates from BSIs by CLSI BMD method using CUB, SDB and RPMI broths as media

Interpretive antifungal susceptibility classification, essential and CAs were also reported.

Antifungal agent	Medium	Range	Modal MIC	No. of isolates by category (%) [*]			EA-CA [†]		
				S	SDD	R	CUB vs SDB	CUB vs RPMI	SDB vs RPMI
AMB	CUB	1–16	16	3 (8.3)	0	33 (91.7)	97.3–88.8	91.7–88.8	94.4–94.4
	SDB	1–16	8	2 (5.5)	0	34 (94.5)			
	RPMI	16 >16	16	0	0	36 (100)			
FLC	CUB	2– >128	128	9 (25)	15 (41.7)	12 (33.3)	69.4–50	86.7–66.7	86.7–66.7
	SDB	8– >128	128	1 (2.8)	9 (25)	26 (72.2)			
	RPMI	8– >128	32	3 (8.3)	15 (41.7)	18 (50)			
ITC	CUB	0.03– >0.25	0.125	33 (91.7)	3 (8.3)	0	86.7–27.8	75–5.5	69.4–33.3
	SDB	0.03–8	0.25	12 (33.3)	18 (50)	6 (16.7)			
	RPMI	0.125–2	0.25	3 (8.3)	26 (72.2)	7 (19.4)			
POS	CUB	0.03–0.125	0.06	36 (100)	0	0	67–88.9	67–100	91.7–88.9
	SDB	0.03–8	0.25	32 (89)	2 (5.5)	2 (5.5)			
	RPMI	0.125–0.5	0.5	36 (100)	0	0			
VRC	CUB	0.03–1	0.25	36 (100)	0	0	88.9–69.4	91.7–83.3	83.3–75
	SDB	0.06–8	1	24 (66.7)	7 (19.4)	5 (13.9)			
	RPMI	0.125–4	1	30 (83.3)	0	6 (16.7)			

^{*}Percentage of isolates classified as S, SDD or R.

[†]EA reflects the percentage of isolates with MICs with no more than twofold dilutions between the different media; CA reflects the percentage of isolates classified in the same category using different media.

Table 2. AMB, FLC, ITC, POS and VRC MIC (mg l⁻¹) data of 36 *M. furfur* isolates from patients before AMB therapy (group I), after AMB therapy (group II) and receiving FLC prophylaxis (group III) by CLSI BMD method using CUB, SDB and RPMI broths as media

Antifungal agent	Medium	Group I (before AMB therapy) (n=16)				Group II (after AMB therapy) (n=16)				Group III (with FLC prophylaxis) (n=4)			
		Range	MIC ₅₀	MIC ₉₀	MICm* (SD)	Range	MIC ₅₀	MIC ₉₀	MICm* (SD)	Range	MIC ₅₀	MIC ₉₀	MICm* (SD)
AMB	CUB	1–16	16	16	11.37 (6.34)	1–16	16	16	15.06 ^A (3.75)	2–16	4	16	6.5 ^A (6.4)
	SDB	1–16	8	16	10.06 (5.14)	4–16	8	16	11.31 (5.17)	4–16	4	16	7 (6)
	RPMI	16– >16	>16	>16	16 (0)	16– >16	>16	>16	16 (0)	>16	>16	>16	16 (0)
FLC	CUB	2– >128	16	>128	34.62 ^b (47.21)	16– >128	32	>128	56.5 ^b (50.78)	4– >128	>128	>128	73 (64.52)
	SDB	16– >128	64	128	75.00 (50.16)	8– >128	128	>128	100.5 (43.91)	64– >128	128	>128	96 (36.95)
	RPMI	8– >128	32	128	47.00 ^c (42.22)	16– >128	128	>128	84.5 ^c (48.17)	32–64	64	64	48 (18.47)
ITC	CUB	0.03–0.25	0.06	0.125	0.09 ^D (0.05)	0.03–0.125	0.125	0.125	0.13 ^{DE} (0.05)	0.06–0.06	0.06	0.06	0.06 ^E (0)
	SDB	0.03–8	0.125	0.5	0.81 (2.17)	0.06–8	0.25	4	1.45 (2.30)	0.03–0.125	0.125	0.5	0.12 (0.09)
	RPMI	0.25–1	0.25	0.5	0.43 (0.47)	0.125–1	0.5	1	0.58 (0.31)	0.25–0.5	0.5	0.5	0.37 (0.14)
POS	CUB	0.016–0.125	0.03	0.125	0.05 ^F (0.03)	0.03–0.125	0.06	0.125	0.07 ^F (0.03)	0.03–0.06	0.06	0.06	0.05 (0.01)
	SDB	0.016–0.25	0.125	1	0.38 (0.52)	0.06–8	0.25	2	1.1 (2.1)	0.06–0.25	0.06	0.25	0.11 (0.09)
	RPMI	0.125–0.25	0.25	0.5	0.24 ^G (0.14)	0.125–0.5	0.5	0.5	0.4 ^G (0.16)	0.15–0.25	0.25	0.25	0.21 (0.06)
VRC	CUB	0.03–0.5	0.25	0.5	0.27 ^H (0.15)	0.06–1	0.5	1	0.64 ^{HI} (0.31)	0.25–0.5	0.5	0.5	0.37 ^I (0.14)
	SDB	0.06–2	0.5	1	0.62 ^L (0.52)	0.5–8	2	4	2.41 ^{LM} (1.99)	0.25–2	0.5	2	0.75 ^M (0.84)
	RPMI	0.125–4	0.5	1	0.89 ^N (0.88)	0.125–4	1	4	1.42 ^N (1.54)	0.5–1	1	1	0.87 (0.25)

