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Use of Fatty Acid RPMI 1640 Media for Testing Susceptibilities of Eight *Malassezia* Species to the New Triazole Posaconazole and to Six Established Antifungal Agents by a Modified NCCLS M27-A2 Microdilution Method and Etest

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A novel formulation of RPMI 1640 medium for susceptibility testing of *Malassezia* yeasts by broth microdilution (BMD) and Etest is proposed. A modification of the NCCLS M27-A2 BMD method was used to test 53 isolates of *Malassezia furfur* (12 isolates), *M. sympodialis* (8 isolates), *M. slooffiae* (4 isolates), *M. globosa* (22 isolates), *M. obtusa* (2 isolates), *M. restricta* (2 isolates), *M. pachydermatis* (1 isolates), and *M. dermatis* (2 isolates) against amphotericin B, ketoconazole, itraconazole, fluconazole, voriconazole, terbinafine, and posaconazole by BMD and Etest. RPMI and antibiotic medium 3 (AM3) were supplemented with glucose, bile salts, a mixture of fatty acids, and *n*-octadecanoate fatty acids and Tween 20. *M. furfur* ATCC 14521 and *M. globosa* ATCC 96807 were used as quality control strains. Depending on the species, MICs were read after 48 or 72 h of incubation at 32°C. Low azole and terbinafine MICs were recorded for all *Malassezia* species, whereas amphotericin B displayed higher MICs (≥16 μg/ml) against *M. furfur*, *M. restricta*, *M. globosa*, and *M. slooffiae* strains, which were AM3 confirmed. Agreement of the two methods was 84 to 97%, and intraclass correlation coefficients were statistically significant ($P < 0.001$). Because of higher amphotericin B MICs provided by Etest for strains also displaying high BMD MICs (≥1 μg/ml), agreement was poorer. The proposed media are used for the first time and can support optimum growth of eight *Malassezia* species for recording concordant BMD and Etest MICs.

Malassezia yeasts are members of the normal human skin flora and agents of skin disorders and systemic infections in subgroups of severely immunocompromised patients. The obligatory lipophilic nature of human pathogenic *Malassezia* species has delayed developments in susceptibility testing of azoles, as varying results have been reported (7, 13, 20). The alternative approach (20), involving indirect assessment of susceptibility using the metabolic activity of the yeast as a viability marker, was also found unsatisfactory for testing azoles. By contrast, reproducible MIC results have been obtained in urea broth, although the method had not been assessed with solid media (13). Broth microdilution (BMD) methods using Leeming-Notman (LN) medium with Alamar Blue (20) and LN medium (7) have also been employed, but interlaboratory agreement protocols and comparative MIC data using the solid-medium tests have not been determined since.

As the incidence of yeasts in deep-seated infections continues to increase in proportion to the growing numbers of immunocompromised, cancer, and postoperative patients, standardized MIC testing for more yeast genera can become pertinent to clinical practice. Regarding bloodstream *Malassezia* infections, poor response to amphotericin B has led to discontinuation of parenteral therapy as an additional therapeutic measure (18) or modification of therapy in order to increase the amphotericin B concentration in the catheter lumen, aiming at eradicating *Malasssezia* yeasts (1). However, the observed poor clinical response (10, 18, 19, 21) has not been supported by in vitro data, as susceptibility of *Malassezia furfur* (sensu lato) to amphotericin B has been recorded (9). Apart from host and yeast factors responsible for the in vivo-in vitro difference in amphotericin B efficiency, the effect of medium composition on yeast growth and drug availability may have accounted for the observed discrepancies between laboratory and clinical findings.

The aims of our study were to adapt the NCCLS M27-A2 microdilution reference method (14) for testing the susceptibilities of eight *Malassezia* species (5, 22) to amphotericin B, itraconazole, fluconazole, voriconazole, ketoconazole, and terbinafine to the new triazole posaconazole and to formulate a suitable solid medium to achieve concordant microdilution and Etest results.

MATERIALS AND METHODS

***Malassezia* isolates.** A total of 53 type, reference (Centraalbureau voor Schimmecultures [CBS] Utrecht, The Netherlands), and clinical *Malassezia* strains maintained in modified Dixon's agar (3.6% malt extract agar, 0.6% mycological peptone, 1% agar no. 1, 2% ox bile, 1% Tween 40, 0.2% glycerol [all from Oxoid, Basingstoke, United Kingdom], and 0.2% oleic acid) were tested (Table 1). Identification of clinical isolates to the species level was performed by conventional (6, 11) and molecular (3) methods.

