In Vitro Amphotericin B Susceptibility of *Malassezia pachydermatis* Determined by the CLSI Broth Microdilution Method and Etest Using Lipid-Enriched Media

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We determined the *in vitro* amphotericin B susceptibility of 60 *Malassezia pachydermatis* isolates by the CLSI broth microdilution method and the Etest using lipid-enriched media. All isolates were susceptible at MICs of ≤ 1 $\mu\text{g/ml}$, confirming the high activity of amphotericin B against this yeast species. Overall, the essential agreement between the tested methods was high (80% and 96.7% after 48 h and 72 h, respectively), and all discrepancies were regarded as nonsubstantial.

Malassezia pachydermatis is a lipophilic but non-lipid-dependent basidiomycetous yeast which thrives on the skin of most warm-blooded vertebrates (1–3). Although generally regarded as a commensal microorganism, *M. pachydermatis* can become pathogenic under the influence of diverse factors, leading to different clinical forms of dermatitis and/or otitis, mainly in small animals (1, 2, 4). Systemic infections caused by this yeast species are only rarely encountered, but some outbreaks of fungemia have been reported in intensive care nurseries (5, 6). Notably, in one such outbreak, the introduction of the yeast into a nursery was linked to its possible zoonotic transmission from colonized pet dogs to the health care workers' hands, with further spread of the microorganism in the unit through person-to-person contact (5).

Antifungal susceptibility testing of *Malassezia* species remains a challenge, as their growth is not supported (or, in the case of *M. pachydermatis*, only poorly supported) on the standard lipid-free RPMI growth medium recommended for yeast testing by the CLSI and EUCAST (6–8). A variety of alternative procedures, most of which use lipid-enriched media, have been proposed, but published studies have mainly focused on *M. pachydermatis* susceptibility to ketoconazole, itraconazole, and other azole derivatives (e.g., 8–17). On the contrary, information on the *in vitro* susceptibility of *M. pachydermatis* isolates to the polyene amphotericin B, which is commonly used for treating bloodstream infections in human patients (5–7), is scarce; the available studies were based on a single method and/or a low number of isolates (see Table 1), so the reliability of their results cannot be adequately assessed.

The aim of this study was to expand the database of MIC values to amphotericin B for *M. pachydermatis* isolates, as determined by two different procedures using lipid-enriched media, (i) a modified broth microdilution (BMD) method based on CLSI guidelines (18) and (ii) the Etest, which is a commercial agar-based gradient technique that is widely used for antifungal susceptibility testing of yeasts.

Isolates. A total of 60 *Malassezia pachydermatis* isolates obtained from clinical cases of canine otitis and primarily recovered in Sabouraud dextrose agar (SDA) containing 0.005% chloramphenicol (bioMérieux, Marcy l'Etoile, France) were included in this study. They were identified as *M. pachydermatis* on the basis of their macroscopic and microscopic morphologies and their ability

to grow on media without lipid supplementation, such as SDA, at 32°C (19). Furthermore, for a random selection of 14 isolates (23.3% of the total), the phenotype-based identification was confirmed by sequencing the D1/D2 domains of the large subunit (LSU) rRNA gene using the primers F63 and LR3 (20), as described in previous studies for other yeast species (21).

All isolates were stored at -80°C as cell suspensions in Sabouraud dextrose broth (SDB) (bioMérieux) supplemented with 25% glycerol (Panreac, Barcelona, Spain). Prior to antifungal susceptibility testing, each isolate was subcultured for 6 or 7 days at 32°C on SDA supplemented with 1% (vol/vol) Tween 80 (Panreac) (SDA-T₈₀) to ensure its viability and purity. SDA-T₈₀ plates can be stored at 4°C for at least 2 weeks without a significant loss of properties.

Antifungal susceptibility testing. The BMD procedure was performed according to the CLSI guidelines for antifungal susceptibility testing of yeasts (18) but using SDB supplemented with 1% (vol/vol) Tween 80 (SDB-T₈₀) as the test medium instead of lipid-free RPMI 1640, as proposed by Cafarchia et al. (9–11) and Eichenberg et al. (12). Amphotericin B (Sigma-Aldrich, Madrid, Spain) stock solutions were prepared in 100% dimethyl sulfoxide (Sigma-Aldrich), further diluted in SDB-T₈₀, and dispensed into 96-well microdilution trays. Cell suspensions (1×10^6 to 5×10^6 cells/ml) from 6- to 7-day-old cultures were prepared in saline solution (0.85% NaCl; Panreac), diluted in SDB-T₈₀ to a final inoculum concentration of 1×10^4 to 5×10^4 CFU/ml, and dispensed into the microdilution wells. The final concentrations of amphotericin B in the wells ranged from 0.031 to 16 $\mu\text{g/ml}$. Inoculated trays were incubated at 32°C and read at 48 and 72 h. The MIC endpoint was defined as the lowest concentration that completely inhibited yeast growth (18). *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control