*Statistically significant differences (ANOVA) are marked with the same superscript letters (upper case letters= $P<0.01$; lower case letters= $P<0.05$).

Table 3. Interpretive antifungal susceptibility classification of 36 *M. furfur* isolated from patients before AMB therapy (group I), after AMB therapy (group II) and receiving FLC prophylaxis (group III) determined by CLSI BMD using different media

Antifungal agent	Medium	Isolates by category (%) ^{*†}								
		Group I (before AMB therapy) (n=16)			Group II (after AMB therapy) (n=16)			Group III (with FLC prophylaxis) (n=4)		
		S	SDD	R	S	SDD	R	S	SDD	R
AMB	CUB	2 (12.5)	0	14 (87.5)	1 (6.25)	0	15 (93.75)	0	0	4 (100)
	SDB	1 (6.25)	0	15 (93.75)	1 (6.25)	0	15 (93.75)	0	0	4 (100)
	RPMI	0	0	16 (100)	0	0	16 (100)	0	0	4 (100)
FLC	CUB	6 (37.5) ^a	7 (43.75)	3 (18.75)	2 (12.5)	7 (43.75)	7 (43.75)	1 (25)	1 (25)	2 (50)
	SDB	0 ^a	7 (43.75)	9 (56.25)	1 (6.25)	2 (12.5)	13 (81.25)	0	0	4 (100)
	RPMI	2 (12.5)	10 (62.5)	4 (25)	1 (6.25)	3 (18.75)	12 (75)	0	2 (50)	2 (50)
ITC	CUB	15 (93.75) ^{bc}	1 (6.25) ^D	0	14 (87.5) ^{EF}	2 (12.5) ^{GH}	0	4 (100)	0	0
	SDB	8 (50) ^b	6 (37.5)	2 (12.50)	1 (6.25) ^E	11 (68.7) ^G	4 (25)	3 (75)	1 (25)	0
	RPMI	2 (12.5) ^C	12 (75) ^D	2 (12.50)	1 (6.25) ^F	10 (62.5) ^H	5 (31.25)	0	4 (100)	0
POS	CUB	16 (100)	0	0	16 (100)	0	0	4 (100)	0	0
	SDB	15 (93.75)	1 (6.25)	0	13 (81.25)	1 (6.25)	2 (12.5)	4 (100)	0	0
	RPMI	16 (100)	0	0	16 (100)	0	0	4 (100)	0	0
VRC	CUB	16 (100)	0	0	16 (100) ^{Lm}	0 ⁿ	0 ^{Pq}	4 (100)	0	0
	SDB	15 (93.75)	1 (6.25)	0	6 (37.5) ^L	5 (31.25) ^{no}	5 (31.25) ^P	3 (75)	1 (25)	0
	RPMI	15 (93.75)	0	1 (6.25)	11 (68.7) ^m	0 ^o	5 (31.2) ^q	4 (100)	0	0

*Percentage of isolates classified in the given category.

†Statistically significant differences (Fisher's test) are marked with the same superscript letters (upper case letters= $P<0.01$; lower case letters= $P<0.05$).

72 h of incubation, the evaluation of the test results after 48 h incubation might aid time-efficient investigations and treatments of clinical cases of *M. furfur* infections, as previously reported for *M. pachydermatis* (Cafarchia *et al.*, 2012a).

