Clinical Evaluation of the ASTY Colorimetric Microdilution Panel for Antifungal Susceptibility Testing

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A method using a commercially prepared colorimetric microdilution panel (ASTY; Kyokuto Pharmaceutical Industrial Co., Ltd.) was compared in four different laboratories with the National Committee for Clinical Laboratory Standards (NCCLS) reference microdilution method by testing 802 clinical isolates of Candida spp. (C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, C. lusitaniae, C. guilliermondii, C. lipolytica, C. rugosa, and C. zeylanoides) against amphotericin B, 5-fluorocytosine (5FC), fluconazole, and itraconazole. Reference MIC endpoints were established after 48 h of incubation, and ASTY endpoints were established after 24 and 48 h of incubation. ASTY endpoints were determined to be the time at which the color of the first well changed from red (indicating growth) to purple (indicating growth inhibition) or blue (indicating no growth). Excellent agreement (within 2 dilutions) between the reference and colorimetric MICs was observed. Overall agreement was 93% at 24 h and 96% at 48 h. Agreement ranged from 90% with itraconazole and 5FC to 96% with amphotericin B at 24 h and from 92% with itraconazole to 99% with amphotericin B and 5FC at 48 h. The ASTY colorimetric microdilution panel method appears to be comparable to the NCCLS reference method for testing the susceptibilities of Candida spp. to a variety of antifungal agents.

The National Committee for Clinical Laboratory Standards (NCCLS) reference method for antifungal susceptibility testing has been successfully adapted to a microdilution format (4, 7). The microdilution broth method has been validated in numerous comparative studies whose results have been published in peer-reviewed journals (3, 5, 9, 15, 16) and is now detailed in the recently approved NCCLS document M27-A (7). Further refinements have included agitation of the microdilution trays prior to the reading of the MICs (1, 4, 11) and the use of other methods designed to improve MIC endpoint determinations such as spectrophotometric (2, 11, 14) and colorimetric (8–10, 13, 17) reading methods. These modifications have been shown to improve the accuracy and reproducibility of the microdilution method (4, 10, 11–13, 17).

The utilization of commercially prepared frozen or dried microdilution panels is now standard in clinical microbiology laboratories performing antimicrobial susceptibility testing of bacteria. Commercial preparation of panels containing an array of antimicrobial agents at appropriate concentrations for clinical testing is not only convenient but also provides additional standardization and quality assurance to a process that is both clinically important and technically demanding (6, 12).

The ASTY colorimetric antifungal panel (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) is a commercially prepared microdilution panel which uses an oxidation-reduction colorimetric indicator to aid in antifungal MIC endpoint determination. The ASTY panel has been developed by the Kyokuto Pharmaceutical Industrial Co., Ltd., for in vitro susceptibility testing of Candida isolates. The purpose of this study was to evaluate the performance of ASTY microdilution panels containing amphotericin B, 5-fluorocytosine (5FC), fluconazole, and itraconazole against a broad spectrum of Candida species in four different laboratories and to compare the results from these panels with those from a reference microdilution method performed according to NCCLS guidelines.

MATERIALS AND METHODS

Test organisms. Approximately 200 clinical isolates of Candida spp. were tested in each of four participating laboratories (a total of 802 isolates; range, 196 to 205) by both the ASTY and the reference microdilution method. The collection included the following numbers of isolates: 358 for C. albicans, 151 for C. glabrata, 83 for C. parapsilosis, 79 for C. tropicalis, 66 for C. krusei, 36 for C. lusitaniae, 14 for C. guilliermondii, and 15 for Candida spp. (5. C. lipolytica, 4. C. rugosa, 3. C. zeylanoides, and 3. Candida spp.). The isolates were identified by standard methods (18) and stored in water suspensions or on agar slants until they were used in the study. Prior to testing, each isolate was passaged at least twice on Sabouraud dextrose agar to ensure optimal growth characteristics.

Antimicrobial drugs and microdilution trays. Amphotericin B, 5FC, fluconazole, and itraconazole were obtained as reagent-grade powders from their respective manufacturers. A single lot of dried microdilution trays containing serial dilutions of the antifungal agents was prepared by Kyokuto Pharmaceutical Industrial Co., Ltd. The microdilution trays and RPMI 1640 medium were shipped by the manufacturer to each participating laboratory. The trays were stored at 4°C until they were used in the study.