Received 15 January 2014 Returned for modification 18 March 2014

Accepted 12 April 2014

Published ahead of print 21 April 2014

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doi:10.1128/AAC.00091-14

TABLE 1 Summary of other reports on amphotericin B susceptibility testing of *Malassezia pachydermatis*

Reference	Test procedure	Test medium	Incubation parameters		Source(s) of isolates	No. of isolates analyzed	Amphotericin B susceptibility (µg/ml)			Comments
			Temp (°C)	Duration(s)			MIC range	MIC ₅₀	MIC ₉₀	
6	Etest	Casitone agar buffered with phosphate buffer	35	72 h	Pediatric patients	17	0.016–0.064	NA ^e	NA	
24	BMD (NCCLS M27–A2) ^a	RPMI 1640 plus supplement 1 ^b	32	48 and 72 h	Unknown	1	0.125			
	Etest	RPMI 1640 plus 1.5% agar plus supplement 1 ^b	32	48 and 72 h	Unknown	1	0.5			
25	Disk diffusion (100-µg amphotericin B disks)	Casitone agar and semisynthetic agar	37	72 h	Dogs with otitis externa	10	NA	NA	NA	Three isolates were reported to be cross-resistant to amphotericin B and other antifungals, but the authors did not provide MIC data or the breakpoints (inhibition diameters) used for defining resistance.
26	Dilution in agar	Sabouraud's agar plus amphotericin B	37	7 days	Dogs (healthy or with dermatitis or otitis externa)	32	0.125–8	0.5 ^d	8 ^d	Results expressed in terms of minimal fungistatic concentrations, although amphotericin B has fungicidal activity.
27	BMD (NCCLS M27–A2)	RPMI 1640 plus supplement 2 ^c	32	48 h	Healthy dogs	20	0.25	0.25 ^d	0.25 ^d	MIC determination required subculturing (for 2–5 days at 32°C) of 1-µl aliquots from each well in petri dishes with potato dextrose agar until visible colonies were counted. MICs for amphotericin B were then defined as the lowest concn of drug that completely inhibited fungal growth.
28	Disk diffusion (50-µg amphotericin B disks)	Sabouraud's agar	37	48 h	Dogs with or without otitis externa	30/19	NA	NA	NA	All isolates were classified as susceptible according to the breakpoints defined by the disk supplier.
29	BMD (NCCLS M27–A2)	RPMI 1640 plus supplement 2 ^c	32	48 h	Dogs (healthy or with dermatitis or otitis)	50	2	2 ^d	2 ^d	MIC determination required subculturing (for 2–5 days at 32°C) of 1-µl aliquots from each well in petri dishes with potato dextrose agar until visible colonies were counted. MICs for amphotericin B were then defined as the lowest concn of drug that completely inhibited fungal growth.
30	BMD (CLSI M27–A3)	RPMI 1640	37	48 h	Dogs and cats with or without otitis	40/40	0.01–1/0.01–0.5	0.25/0.125	0.5/0.5	

^a BMD, broth microdilution; NCCLS, National Committee for Clinical Laboratory Standards (now CLSI).
^b Supplement 1, 20 g/liter glucose, 4 g/liter ox bile, 1 ml/liter glycerol, 0.4 ml/liter Tween 20, and 0.5 g/liter glycerol monostearate.
^c Supplement 2, 20 g/liter glucose, 4 g/liter ox bile, 1 ml/liter glycerol, 0.4 ml/liter Tween 20.
^d These values were estimated from the results provided by the authors.
^e NA, not available.

TABLE 2 Distribution of MICs for amphotericin B determined by the CLSI broth microdilution and Etest methods for 60 isolates of *Malassezia pachydermatis* and essential agreement between the methods

Test method (time results were read [h])	No. (cumulative %) of isolates susceptible at MIC ($\mu\text{g/ml}$) of:					MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	EA ^a (%)	No. (%) of isolates showing discrepancy ^b	
	0.063	0.125	0.25	0.5	1				NSD	SD
BMD ^c (48)	7 (11.7)	34 (68.3)	19 (100)		14 (100)	0.125	0.25			
Etest (48)			7 (11.7)	39 (76.7)		0.5	1	80	12 (20)	0 (0)
BMD (72)		29 (48.3)	29 (96.7)	2 (100)		0.25	0.25			
Etest (72)			7 (11.7)	41 (80)	12 (100)	0.5	1	96.7	2 (3.3)	0 (0)

^a EA, essential agreement.^b Nonsubstantial differences (NSDs) or substantial differences (SDs) with respect to the BMD method.^c BMD, broth microdilution.

strains, and all of the *M. pachydermatis* isolates were tested at least twice on different days.