Susceptibility testing. Stock solutions of itraconazole (Janssen, Beerse, Belgium), fluconazole, voriconazole (Pfizer Inc., Sandwich, Kent, United Kingdom), ketoconazole (Janssen), terbinafine (Novartis, Basel, Switzerland), posaconazole (Schering Plough Research Institute, Kenilworth, N.J.), and amphotericin B (Sigma, St. Louis, Mo.) were prepared as 100× stocks in dimethyl sulfoxide (Merck, Darmstadt, Germany) or water (fluconazole) and stored at −70°C until they were used. The final concentrations for all drugs were 0.031 to 16 μg/ml, except for fluconazole, where the highest concentration was 64 μg/ml.

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TABLE 1. MIC ranges, MICs, and geometric means obtained by the modified NCCLS M27-A2[a] BMD and Etest for 53 *Malassezia* species

Malassezia species (no. of isolates)	Method	ITZ[b]			FCZ			VOR			KET			POS			TER			AmB	
		Range	MIC_{90}	GM[c]	Range	MIC_{90}	GM	Range	MIC_{90}	GM	Range	MIC_{90}	GM	Range	MIC_{90}	GM	Range	MIC_{90}	GM	Range	MIC_{100}	GM
M. furfur (12)	NCCLS	0.03–0.06	0.06	0.03	0.5–32	8	1.89	0.03–16	1	0.10	0.03–1	0.25	0.05	0.03–32	2	0.05	0.03–0.50	0.12	0.10	0.12–16	1	1.41
	Etest	0.03–0.12	0.12	0.04	0.5–64	16	3.17	0.03–32	2	0.15	0.04–2	0.12	0.09	0.03–32	1	0.09	N/A[g]			0.50–64	1	2.38
M. dermatis (2)[f]	NCCLS	0.03[d]	0.03	0.03	2[d]	2	2	0.12[d]	0.12	0.12	0.06–0.25	0.12	0.12	0.03–0.50	0.25	0.12	0.03–4	0.5	0.35	0.03–0.12	0.12	0.06
	Etest	0.03[d]	0.03	0.03	2[d]	2	2	0.25–0.50	0.25	0.35	0.25–1.00	0.5	0.50	0.06–0.25	0.12	0.12	N/A			0.06–0.5	0.5	0.17
M. globosa (22)	NCCLS	0.03–0.12	0.12	0.05	1–32	4	2.34	0.03–0.12	0.06	0.04	0.03–0.12	0.06	0.04	0.03–0.06	0.06	0.03	0.03–0.12	0.12	0.05	0.10–4	1	0.92
	Etest	0.03–0.5	0.12	0.10	1–64	6	3.76	0.01–0.5	0.12	0.05	0.02–0.12	0.06	0.04	0.03–0.12	0.06	0.04	N/A			0.5–32	4	3.02
M. obtusa (2)[f]	NCCLS	0.03–0.25	0.12	0.09	2	2	2	0.03–0.06	0.06	0.04	0.03–0.06	0.06	0.04	0.03[d]	0.03	0.03	0.03–0.12	0.12	0.06	0.03–0.06	0.06	0.04
	Etest	0.03–1	0.12	0.17	2–4	2	2.83	0.03–0.06	0.03	0.04	0.06–0.12	0.12	0.08	0.06–0.12	0.12	0.08	N/A			0.03–0.12	0.06	0.06
M. restricta (2)[f]	NCCLS	0.03[d]	0.03	0.03	0.5–1	1	0.71	0.03[d]	0.03	0.03	0.03[d]	0.03	0.03	0.03[d]	0.03	0.03	0.12–1	0.5	0.35	4–8	8	5.66
	Etest	0.03[d]	0.03	0.03	1–2	2	1.41	0.03–0.12	0.12	0.06	0.12[d]	0.12	0.12	0.03–0.06	0.06	0.04	N/A			16–32	32	22.63
M. slooffiae (4)[f]	NCCLS	0.03–0.06	0.06	0.04	1–4	2	2	0.03–0.06	0.03	0.04	0.03–0.06	0.03	0.04	0.03[d]	0.03	0.03	0.12–4	0.5	0.35	0.50–8	2	1.41
	Etest	0.03–0.05	0.05	0.03	2–8	4	3.36	0.03–0.06	0.03	0.04	0.03–0.12	0.12	0.06	0.03–0.06	0.03	0.04	N/A			1–32	4	3.36
M. sympodialis (8)[f]	NCCLS	0.03–0.06	0.06	0.04	0.5–16	8	2	0.03–0.06	0.04	0.03	0.03–0.06	0.03	0.04	0.03–0.06	0.03	0.04	0.03–0.06	0.06	0.04	0.06–0.5	0.25	0.13
	Etest	0.03–0.25	0.12	0.07	1–32	4	3.67	0.03–0.06	0.03	0.04	0.03–0.12	0.03	0.04	0.03–0.06	0.03	0.03	N/A			0.25–1.50	1	0.44
M. pachydermatis (1)[f]	NCCLS	0.06[e]	0.06	0.06	16[e]	16	16	0.03[e]	0.03	0.03	0.06[e]	0.06	0.06	0.12[e]	0.12	0.12	0.12[e]	0.12	0.12	0.12[e]	0.12	0.12
	Etest	0.12[e]	0.12	0.12	32[e]	32	32	0.06[e]	0.06	0.06	0.12[e]	0.12	0.12	0.25[e]	0.25	0.25	N/A			0.5[e]	0.5	0.5

