

Susceptibility Testing of *Malassezia* Species Using the Urea Broth Microdilution Method

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A urea broth microdilution method to assay the susceptibilities of seven *Malassezia* species was developed. This method indicated the same sensitivities as the agar plate dilution method for isolates of *Malassezia furfur*, *M. pachydermatis*, *M. slooffiae*, and *M. sympodialis*.

Malassezia species may be etiological agents of systemic infection as well as of skin disorders such as pityriasis versicolor and *Malassezia* folliculitis (2, 8, 14). The genus *Malassezia* has been recently revised to include seven species, *Malassezia furfur*, *M. pachydermatis*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae* (5, 6). Numerous susceptibility testing methods for determination of the MICs against *Candida* spp. and other yeast isolates have been developed (1, 3, 4). In 1992, the National Committee for Clinical Laboratory Standards recommended a broth microdilution method for susceptibility testing of yeasts (11). However, this method was not applicable to *Malassezia* species other than *M. pachydermatis*, because the organism could not develop without lipoidal substances in the medium (5, 8). Previously, we devised a urea broth microdilution method for antifungal testing with *Cryptococcus neoformans* (10). *Malassezia* species were confirmed to be urease positive, as is *C. neoformans* (5, 13). We attempted to develop a practical method of susceptibility testing to obtain reliable information on the antifungal activities of various drugs against seven *Malassezia* species which possesses urease activity.

Forty-two isolates of *Malassezia* (13 isolates of *M. furfur*, 1 isolate of *M. globosa*, 6 isolates of *M. obtusa*, 1 isolate of *M. restricta*, 2 isolates of *M. slooffiae*, 7 isolates of *M. sympodialis*, and 12 isolates of *M. pachydermatis*) were used in this study. Clinical isolates of *M. furfur*, *M. obtusa*, *M. sympodialis*, and *M. pachydermatis* from humans and animals were identified by a

conventional method (5) and molecular analysis (7). These isolates were maintained by culturing on modified Dixon agar (5) at 30°C and were subcultured every week more than three times before use. The ivory colonies grown on modified Dixon agar were collected by scraping and were suspended with 0.04% Tween 80 in distilled water (pH 7.4) with a glass homogenizer, and the suspension was adjusted to an absorbance (optical density [OD] at 660 nm) of 1.0 (2.5×10^6 cells/ml) with an automatic absorbance meter (UV-160A; Shimadzu, Kyoto, Japan). The antifungal drugs tested were bifonazole, itraconazole, amorolfine, and terbinafine. Every drug was dissolved in dimethyl sulfoxide solution and gradually diluted with distilled water. The highest test concentration of the drug was 50 µg/ml, as recommended in previous reports for the other yeasts (1, 3, 4, 15). The urea broth microdilution method of susceptibility testing (urease method) was performed in 96-well microtiter plates. To these wells were added modified Christensen's urea broth (100 µl), fungal suspension (50 µl), and the test drugs (50 µl). The 96-well microtiter plates were arranged in twofold dilution from left to right and in triplicate. They were incubated at 30°C for 48 h. The results were measured by an automatic microtiter plate reader (AUTO READER II; Sanko Junyaku, Tokyo, Japan) at 545 nm (10). The final mean OD obtained for each antifungal concentration was expressed as a percentage of the control growth. The agar plate dilution method for determining colony development was

TABLE 1. Antifungal activities against *Malassezia* species by the urease method

| Species (no. of isolates) | MIC (µg/ml) ^a | | | | | | | |
|------------------------------|--------------------------|----------|--------------|-----------|------------|----------|-------------|----------|
| | Bifonazole | | Itraconazole | | Amorolfine | | Terbinafine | |
| | Mean | Range | Mean | Range | Mean | Range | Mean | Range |
| <i>M. furfur</i> (13) | 12.5 | 6.3–25 | 6.3 | 3.2–25 | 12.5 | 3.2–25 | 12.5 | 3.2–50 |
| <i>M. globosa</i> (1) | 1.6 | 0.8–6.3* | 1.6 | 0.8–6.3* | 6.3 | 3.2–6.3* | 1.6 | 0.8–6.3* |
| <i>M. obtusa</i> (6) | 0.4 | 0.1–3.2 | 0.4 | 0.1–1.6 | 6.3 | 3.2–12.5 | 1.6 | 0.8–6.3 |
| <i>M. pachydermatis</i> (12) | 6.3 | 3.2–25 | 1.6 | 0.8–6.3 | 25 | 3.2–50 | 6.3 | 3.2–25 |
| <i>M. restricta</i> (1) | 0.4 | 0.1–0.8* | 6.3 | 1.6–6.3* | 12.5 | 6.3–25* | 12.5 | 6.3–25* |
| <i>M. slooffiae</i> (2) | 0.4 | 0.1–0.8* | 0.4 | 0.4–0.8* | 3.2 | 0.8–3.2* | 0.2 | 0.1–0.8* |
| <i>M. sympodialis</i> (7) | 0.1 | 0.1–0.2 | 0.05 | 0.025–0.1 | 1.6 | 0.2–6.3 | 0.2 | 0.05–0.8 |