The Etest method was performed in accordance with the manufacturer's instructions (bioMérieux) but following the modifications for *M. pachydermatis* suggested by Cafarchia et al. (10). Briefly, 90-mm-diameter plates containing SDA-T₈₀ medium were inoculated by dipping a cotton-tipped sterile swab (Nuova Aptaca, Canelli, Italy) into a cell suspension (prepared as for the BMD method) and streaking it across the surface of the agar in three directions. The inoculated plates were left to stand at room temperature for 15 min, or until the excess moisture was completely absorbed, before applying amphotericin B Etest strips. The plates were incubated at 32°C and read at 48 and 72 h. The MIC endpoint was the drug concentration that resulted in complete inhibition of growth, including all microcolonies, hazes, and isolated colonies.

Data analysis. Essential agreement between the MIC values determined by the BMD and the Etest procedures was defined as a discrepancy of no more than ± 2 2-fold dilutions (22). Since Etest strips provide a continuous gradient of antifungal concentrations, the MICs falling between 2-fold dilutions were elevated to the next 2-fold dilution of the reference method for comparison (23). In the absence of clinically validated MIC breakpoints for antifungal susceptibility testing of *M. pachydermatis*, discrepancies were classified as nonsubstantial differences (discrepancies in MIC results of 3 or 4 2-fold dilutions) or substantial differences (discrepancies of > 4 2-fold dilutions) (22). The Mann-Whitney rank sum test was used to evaluate the differences among the MIC data, and *P* values of ≤ 0.05 were considered to be statistically significant. Statistical analyses were performed with SigmaPlot v.11 software (Systat Software, Inc., San Jose, CA, USA).

Table 2 displays the amphotericin B MIC results for the studied isolates. These results confirm that amphotericin B is very active against *M. pachydermatis* (100% of isolates were susceptible at MICs of ≤ 1 $\mu\text{g/ml}$), as observed in most previous studies testing the same compound (see Table 1 for comparison). Nevertheless, the Etest MICs of our clinical isolates were at least 2 2-fold dilutions higher than those obtained by Chrysanthou et al. (6) with the same method but using lipid-free Casitone agar instead of SDA-T₈₀ as the test medium and 35°C rather than 32°C as the incubation temperature. The lipid supplementation of the solid medium in our Etest protocol may have enhanced yeast growth with a consequent increase in MIC values. In any case, the MIC values obtained in this and most of the previous studies (Table 1) (6, 24–30), as well as the good response observed in bloodstream-

infected patients treated with amphotericin B (6), are not suggestive of clinical resistance of *M. pachydermatis* to this polyene. Notably, this contrasts with the decreased *in vitro* susceptibility to amphotericin B detected by Velegraki et al. (24) for a significant proportion of isolates belonging to other *Malassezia* species, such as *Malassezia furfur*, *Malassezia restricta*, *Malassezia globosa*, and *Malassezia slooffiae*.

MIC results determined after 48 h of incubation by either the Etest or the BMD method were equal (for 93.3% and 65% of isolates, respectively) or within a ± 2 2-fold dilutions difference (for the remaining 6.7% and 35%, respectively) of those determined after 72 h, and the difference in median values was statistically significant for only the BMD method ($P < 0.01$). In addition, there was a highly significant difference between the tested methods in the MICs determined after 48 h or 72 h of incubation ($P < 0.001$ in both cases), with the essential agreement being higher for the second reading (80% versus 96.7%, respectively; Table 2). When discrepancies in the MICs were noted between the Etest and BMD, the Etest tended to give higher values. Prolonged incubation seemed to reduce the percentage of isolates showing any discrepancy (20% at 48 h versus 3.3% at 72 h); nonetheless, all discrepancies were classified as nonsubstantial differences (Table 2). Further evaluation of the categorical agreement between the tested methods was not attempted, as interpretive breakpoints for antifungal susceptibility testing are not yet available for *M. pachydermatis* or any other *Malassezia* spp.

A similar excellent agreement between the Etest and the CLSI reference procedure was reported by Cafarchia et al. (10) for fluconazole, itraconazole, ketoconazole, posaconazole, and voriconazole testing ($> 87\%$ essential agreement in all cases). However, these authors based their comparison of methods on the MIC values determined after 48 h of incubation at 32°C. In our experience, as some *M. pachydermatis* isolates display slow growth in culture, MIC results for amphotericin B are more reliably determined after a 72-hour incubation period.

In conclusion, the results of this study provide further evidence of the high *in vitro* activity of amphotericin B against *M. pachydermatis*. Furthermore, we demonstrate the reliability of the CLSI and Etest methods, after accounting for particular growth requirements, for routine amphotericin B susceptibility testing of this yeast species.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences reported here are [KJ610814](#) to [KJ610827](#).

ACKNOWLEDGMENTS

Sergio Álvarez-Pérez is supported by a Juan de la Cierva postdoctoral contract from the Spanish Ministry of Economy and Competitiveness (JCI-2012-12396).

We thank Cristina Orden for providing invaluable technical and moral support. The constructive comments provided by two anonymous referees are also acknowledged.

We have no financial or personal relationships that inappropriately influence or bias the content of this paper. We are responsible for the writing and the content of the paper.

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