[a] For the NCCLS M27-A2 BMD method, see reference 14.
[b] ITZ, itraconazole; FCZ, fluconazole; VOR, voriconazole; KET, ketoconazole; POS, posaconazole; TER, terbinafine; AmB, amphotericin B. Values are micrograms per milliliter.
[c] GM, geometric mean.
[d] Only one value; MICs for both strains were identical.
[e] No range; only one *M. pachydermatis* strain was indicatively tested, because growth is enhanced by lipids but is not lipid dependent.
[f] For <10 strains tested, the MIC_{50} is reported.
[g] N/A, not applicable; no Etest strips were available for terbinafine.

(i) BMD method. Susceptibility testing using RPMI 1640 (Sigma) as a diluent (14) supplemented, per liter, with 20 g of glucose, 4 g of ox bile (Oxoid), 1 ml of glycerol (Sigma), 0.5 g of glycerol monostearate (Sigma), and 0.4 ml of Tween 20 (Sigma). Itraconazole, ketoconazole, fluconazole, voriconazole, posaconazole, and amphotericin B suspensions and the final concentrations of antifungal drugs were prepared according to the NCCLS M27-A2 guidelines (14).

All stock inoculum suspensions were prepared in 5 ml of 50% (vol/vol) sterile saline-Tween 20 (Sigma) containing sterile glass beads 1.1 to 1.2 mm in diameter (Sherwood, St. Louis, Mo.). After 20 s of vortexing to disperse *Malassezia* clumps, the inoculum was standardized spectrophotometrically at 530 nm and adjusted with the modified RPMI medium to a final inoculum size of 2.0×10^3 to 3.5×10^3 CFU/ml, as validated by quality control (QC) quantitative plate counts of CFU in LN agar. Quantitative plate counts were not attainable with all *Malassezia* species, as CFU clumps, glistening to dull and brittle and butyrous in texture, were occasionally formed. In those cases, plate counts were semiquantitative, thus resulting in higher inoculum size estimates ranging from 3.0×10^3 to 4.0×10^3 CFU/ml.

Microtiter plates were incubated at 32°C and were read at 48 h for *M. furfur* and *M. pachydermatis* and at 72 h for *M. sympodialis*, *M. slooffiae*, *M. globosa*, *M. obtusa*, *M. restricta*, and *M. dermatis*. For azoles, the MIC endpoint was defined as the lowest concentration which produced a predominant decrease (90% inhibition) of turbidity compared with that of the growth control. These stringent 90% endpoints were employed because, using reference strains and 20 of 53 haphazardly selected clinical strains in pilot experiments, it was established that intense trailing phenomena, expressed as persistent turbidity for drug concentrations above the MIC, were not observed. However, to allow for statistical discrepancies, an endpoint of 50% was employed when the number of *Malassezia* species tested was <10. For amphotericin B, the MIC endpoint was defined as the lowest concentration which completely inhibited growth (100% inhibition) because pilot BMD results had shown that trailing endpoints were not encountered. Results for *M. furfur* and *M. pachydermatis* tests were also read at 72 h to assess the extent to which trailing phenomena contribute to MIC endpoint variability.

Microtiter plates containing drug dilutions can be stored at −70°C for 6 months, as testing of QC strains showed that MIC readings were within the expected range.

(ii) Etest. The agar formulations used for Etest (AB Biodisk, Solna, Sweden) comprised RPMI 1640 (Angus Buffers and Biochemicals) containing 1.5% agar (Difco, Detroit, Mich.) and 2% glucose buffered with MOPS (morpholinepropanesulfonic acid) and was prepared according to the manufacturer's instructions. The solid medium was supplemented with the aforementioned mixture of ox bile, glycerol, glycerol monostearate, and Tween 20, which was autoclaved at 121°C for 15 min, allowed to reach ~50°C, and added to the sterile buffered RPMI 1640 agar suspension. The prepared plates can be stored at 6 to 8°C for not more than 2 weeks, as the medium dehydrates while the fatty acid supplements form exudates concentrated on the agar surface. Before being tested, each isolate was subcultured at least twice in LN agar (8) to ensure optimum growth.

