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A Modified Christensen's Urea and CLSI Broth Microdilution Method for Testing Susceptibilities of Six *Malassezia* Species to Voriconazole, Itraconazole, and Ketoconazole

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Two supplemented broths (Christensen's urea with 0.1% Tween 80 and 0.5% Tween 40 and RPMI 1640 with 1% glycerol, 1% peptone, 1.8% glucose, and 0.05% Tween 80) were evaluated to determine voriconazole, itraconazole, and ketoconazole MICs for 200 *Malassezia* sp. isolates. *Malassezia globosa* and *M. restricta* were the least susceptible species (MICs at which 90% of the isolates tested were inhibited, 1 to ≥8 μg/ml versus 0.25 to 1 μg/ml).

An increased incidence of severe dermatological and systemic infections by *Malassezia* spp. has been reported among immunosuppressed patients. Standardized assays are not available to determine the in vitro susceptibilities of these yeasts to any antifungal due to their complex nutritional requirements. Recently, Christensen's urea (measures metabolic activity) and supplemented RPMI 1640 (measures growth inhibition) broths were evaluated (16, 21). We investigated (i) several medium formulations, (ii) inoculum preparations, and (iii) incubation times of modified urea and Clinical and Laboratory Standards Institute (CLSI) broth microdilution techniques to determine voriconazole, itraconazole, and ketoconazole MICs for 200 isolates (74 *Malassezia globosa*, 50 *M. sympodialis*, 52 *M. furfur*, 16 *M. restricta*, 6 *M. obtusa*, and 2 *M. pachydermatis*).

The 200 isolates were cultured from 77 patients (human immunodeficiency virus positive and negative) with dermatological pathologies (3) and 33 healthy volunteers. Isolates were identified by following conventional standard guidelines (10, 11, 15). Six reference *Malassezia* strains (see Table 2), CLSI quality control strain *Candida krusei* ATCC 6258 (2, 17), and *Cryptococcus neoformans* ATCC 90112 were tested as controls. The identification of representative isolates of each species and of the six reference isolates was confirmed by PCR-restriction fragment length polymorphism (8). For the quality control strain, the MICs of the three azoles were within the expected ranges (2, 17).

CLSI RPMI 1640 medium (17) is not suitable for most *Malassezia* spp.; it lacks the lipid supplements required for their growth. In this study, the evaluation of nine RPMI 1640 medium (Sigma, St. Louis, MO) formulations with the reference isolate of each species (*M. furfur*, *M. globosa*, *M. restricta*, *M. sympodialis*, *M. pachydermatis*, and *M. obtusa*) indicated that no. 7 (Table 1) was clear and provided suitable growth of only *M. furfur* (large buttons in wells). The medium was not

TABLE 1. Different supplemented RPMI 1640 broths evaluated for susceptibility testing of *Malassezia* spp.[a]

Medium	Supplement[b]									Medium property(ies)
	Tween 40 (0.5%)	Glycerol (1 %)	Tween 80 (0.05%)	Oleic acid (0.5%)	Peptone (1%)	Glucose (1.8%)	Olive oil (1%)	Ox bile (0.5%)	Malt extract (0.5%)	
1	+	+		+						Hazy
2			+	+						Hazy
3		+	+	+						Turbid
4		+	+							Clear; middle-sized button growth (++)
5			+							Clear; small button growth (+)
6		+								Clear; small button growth (+)
7		+	+		+	+				Clear; larger button growth (+++) but optimal growth of only *M. furfur*
8		+	+		+	+	+			Hazy
9		+	+		+	+		+	+	Larger button growth (+++) but turbid medium due to ox bile precipitation

[a] Each of the nine medium formulations (pH 7.0 for each) was evaluated by testing each reference isolate of the six species evaluated.
[b] +, supplement added to RPMI 1640 medium.