Antifungal susceptibility test methods. Broth microdilution testing was performed according to NCCLS guidelines (7). The wells of each microdilution tray were reconstituted by the addition of the inoculum suspension (0.5 × 109 to 2.5 × 109 cells per ml) in RPMI 1640 medium with and without the colorimetric indicator (0.1 ml per well). Final concentrations of the antifungal agents were 0.05 to 16 μg/ml for amphotericin B, 0.12 to 64 μg/ml for 5FC, 0.12 to 64 μg/ml for fluconazole, and 0.015 to 8 μg/ml for itraconazole. The trays were incubated at air at 35°C and were observed for the presence or absence of growth at 24 and 48 h.

The colorimetric MIC endpoints were read with the aid of a reading mirror. Growth in each well was indicated by a color change from blue (no growth) to red (growth). With all drugs the MIC was defined as the lowest concentration of antifungal agent that prevented the development of a red color (the first blue or purple well). Unlike the NCCLS guidelines, a less stringent amphotericin B MIC endpoint criterion was used for the colorimetric part of the
study. This criterion was based on the results obtained in preliminary studies in which most of the isolates failed to yield a blue color. This was particularly evident with the 48-h readings, which resulted in amphotericin B MICs significantly discrepant from those of their turbidimetric counterparts. However, these discrepancies were neither drug nor isolate related, as the blue color was not achieved even in the sterility control wells. This lack of color development was thus accepted to be due to color deterioration of the reagent, and the amphotericin B MIC definition was handled less restrictively, i.e., as the first blue or purple well.

The reference (noncolorimetric) MICs were read after 48 h of incubation. The 48-h reference MIC endpoint for each antifungal agent was determined according to NCCLS recommendations (complete inhibition for amphotericin B and 80% reduction in turbidity for 5FC, fluconazole, and itraconazole) (7).

QC and reproducibility. Quality control (QC) and reproducibility of the results of the reference and ASTY methods was assessed by testing the QC strains listed in the NCCLS M27-A document, C. krusei ATCC 6258 and C. parapsilosis ATCC 22019, plus four additional reference strains, C. lusitaniae 5W31, C. albicans 707, C. tropicalis ATCC 750, and C. parapsilosis ATCC 90018. These isolates were sent to the four participating laboratories and were each tested between 4 and 14 times in each of the four laboratories.

Study design and analysis. Four laboratories (referred to as laboratories 1 to 4) in three different countries (the United States, Canada, and Japan) participated in the study. Each laboratory received individual subcultures of the QC and reference isolates for reproducibility studies, microdilution trays, and sufficient buffered RPMI 1640 test medium with and without indicator to perform the microdilution reference and ASTY methods. Thus, 21 to 38 MICs of each antifungal agent were available for each reference isolate tested by the QC and reference strains and the four antifungal agents. Generally, the MICs read at 24 h and 80% reduction in turbidity for 5FC, fluconazole, and itraconazole) (7).

Additionally, each laboratory tested approximately 200 (range, 196 to 205) recent clinical isolates of Candida spp. by both the reference and ASTY methods. The ASTY MICs read at 24 and 48 h for the 302 clinical isolates were compared with the reference microdilution MICs read at 48 h. Both on-scale and off-scale results were included in the analysis. As with previous studies, the high off-scale MICs were converted to the next highest concentrations and the low off-scale MICs were left unchanged. Overall, >95% of MICs were on-scale. Discrepancies among MIC endpoints of no more than 2 dilutions (two wells) were used to calculate the percent agreement between methods. Essential agreement between the results of the two methods based on comparison of interpretive categories for fluconazole, itraconazole, and 5FC was calculated by NCCLS criteria for susceptible and resistant strains (7). In performing this analysis, the susceptible and susceptible–dose-dependent categories were combined. For susceptible and resistant strains, the MICs of each agent were as follows: ≤32 μg/ml for fluconazole-resistant strains, ≤16 μg/ml for itraconazole-resistant strains, and ≤8 μg/ml for fluconazole-susceptible strains.