For the Etest, 90-mm-diameter plates with 4-mm agar depth were used. The agar surface was inoculated using the Retro C80 inoculator (Biotools; AB Biodisk) with a nontoxic swab dipped in each suspension of *Malassezia* cells adjusted spectrophotometrically at 530 nm to a turbidity of 0.5 McFarland standard. The inoculated plates were left to stand for 15 min until the excess moisture was completely absorbed into the agar before the Etest strips (Nema C88; AB Biodisk) were applied to the agar surface of each plate. The plates were incubated at 32°C in a humid atmosphere, and MICs were recorded after 48 and 72 h. The MIC was taken as the lowest concentration at which the zone of complete inhibition intersected the strip. For the trailing *Malassezia* isolates, discernible (hazy) growth within the Etest inhibition ellipse was ignored.

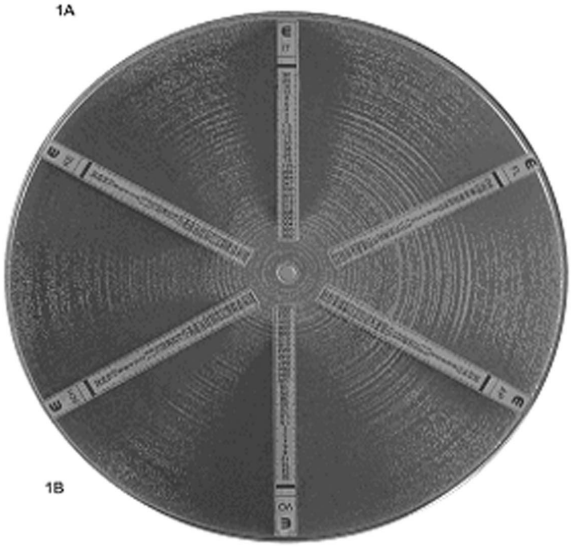
For comparison purposes, Etest MIC assays were also performed using LN medium (8).

(iii) AM3 MIC determinations. To confirm that amphotericin B increased MICs, antibiotic medium 3 (AM3) broth and agar (Unipath, Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with 2% dextrose was used for BMD and Etest assays, respectively. The medium was also supplemented with the same quantities of bile salt, fatty acid, and Tween 20 per liter. Incubation temperatures and reading of results were as described for the RPMI media.

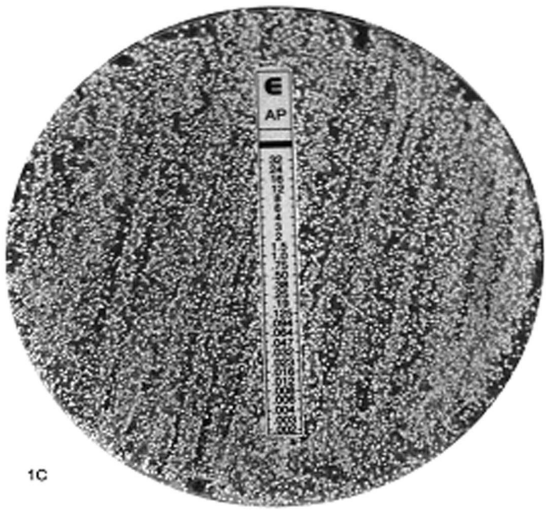
(iv) Reference strains for QC. QC was performed throughout with *M. furfur* ATCC 14521 and *M. globosa* ATCC 96807. *M. sympodialis* ATCC 96803 was used as a reference strain. QC determinations were made on each test occasion and read at 48 h for *M. furfur* and at 72 h for *M. globosa* and *M. sympodialis*. The MICs on each test occasion were always within the same range.

(v) Analysis of results. Both on-scale and off-scale results were included in the analysis. The low off-scale MICs were left unchanged, and the high off-scale MICs were converted to the next-highest concentration. The data were log

1A



1B



1C

FIG. 1. *M. furfur* ATCC 15521 and *M. globosa* ATCC 96807 susceptibility testing by Etest. Both were used as QC strains in this study. (A) *M. furfur* type strain CBS 1878/ATCC 15521 tested against posaconazole (strip SCH), fluconazole (strip FL), voriconazole (strip VO), ketoconazole (strip KE), amphotericin (strip AP), and itraconazole (strip IT). Trailing is evident with KE, VO, and IT. (B) *M. globosa* type strain CBS 7966/ATCC 96807. Similar degrees of trailing were not present with all azoles; less trailing was observed with posaconazole. (C) Confirmation of resistance of an *M. furfur* clinical isolate (BMD and Etest MICs, ≥2 μg/ml) to amphotericin B in AM3.

transformed before analysis and expressed as geometric means. Agreement was evaluated by concordance between the MICs determined by the two different susceptibility-testing methods. The BMD and Etest methods were considered to be in agreement when the MICs differed by no more than one twofold dilution. Intraclass correlation coefficients (ICCs) and 95% confidence intervals were calculated on $\log_2$-transformed data. The ICC was calculated to determine the variability of MICs and the reproducibility of each of the methods in the various subgroups. All values indicated average ICCs and showed absolute agreement with a maximum value of 1. All *P* values were two sided, and a *P* value of <0.05 was considered to indicate statistical significance. The statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) software (version 10.0; SPSS Inc., Chicago, Ill.).