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TABLE 2. In vitro susceptibilities of reference type *Malassezia* isolates to three antifungal agents as determined by the modified urea and CLSI broth microdilution methods[a]

Isolate	MIC range (μg/ml)		
	Voriconazole	Itraconazole	Ketoconazole
M. furfur CBS 7019[b]	0.125–0.5	0.125–0.25	0.125–0.5
M. globosa CBS 7966[c]	0.5–2.0	2.0–8.0	1.0–4.0
M. restricta CBS 7877[c]	0.06–0.25	0.5–4.0	1.0–2.0
M. sympodialis CBS 7222[b]	0.125–0.5	0.06–0.25	0.25–1.0
M. pachydermatis CBS 1879[b]	0.25	0.06	0.25
M. obtusa CBS 7876[b]	≤0.03–0.03	0.125–0.25	0.03–0.125

[a] Results were obtained on two to nine different days for the six species with Christensen's urea supplemented with 0.1% Tween 80 and 0.5% Tween 40, pH 5.2.
[b] Results were obtained at 72 h.
[c] Results were obtained at 96 h.

suitable forgrowth of the other species (small buttons), or it was turbid (Table 1); all nine formulations were adjusted to pH 7.0 (17). Because of that, only 41 *M. furfur* isolates were tested with RPMI 1640 medium no. 7 but all isolates were tested with Christensen's urea (Difco, Detroit, MI) supplemented with 0.1% Tween 80 and 0.5% Tween 40 (Sigma), pH 5.2, and by following CLSI guidelines (17).

Yeasts were grown on modified Dixon agar (3.6% malt extract [Oxoid, Basingstoke, United Kingdom], 0.6% peptone [Difco], 1% Tween 40, 2% desiccated ox bile, 0.2% glycerol, 0.2% oleic acid [all from Sigma]) for 72 h (32°C ± 2°C), and uniform suspensions were prepared with the aid of sterile swabs in sterile distilled water (0.04% Tween 80 [Sigma]). Inoculum suspensions (10^6 CFU/ml) were adjusted by spectrophotometer (SP-850; Turner, Dubuque, IA) to an absorbance of either 1.0 (660-nm wavelength, *M. globosa* and *M. restricta*) or 0.425 to 0.435 (530-nm wavelength, other species). Prior inoculum sizes have ranged from ~10^3 to ~10^6 CFU/ml (9, 12, 13, 16, 20, 21). Although our inoculum density was higher than that recommended for other yeasts (~10^3 CFU/ml) (17), it provided suitable growth for all six species between 72 and 96 h. The inoculum preparation procedure yielded reproducible results (>92%, 0.5 to 6×10^5 CFU/ml after dilution); the others had slightly lower (0.2×10^5 CFU/ml) or higher (7×10^5 CFU/ml) densities (viable colony counts).

Stock suspensions of voriconazole (Pfizer Pharmaceuticals, New York, N.Y.), itraconazole, and ketoconazole (Janssen Pharmaceuticals, Beerse, Belgium) were prepared in dimethyl sulfoxide; the final drug concentrations in each medium were 0.03 to 16 μg/ml. Microdilution trays were incubated at 32°C ± 2°C for 96 (*M. globosa* and *M. restricta*) and 72 h (other species). Azole MICs were the lowest drug concentrations that showed an optical density of ≤50% (Labsystems Multiskan, Franklin, Mo.; 550-nm filter) of that of the (drug-free) growth control.

Both on-scale and off-scale MICs were considered in agreement when results from the different testing days for each isolate and with each antifungal agent were within a 3-dilution range (17). The comparison of duplicate MICs obtained with the six reference strains and 10% of the 200 isolates evaluated with each antifungal agent indicated that MICs were reproducible (within 1 to 2 dilutions) (Table 2), including the consistently high MICs for *M. globosa* and *M. restricta*.

Excellent activity was demonstrated with the three azoles for *M. furfur* with RPMI medium (MIC_{90}s [MICs at which 90% of the isolates tested were inhibited], 0.125 to 0.5 μg/ml; results not included in Table 3) as previously reported (MIC_{90}s, 0.03 to 1 μg/ml) by using a similarly modified RPMI 1640 medium (21) and other methods (9, 12, 20). Results were comparable to those obtained with the urea broth (Table 3).