RESULTS AND DISCUSSION

In the QC tests performed in each laboratory, the MICs of amphotericin B, 5FC, and fluconazole for both of the QC isolates were within the ranges of the NCCLS reference method. The MICs of itraconazole for both QC strains tended to be 1 dilution higher than the MICs in the NCCLS reference range. The QC range for itraconazole was expanded by 1 dilution, then 95% of MIC results for C. parapsilosis ATCC 22019 and 100% of results for C. krusei ATCC 6258 were encompassed by the QC reference range.

A tight distribution of MICs was observed for the set of six isolates of Candida spp. to amphotericin B, 5FC, fluconazole, and itraconazole as determined by the NCCLS reference microdilution method. A broad range of MICs was observed with each antifungal agent. The MICs of each antifungal agent were also typical for the individual Candida species tested (4, 8, 12).

Table 2 summarizes the in vitro susceptibilities of 802 isolates of Candida spp. to amphotericin B, 5FC, fluconazole, and itraconazole as determined by the NCCLS reference microdilution method. A broad range of MICs was observed with each antifungal agent. The MICs of each antifungal agent were also typical for the individual Candida species tested (4, 8, 12).

The overall agreement between the results of the reference method and the ASTY colorimetric method was 93% when the ASTY MICs were read at 24 h and 96% when they were read at 48 h (Table 3). Agreement ranged from 90% with itraconazole and 5FC to 96% with amphotericin B at 24 h and 92% with itraconazole to 99% with amphotericin B and 5FC at 48 h. Similar high levels of agreement were observed for results from the four study sites at both 24 and 48 h. The essential categorical agreement observed between 2,406 pairs of MIC determinations was 91.7% (0.7% M errors and 7.6% VM errors) when the ASTY results were read at 24 h and 92.6% (5.1% M errors and 2.3% VM errors) when they were read at 48 h.

Regarding individual species of Candida, the agreement in the MICs of all drugs for all species was ≥80% at 24 h of incubation, with the exception of the MICs of 5FC for C. krusei (48%) and the MICs of the triazoles, fluconazole and itraconazole, for C. guilliermondii (79% agreement) (Table 4). At 48 h of incubation the agreement between ASTY MICs and those
of Candida species. Importantly, results similar to those obtained by the reference method were observed by technicians in four different laboratories in three different countries. Not only did these four laboratories find similar levels of agreement between results of the ASTY and reference methods, they also demonstrated very good interlaboratory agreement in results from testing a common set of reference isolates. It must be emphasized that when a new antifungal susceptibility test method is evaluated, it is important to demonstrate that the method performs comparably to the reference method when clinical isolates representing several different species of Candida are tested. Simply showing that the new method performs well with the QC strains is not sufficient to establish comparability with the NCCLS reference method.

Although the level of agreement between the results of the ASTY colorimetric and reference methods was slightly better after 48 h of incubation than after 24 h, the data suggest that an earlier reading is certainly possible for many drug-organism combinations. This is most true for C. tropicalis and the triazoles, with which the colorimetric method gave falsely high MICs of both fluconazole and itraconazole when the tests were read at 48 h but gave MICs in excellent agreement with those of the reference method when the tests were read after 24 h of incubation. Similar discrepancies with fluconazole and C. tropicalis were reported by Pfaller and Barry (8) in a study of an Alamar Blue microdilution method. Curiously, the discrepancy with C. tropicalis in the present study was observed only at two of the four study sites. The reasons for such species-specific discrepancies between colorimetric and reference MICs remain unclear but may be obviated by reading the tests at the earlier time point. The importance of contrasting 24- and 48-h MICs was underscored in a recent study by Rex et al. (15a) that...
demonstrated that the lower 24-h MICs correlated better with outcome in vivo than the higher 48-h MICs.

In summary, we have provided evidence that the commercially prepared ASTY colorimetric antifungal susceptibility panel method exhibits excellent reproducibility and comparability of results when it is compared with the NCCLS reference method. The availability of a commercially prepared microdilution panel that provides a colorimetric endpoint and can be read within 24 h for most isolates will certainly be attractive to those laboratories currently using in-house-prepared panels or macrodilution methods for performing antifungal susceptibility testing. Additional benefits include improved standardization, reduced costs associated with production and quality assurance, and the ability to test simultaneously several antifungal agents.

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REFERENCES