RESULTS

In order to fulfill the demanding physiological requirements of the eight *Malassezia* species tested, the amino acid- and vitamin-rich RPMI 1640 medium was supplemented with fatty acids, bile salt lipids, oleic acid, Tween 20 as a source of lipids, and the *n*-octadecanoate fatty acid glycerol monostearate.

Susceptibility testing by BMD and Etest. The fatty acid broth and solid RPMI medium allowed good *Malassezia* growth and MICs that were readily read after incubation at the optimum *Malassezia* incubation temperature (32°C) for 48 and 72 h. Reading times for microtiter plates and the Etest were species dependent. MICs of all drugs against *M. furfur* and *M. pachydermatis* could be recorded after 48 h, whereas MICs against all other *Malassezia* species were read after 72 h. For most *M. furfur* isolates and the *M. pachydermatis* strain, the difference between readings at 48 and 72 h was minimal (1 dilution). Certain *M. furfur* isolates (2 of 12) showed a rise in azole MICs by the BMD method between 48 and 72 h due to partial inhibition of growth over a range of antifungal concentrations (trailing phenomenon). On these rare occasions, persistent turbidity was encountered in wells 1 to 2 dilutions above the MIC endpoint at 72 h of incubation. Dispersion of clumps by pipetting made 48- and 72-h endpoint determinations reproducible. The formation of clumps, resulting from the butyrous-textured *Malassezia* colonies, was common in the course of testing the yeast, and it was also the reason for attaining semiquantitative, rather than quantitative, plate counts during estimations of the inoculum size. *M. furfur* clinical isolates also presented the trailing phenomenon when tested by the Etest (Fig. 1A). Trailing growth was recorded with *Malassezia* species (~4% of the isolates), including the QC type strain *M. globosa* ATCC 96807. No trailing was observed with terbinafine, which was tested only by the BMD method.

The susceptibilities of 53 *Malassezia* species are summarized in Table 1. Azole and terbinafine MICs were low, but amphotericin B MICs at which 100% of the isolates were inhibited (MIC_{100}) of 1 to 32 μg/ml were recorded for *M. furfur*, *M. restricta*, *M. globosa*, and *M. slooffiae* strains with both methods (Table 1). High amphotericin B MICs (2 μg/ml) (Fig. 1B) were confirmed by testing in the *Malassezia*-modified AM3 (Fig. 1C). The only isolate tested from a confirmed bloodstream

TABLE 2. Agreement and ICCs between BMD method and Etest

Antifungal drug[a]	% agreement	ICC	*P*
ITZ	84	0.64	$<10^{-4}$
FCZ	97	0.88	$<10^{-4}$
VOR	90	0.90	$<10^{-4}$
KET	87	0.73	$<10^{-4}$
POS	91	0.88	$<10^{-4}$
AmB	49	0.88	$<10^{-4}$

[a] ITZ, itraconazole; FCZ, fluconazole; VOR, voriconazole; KET, ketoconazole; POS, posaconazole; TER, terbinafine; AmB, amphotericin B.

infection was the *M. furfur* CBS 7983 reference strain, deposited in the CBS culture collection by E. Guého in 1991. That strain displayed BMD and Etest amphotericin B MICs of 2 μg/ml. The amphotericin B resistance of this strain was confirmed by testing in AM3. Generally, the smaller amphotericin B Etest ellipse (Fig. 1A and B), compared with that generated by the azoles, was similar to those we have observed for many ascomycetous (*Candida*, *Saccharomyces*, and *Yarrowia*) and basidiomycetous yeasts, such as *Cryptococcus* and *Trichosporon* species (data not shown).

Azole MICs tested by Etest in LN medium could be obtained for *M. furfur*, *M. slooffiae*, *M. dermatis*, *M. sympodialis*, and *M. pachydermatis*, but as growth was slow, readings could only be recorded after 4 to 5 days (data not shown). Concordant amphotericin B MICs using RPMI 1640-fatty acid substrate and LN agar were not obtained, as MICs for the majority (85%) of strains showed low susceptibility (8 to ≥32 μg/ml) in LN medium. These high MICs could not be confirmed by AM3 (data not shown).