In contrast to the numerous antifungal susceptibility profiles reported for other yeasts (5), few are available for the different *Malassezia* spp. Earlier data were for either *M. furfur* or *Pityrosporum* spp. (1, 6, 7, 14, 18, 19). Susceptibility to the three azoles was species dependent, with low MICs (<1 μg/ml) for most *Malassezia* spp. and high endpoints (MIC_{90}s, 1 to ≥8 μg/ml) for *M. globosa* and *M. restricta* (Table 3). Itraconazole MICs have been similar (0.8 to 6.3 μg/ml) to ours for these two species with the urea broth (16) and discrepant (<1 μg/ml) by other methods (12, 13, 20, 21). These contradictory results were obtained by using nonstandardized methodologies with either <10 isolates per species (12, 13, 16, 20, 21) or testing with a much lower inoculum (21) or by an agar dilution method (20). We evaluated 16 to 74 isolates of four of the six species and evaluated voriconazole for the first time with the urea broth. Unfortunately, the effect of testing variables on MICs, including their reproducibility, has not been evaluated as it has been for other yeasts (17). All of these factors could preclude a reliable comparison of MIC data, which a standardized method should clarify.

TABLE 3. In vitro susceptibilities of 200 *Malassezia* sp. isolates to three antifungal agents as determined by the modified urea and CLSI broth microdilution methods[a]

Species (no. of isolates)	Voriconazole			Itraconazole			Ketoconazole		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
M. furfur (52)[b]	0.03–1	0.25	0.5	0.03–0.5	0.125	0.25	0.03–1	0.25	0.5
M. globosa (74)[c]	0.03–≥8	2	≥8	0.015–≥8	4	≥8	0.0015–≥8	8	≥8
M. obtusa (6)[b]	0.03–0.25	0.125	NA[d]	0.015–0.25	0.06	NA	0.125–1	0.25	NA
M. pachydermatis (2)[b]	0.125–0.25	NA	NA	0.03–0.125	NA	NA	0.06–0.25	NA	NA
M. restricta (16)[c]	0.06–8	0.125	2.0	0.015–>8	0.125	2.0	0.015–≥8	0.125	1.0
M. sympodialis (50)[b]	0.015–1	0.06	0.25	0.015–2	0.06	1.0	0.015–4	0.125	0.5

[a] Results were obtained for the six species with Christensen's urea supplemented with 0.1% Tween 80 and 0.5% Tween 40, pH 5.2. MICs are in micrograms per milliliter.
[b] Results were obtained at 72 h.
[c] Results were obtained at 96 h.
[d] NA, not applicable.

The urease method has identified the possible species azole activity dependency in our and a previous study (16); resistance detection is the most important goal of antifungal susceptibility testing. This fact further supports the importance of performing susceptibility testing of these isolates by a standardized method. The poor activity demonstrated by the three azoles against *M. globosa* and to a certain extent against *M. restricta* could be an important finding. *M. globosa* is the etiological agent of pityriasis versicolor, which has a high rate (60 to 80%) of recurrence (4, 10). Based on pharmacokinetics, pharmacodynamics, and in vitro correlations with clinical response in candidal infections, voriconazole MICs of ≤ 1 μg/ml (susceptibility breakpoint) have correlated with a clinical therapeutic response while voriconazole MICs of ≥ 4 μg/ml and itraconazole MICs of ≥ 1 μg/ml are indicators of a poor response to therapy (resistant breakpoints) with these two agents (2). Although the correlation between in vitro and in vivo results for *Malassezia* spp. has not been established for any antifungal agent, high azole MICs could also indicate a poor therapeutic response, as they have for *Candida* spp.

In conclusion, the optimum testing conditions for the *Malassezia* spp. evaluated were Christensen's urea broth with 0.1% Tween 80 and 0.5% Tween 40, stock inoculum suspensions adjusted to an optical density of either 0.425 to 0.435 (530 nm) or 1.0 (660 nm, *M. globosa* and *M. restricta*), and incubation times of 72 to 96 h. Collaborative studies are essential to investigate the interlaboratory reproducibility of our modified method.

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