^a *, results from isolates cultured more than three times.

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TABLE 2. Antifungal activities against *Malassezia* species by the agar plate dilution method

| Species (no. of isolates) | MIC ($\mu\text{g/ml}$) ^a | | | | | | | |
|------------------------------|---------------------------------------|----------|--------------|-----------|------------|----------|-------------|---------|
| | Bifonazole | | Itraconazole | | Amorolfine | | Terbinafine | |
| | Mean | Range | Mean | Range | Mean | Range | Mean | Range |
| <i>M. furfur</i> (13) | 25 | 6.3–50 | 6.3 | 0.8–12.5 | 50 | 6.3–50 | 25 | 3.2–50 |
| <i>M. pachydermatis</i> (12) | 12.5 | 3.2–25 | 3.2 | 0.4–6.3 | 50 | 6.3–50 | 12.5 | 3.2–50 |
| <i>M. slooffiae</i> (2) | 1.6 | 0.8–6.3* | 0.8 | 0.4–0.8* | 12.5 | 3.2–50* | 6.3 | 1.6–25* |
| <i>M. sympodialis</i> (7) | 0.4 | 0.1–1.6 | 0.1 | 0.025–0.2 | 3.2 | 0.8–12.5 | 1.6 | 0.1–6.3 |

^a *, results from isolates cultured more than three times.

also performed as described in previous reports (9, 12), and the MIC results were compared with those by the urease method. The plates were incubated at 30°C for 4 days.

All *Malassezia* species were positive for urease activity when modified Christensen's urea broth was used. A good correlation between the number of fungal cells and ODs was shown. It was confirmed in these experiments that the urease activity could be measured instead of counting viable cells of *Malassezia*. Phenol red used as an indicator was very sensitive to fungal cell activity. The antifungal activities of the four drugs are shown in Tables 1 and 2. Every result obtained using this urease method demonstrated good reproducibility.

The National Committee for Clinical Laboratory Standards has recommended a broth microdilution method for the susceptibility testing of yeasts. However, this method was not suitable for testing the susceptibilities of *Malassezia* species because they were lipophilic except for *M. pachydermatis*. Previous reports suggested that *Malassezia* species were urease positive (5, 13). Therefore, we confirmed that all seven species of *Malassezia* were positive for urease activity by a method using Christensen's urea broth modified to grow these lipophilic yeast-like fungi by the addition of Tween 40 (0.5%) and Tween 80 (0.1%).

Seven *Malassezia* species examined were susceptible to four antifungal drugs (bifonazole, itraconazole, amorolfine, and terbinafine) in the following decreasing order: *M. sympodialis*, *M. slooffiae*, *M. obtusa*, *M. globosa*, *M. pachydermatis*, *M. restricta*, and *M. furfur*. Moreover, they were divided into two groups according to their susceptibilities to the antifungal drugs: *M. sympodialis*, *M. slooffiae*, and *M. obtusa* were included in the more-susceptible group, and *M. globosa*, *M. pachydermatis*, *M. restricta*, and *M. furfur* composed the less-susceptible group. In addition, bothazole derivatives were found to be more active than amorolfine and terbinafine against each *Malassezia* isolate. The most susceptible isolate was an isolate of *M. sympodialis*, and the least susceptible isolate was an isolate of *M. pachydermatis*. The urease method was also able to assess the antifungal activity at each concentration of the drug, even at a concentration lower than the MIC, because the inhibition, expressed as a percentage of the control, was determined at each drug dilution step.

The antifungal activities of amorolfine and terbinafine measured using the urease method were higher than those measured using the agar plate dilution method against *M. slooffiae* and *M. sympodialis*. This higher sensitivity of the urease method might have been obtained as a result of measuring a

single metabolic activity instead of the number of cells developed.

Furthermore, this urea broth microdilution method should be evaluated for the antifungal testing of drugs against other urease-positive fungi.

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