For the *M. furfur* susceptibility profile, the results showed that isolates were highly susceptible to triazole, except for FLC, as reported in isolates from pityriasis versicolor lesions and from a single BSI patient (Velegriki *et al.*, 2004; Carrillo-Muñoz *et al.*, 2013). The high FLC MIC values shown here might be due to the drug's hydrophilic nature and this might reduce its permeability in *Malassezia* cells, whose membranes are very rich in lipids. Even if the MIC values varied according to the media employed in the CLSI BMD protocol, no statistically significant differences were registered in susceptibility to ITC, POS and VRC; thus, suggesting that all the media tested here might be useful for the *in vitro* testing of these drugs. However, the highest EA and CA registered when comparing results obtained using RPMI and SDB for all drugs, except for VRC, suggests the usefulness of the above media in the susceptibility tests. Although previous studies have already proposed SDB as the standard medium for CLSI BMD testing in *Malassezia* spp. (Cafarchia *et al.*, 2012b), the correlation between MIC values of the isolates and the clinical outcome of treatment protocols has never previously been assessed.

In this study, SDB seems to be the only medium in which it is possible to detect a FLC MIC ≥ 64 mg l⁻¹ in the isolates (100 %) of BSI patients receiving FLC prophylaxis (group III); thus indicating that this medium might be useful for detecting FLC-resistant isolates. These results are in agreement with a previous study in which resistance was detected in SDB for one strain of FLC-induced resistant *M. pachydermatis* (Cafarchia *et al.*, 2012a). Interestingly, using the same medium, the *M. furfur* isolates of groups II and III with high FLC MIC values showed high MICs also for ITC, POS and VRC; thus suggesting a possible cross-resistance. Since cross-resistance is a well-documented phenomenon in *M. pachydermatis* (Jesus *et al.*, 2011; Cafarchia *et al.*, 2012a), results presented here indicate that SDB might be the best medium in which to test the antifungal susceptibility of *M. furfur*. The emergence of a possible azole-resistant *M. furfur* could result in the need for standardized antifungal tests and novel therapies. Additionally, the finding of a higher level of resistance to all the tested drugs using SDB in strains from group II (receiving AMB therapy) than in group I (before AMB therapy) suggests that BSI isolates might have acquired antifungal resistance due to biofilm formation, as already demonstrated for *M. pachydermatis* (Figueredo *et al.*, 2012, 2013). Further studies are needed to corroborate this hypothesis.

The large number of *M. furfur* isolates with high AMB MIC values suggests that the media or the *Candida* breakpoints may not be useful for assessing the susceptibility of *M. furfur* from BSIs. In addition, the observation that all the *M. furfur* isolates show *in vitro* resistance to AMB when

cultured in RPMI indicates that this medium might be not suitable for antifungal susceptibility testing of this yeast. However, these results might also be accounted for by the fact that the formulation of AMB used here might be not appropriate to test for *M. furfur* susceptibility, since this yeast is lipophilic in nature. Indeed, although *Aspergillus flavus* is a different organism, clinical isolates have recently shown that the AMB MIC values differ according to the formulations of AMB (Barchiesi *et al.*, 2013). Moreover, the few isolates ($n=5$) with an AMB MIC ≤ 1 mg l⁻¹ were revealed when tested in CUB or SDB media, which might be potentially useful for AMB susceptibility.

Finally, the findings of lower AMB MIC₅₀ (4 mg l⁻¹) and MIC_m (6.5 and 7 mg l⁻¹) in patients receiving FLC prophylaxis (i.e. group III) than in those from other groups might suggest a higher efficacy of AMB in *M. furfur* strains coming from patients pre-treated with FLC. The combination of FLC plus AMB seems to be more effective towards a more rapid clearance of the BSI (Rex *et al.*, 2003). In addition, the staggered administration of FLC might result in higher *in vitro* AMB susceptibility. Further studies need to be performed in order to validate this hypothesis.

Although AMB is one of the most common antifungal drugs used in the treatment of invasive infections, including in neonatal patients (Arendrup *et al.*, 2014), there is little data regarding the susceptibility of BSI *M. furfur* isolates (Velegriki *et al.*, 2004), and this deserves further investigation.

Based on the findings described here, SDB is suitable as a culture medium in the CLSI BMD method to determine the antifungal susceptibility of *M. furfur* to triazole drugs. However, the effectiveness of SDB or CUB for testing the susceptibility to AMB requires further studies in which *in vitro* and *in vivo* correlation should be compared.

ACKNOWLEDGEMENTS

We kindly thank Dr Bronwyn Campbell for revising the English text.

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