QC. Azole MICs, except fluconazole, in a series of 20 consecutive tests by BMD ranged from 0.03 to 0.125 μg/ml; terbinafine and amphotericin B MICs ranged from 0.03 to 0.25 μg/ml. Fluconazole MICs were typically higher than those of the other azoles and ranged between 4 and 16 μg/ml. Etest azole MICs, except that of fluconazole, in a series of 20 consecutive tests ranged from 0.03 to 0.25 μg/ml, and amphotericin B MICs ranged from 0.03 to 0.5 μg/ml. Etest fluconazole MICs ranged from 6 to 32 μg/ml. Only 1 out of 20 azole and terbinafine MICs was outside the recorded BMD and Etest range. No amphotericin B measurement was out of the control with either method.

Analysis of results. Overall, agreement within ±1 dilution between the BMD method and Etest was 84 to 97% for all drugs tested except amphotericin B (49%). ICCs were statistically very significant ($P < 0.001$) (Table 2). The correlation between levels of MICs obtained by the two methods is shown in Fig. 2 on $\log_2$-transformed data.

DISCUSSION

The results of the present study provide the first documentation of the applicability of fatty acid-supplemented RPMI 1640 medium in testing susceptibilities of eight *Malassezia* species to azoles and amphotericin B by BMD and Etest. Both methods produced generally low azole MICs, whereas high amphotericin B MICs were obtained for strains of *M. furfur*, *M. globosa*, *M. restricta*, and *M. slooffiae*. On the whole, the susceptibility data generated by both methods showed good reproducibility with high overall agreement.

As previously recorded in Etest evaluation studies of other yeasts (15–17), the problem of trailing endpoints due to partial inhibition of growth by the azoles was also observed with *Malassezia* species. However, compared with *Candida* species, *Malassezia* yeasts displayed limited trailing phenomena after 48 h of incubation. In the BMD method, reading of MICs after 48 h of incubation was facilitated by dispersing the clumps formed in the one or two wells above the 48-h MICs. In addition, precise implementation of the specific criteria for reading Etest MICs, as explained in the Etest package insert and technical guide for yeasts (AB Biodisk), facilitates the reading of *Malassezia* endpoint MICs. In that respect, good agreement with BMD MICs was recorded when discernible growth within an established ellipse was ignored.

Although terbinafine, amorolfine, and established and newer azole compounds have been tested against *Malassezia* skin isolates by BMD methods (4, 12), data on the in vitro performance of amphotericin B are almost exclusively associated with cases of bloodstream infections (1, 9, 18). However, these studies were performed prior to the resolution of the taxonomic status of *Malassezia* species and the recognition of new species (5). Before 1996, *Malassezia* isolates were identified in a broad sense (sensu lato) and susceptibility testing was performed by a variety of methods in dissimilar media, while AM3 was not used before for confirmation of amphotericin B resistance. Therefore, the association between the amphotericin B resistance data presented here and the therapeutic outcomes reported in earlier studies is obscured.

In addition, there are limited data on amphotericin B susceptibility relating to a large sample size of *Malassezia* isolates from human skin, which, in immunocompromised hosts, constitutes the reservoir for bloodstream infections (1, 2, 6, 12, 21). Interestingly, of our clinical isolates, 3 of 12 *M. furfur*, 2 of 2 *M. restricta*, 7 of 22 *M. globosa*, and 4 of 4 *M. slooffiae* isolates showed high amphotericin B MICs by BMD and Etest, which in all cases were confirmed by testing in AM3. Of these strains, the single bloodstream isolate tested displayed AM3-confirmed

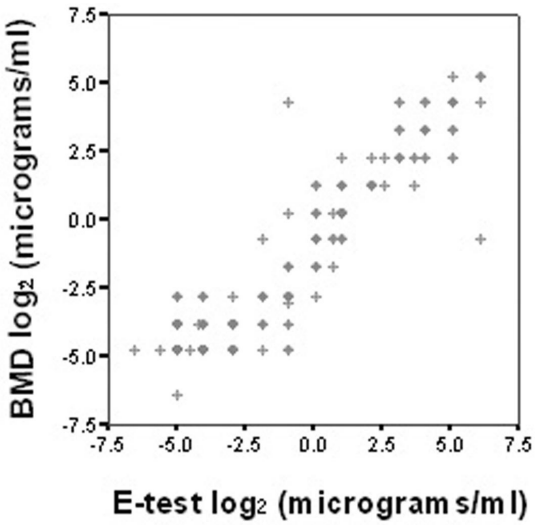


FIG. 2. Scatter diagram indicating correlation between $\log_2$ MICs obtained by BMD and Etest ($R^2 = 0.89$ by Pearson correlation coefficient).

resistance to amphotericin B. Although comparing MICs for skin and bloodstream isolates was beyond the aims of our study, this finding shows that data on the MIC ranges of *Malassezia* bloodstream isolates can be helpful in the clinical setting. The scarcity of recovery of *Malassezia* species from blood cultures has so far impaired antifungal susceptibility studies of a significant number of isolates and consequently correlation of amphotericin B in vitro response with the in vivo outcome. Despite that, the few published case reports give evidence for the poor therapeutic outcome of amphotericin B against *Malassezia* bloodstream infections. Amphotericin B is used empirically for management of *Malassezia* bloodstream infections, but with low response to therapy irrespective of the patient's immune status (1, 2, 10, 18, 19, 21). Therefore, testing the susceptibilities of these isolates by methods with established reproducibility may be indicated.

Despite the fact that growth of *Malassezia* species is well supported by the liquid and solid LN media, our study indicated that LN media may not be suitable for susceptibility testing of azoles. This was due to the long incubation time required before growth was adequate for MIC reading, thus enhancing trailing phenomena. Long incubation required for sharp MIC readings in LN media has been documented before (7), whereas the low growth rates of *M. restricta*, *M. obtusa*, and *M. globosa* recorded in LN-Alamar Blue broth (4) disqualifies the medium for azole MIC testing, at least for these three *Malassezia* species. In the present study, consistently higher amphotericin B MICs were recorded in LN media. The absence of MIC concordance between the LN and the fatty acid RPMI media and the subsequent unconfirmed resistance in AM3 indicates that the proposed RPMI media may be better candidates for amphotericin B MIC testing.

The fatty acid RPMI BMD and Etest susceptibility data reported here are not in agreement with those recorded using the urea BMD method (13), although they are not directly comparable. The utilization of a single metabolic marker as a growth indicator and the use of a large inoculum size (2.5×10^6 cells/ml), as opposed to an inoculum corresponding to 2.0×10^3 to 4.0×10^3 CFU/ml adopted in this study, may have accounted for the low susceptibilities to itraconazole and terbinafine of the seven *Malassezia* species tested in urea broth.

Our data indicated low agreement (49%) between the BMD method and Etest concerning amphotericin B. This is attributed to the consistently higher Etest MICs (1 to 2 dilutions) obtained for strains with BMD MICs of ≥ 4 μg/ml, thus influencing the agreement between the two methods. In real terms, this does not appear to constitute significant divergence of Etest MICs from those obtained by BMD, because all higher amphotericin B Etest MICs were obtained only for strains with high BMD MICs. This indicates that the low amphotericin B susceptibilities of certain *Malassezia* strains reflect a real phenotypic trait, which is possibly enhanced by testing it on solid fatty acid RPMI medium. Moreover, the observed low amphotericin B *M. restricta* and *M. slooffiae* susceptibilities may be representing a random event rather than a medium- or species-associated phenomenon, due to the small number of strains tested.

Overall, the agreement of BMD and Etest under the stringent conditions of ±1 dilution was 88%. This provides confidence that reproducible MICs can be obtained by using fatty acid RPMI for BMD and Etest. Both methods appear reliable and have good correlation ($r = 0.89$), with a highly significant intraclass correlation coefficient ($P < 0.001$). Nonetheless, the clinical relevance of MICs for the eight *Malassezia* species tested, and MICs for new species (23) yet to be determined, remains to be ascertained.

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REFERENCES

1. **Arnow, P., and R. Kushner.** 1991. *Malassezia furfur* catheter infection cured with antibiotic lock therapy. Am. J. Med. **90:**128–130.
2. **Brooks, R., and L. Brown.** 1987. Systemic infection with *Malassezia furfur* in an adult receiving long-term hyperalimentation therapy. J. Infect. Dis. **156:** 410–411.
3. **Gaitanis, G., A. Velegraki, E. Frangoulis, A. Mitroussia, A. Tsigonia, A. Tzimogianni, A. Katsambas, and N. J. Legakis.** 2002. Identification of *Malassezia* species from patient skin scales by PCR-RFLP. Clin. Microbiol. Infect. **8:**162–173.
4. **Garau, M., M. Pereino, Jr., and A. del Palacio.** 2003. In vitro susceptibilities of *Malassezia* species to a new triazole, albaconazole (UR-9825), and other antifungal compounds. Antimicrob. Agents Chemother. **47:**2342–2344
5. **Guého, E., G. Midgley, and J. Guillot.** 1996. The genus *Malassezia* with description of four new species. Antonie Leeuwenhoek **69:**337–355.
6. **Guillot, J., E. Guého, M. Lesourd, G. Midgley, G. Chevrier, and B. Dupont.** 1996. Identification of *Malassezia* species. A practical approach. 1996. J. Mycol. Med. **6:**103–110.
7. **Gupta, A. K., Y. Kohi, I. Li, J. Faergemann, and R. C. Summerbell.** 2000. *In vitro* susceptibility of the seven *Malassezia* species to ketoconazole, voriconazole, itraconazole and terbinafine. Br. J. Dermatol. **142:**758–765.
8. **Leeming, J. P., and F. H. Notman.** 1997. Improved methods for isolation and enumeration of *Malassezia furfur* from human skin. J. Clin. Microbiol. **25:** 2017–2019.
9. **Marcon, M. J., D. E. Durrell, D. A. Powell, and W. J. Buesching.** 1987. *In vitro* activity of systemic antifungal agents against *Malassezia furfur*. Antimicrob. Agents Chemother. **31:**951–953.
10. **Masure, O., C. Leostic, M. L. Abalain, C. Chastel, I. Yahoub-Agha, C. Berthou, and J. Brieve.** 1991. *Malassezia furfur* septicemia in a child with leukemia. J. Infect. **23:**335–336.
11. **Mayser, P., P. Haze, C. Papavassilis, M. Pickel, K. Gruender, and E. Guého.** 1997. Differentiation of *Malassezia* species: selectivity for cremophor El, castor oil and ricinoleic acid for *M. furfur*. Br. J. Dermatol. **137:**208–213.
12. **Murai, T., Y. Nakamura, R. Kano, S. Watabane, and A. Hasegawa.** 2002. Susceptibility testing of *Malassezia pachydermatis* using the urea broth microdilution method. Mycoses **28:**84–87.
13. **Nakamura, A., A. Kano, T. Murai, S. Watabane, and A. Hasegawa.** 2000. Susceptibility testing of *Malassezia* species using the urea broth microdilution method. Antimicrob. Agents Chemother. **44:**2185–2186.
14. **NCCLS.** 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2. NCCLS, Wayne, Pa.
15. **Pfaller, M. A., S. A. Messer, A. Houston, K. Mills, and A. Bolmström.** 2000. Evaluation of the Etest method for determining voriconazole susceptibilities of 312 clinical isolates of *Candida* species by using three different agar media. J. Clin. Microbiol. **38:**3715–3717.
16. **Pfaller, M. A., S. A. Messer, A. Karlsson, and A. Bolmström.** 1998. Evaluation of the Etest method for determining fluconazole susceptibilities of 402 clinical yeast isolates by using three different agar media. J. Clin. Microbiol. **36:**2586–2589.
17. **Pfaller, M. A., S. A. Messer, K. Mills, A. Bolmström, and R. N. Jones.** 2001. Evaluation of Etest method for determining posaconazole MICs for 314 clinical isolates of *Candida* species. J. Clin. Microbiol. **39:**3952–3954.
18. **Redline, R. W., S. S. Redline, B. Boxerboum, and B. Dahms Barett.** 1985. Systemic *Malassezia furfur* infections in patients receiving intralipid therapy. Hum. Pathol. **16:**815–822.
19. **Schleman, K., G. Tullis, and R. Blum.** 2000. Intracardiac mass complicating *Malassezia furfur* fungemia. Chest **118:**1828–1829.
20. **Schmidt, A., and B. Rühl-Hörster.** 1996. *In vitro* susceptibility of *Malassezia furfur* against azole compounds. Mycoses **39:**309–312.
21. **Schoepfer, C., H. Carla, M. J. Bezou, M. Cambon, D. Girault, F. Demeocq, and G. Malpuech.** 1995. *Malassezia furfur* septicemia after bone marrow graft. Arch. Pediatr. **2:**245–248.
22. **Sugita, T., M. Takashima, T. Shinoda, H. Suto, T. Unno, R. Tsuboi, H. Ogawa, and A. Nishikawa.** 2002. New yeast species, *Malassezia dermatis*, isolated from patients with atopic dermatitis. J. Clin. Microbiol. **40:**1363–1367.
23. **Sugita, T., M. T. Takashima, M. Kodama, R. Tsuboi, and A. Nishikawa.** 2003. Description of a new yeast species, *Malassezia japonica*, and its detection in patients with atopic dermatitis and healthy subjects. J. Clin. Microbiol. **41:**4695–4699.

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ERRATUM

Use of Fatty Acid RPMI 1640 Media for Testing Susceptibilities of Eight *Malassezia* Species to the New Triazole Posaconazole and to Six Established Antifungal Agents by a Modified NCCLS M27-A2 Microdilution Method and Etest

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Volume 42, no. 8, p. 3589–3593, 2004. Page 3590, column 2, line 7 should read as follows: "All stock inoculum preparations were prepared in 5 ml of 0.5% (vol/vol) . . . ."