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Proficiency Testing Program for Clinical Laboratories Performing Antifungal Susceptibility Testing of Pathogenic Yeast Species

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Antifungal susceptibility testing is expected to facilitate the selection of adequate therapy for fungal infections. The general availability of antifungal susceptibility testing in clinical laboratories is low, even though a number of standard methods are now available. The objective of the present study was to develop and evaluate a proficiency testing program (PTP) for the antifungal susceptibility testing of pathogenic yeasts in laboratories licensed by the New York State Department of Health. A number of quality control standards, and methods for documenting laboratory performance, were developed in consultation with the laboratory directors. The participating laboratories were provided with five American Type Culture Collection strains of pathogenic yeasts for which the minimum inhibitory concentrations (MICs) of amphotericin B and fluconazole were well defined. A majority of laboratories (14 of 17) used broth microdilution, and these were evenly split between the NCCLS M-27A protocol and the Sensititre YeastOne method. The other three laboratories performed susceptibility testing with Etest. Overall, the levels of agreement between MIC reference ranges and the reported MICs were 85 and 74% for amphotericin B and for fluconazole, respectively. All laboratories except one successfully detected fluconazole resistance in a *Candida krusei* strain. However, amphotericin B resistance in a *Candida lusitaniae* strain was not detected by any of the participating labs. It is concluded that a suitably designed PTP could adequately monitor the competence of clinical laboratories performing antifungal susceptibility testing.

Antifungal susceptibility testing of pathogenic fungi is expected to facilitate the selection of adequate therapy for fungal infections (5, 13, 14, 18). The antifungal susceptibility testing may also provide an estimate of antifungal efficacy, prediction of therapeutic outcome, monitoring of the development of drug resistance, and therapeutic potential of untested compounds (5, 13, 14). The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Testing has published a series of documents describing the development of a standard method for the antifungal susceptibility testing of pathogenic yeasts and molds (11, 12, 18). The general availability of antifungal susceptibility testing in clinical laboratories is low, even though the standardized methods are now available. Currently, reference laboratories perform most antifungal testing. Two quality control strains (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258) and four reference strains have been selected by the NCCLS Subcommittee, and minimum inhibitory concentration (MIC) ranges of amphotericin B, 5-flucytosine, fluconazole, itraconazole, and ketoconazole have been reported (11, 15, 16). A number of commercial systems are now under development for antifungal susceptibility testing of yeasts, and Sensititre YeastOne (TREK Diagnostics Systems Inc., Westlake, Ohio) and Etest (AB BIODISK North America Inc., Piscataway, N.J.) have been extensively tested (4, 6, 8, 13). Sensititre YeastOne was recently cleared by the Food and Drug Administration.

The Clinical Laboratory Improvement Amendments of 1988 (CLIA 1988) have brought significant changes in the operations of clinical laboratories in the United States (7). Under the regulations of CLIA 1988, the development of proficiency testing (PT) programs (PTPs) would allow maximum limits on deviations of PT results from peer means of the participating laboratories (7). In CLIA 1988, the number of unknowns analyzed by the participating laboratories was increased from two to five specimens per distribution (2). The New York State Department of Health (NYSDOH) initiated a PTP to monitor the overall quality of testing performed by State permit-holding clinical laboratories, in response to the 1964 legislative mandate (17). The Mycology PTP of the NYSDOH is responsible for ensuring the quality of clinical mycology testing in laboratories that test specimens originating from patients in New York State. Currently, 152 laboratories hold licenses either to identify yeast-like pathogens (yeast only) or to identify all fungi (general) from clinical specimens. The participants in the program are tested thrice yearly with a total of 15 unknown fungal specimens, and they also submit background information about their laboratory operations. These data were previously used to analyze the quality of the participating laboratories (17). The Bacteriology PTP at the NYSDOH has also reported the results of a survey of laboratories performing antimicrobial susceptibility testing and interpretation (9). Similarly, PT and quality control results for antimicrobial susceptibility testing have been reported by the Centers for Disease Control and Prevention in collaboration with the World Health Organization (20). The College of American Pathologists (CAP) currently has an ongoing ungraded program of antifungal susceptibility testing (3, 13). Thus, PTP data could serve as a valuable resource for the analysis of laboratory

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performance in the area of antifungal susceptibility testing. The objective of the present study was to develop and evaluate the PT program for antifungal susceptibility testing of pathogenic yeasts in laboratories licensed by the NYSDOH.

MATERIALS AND METHODS

Questionnaire. In October 2000, a questionnaire was sent to 152 laboratories participating in the Mycology PTP to seek feedback about the scope of antifungal susceptibility testing. The survey requested information about testing performed within the laboratory, specimens sent out to reference laboratories, antifungal agents used for susceptibility testing, methods employed, and any plans for the introduction of antifungal susceptibility testing in the near future.

Test design. Based on the response to the questionnaire, a pilot PT was designed for the laboratories that voluntarily disclosed that they perform antifungal susceptibility testing. It was proposed to use five American Type Culture Collection (Manassas, Va.) (ATCC) strains of pathogenic yeasts for which the MICs of amphotericin B and fluconazole have been published (11, 15, 18). These strains were *Candida albicans* ATCC 24433, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, and *C. lusitaniae* ATCC 200950. Each laboratory was asked to record the method used, the choice of medium, inoculum preparation and concentration, incubation temperature, duration of incubation, and endpoint reading for the two drugs to be tested.

Fungal isolates and preparation. The five yeast isolates were purchased from the ATCC. The samples were prepared according to the NYSDOH protocol for preparation of PT specimens. Briefly, these organisms were removed from frozen glycerol stock (10% sterile glycerol suspensions stored at −70°C), subcultured onto Sabouraud's dextrose plates, and incubated at 35°C. After 48 h of incubation, the plates were inspected for purity. Broth microdilution tests for amphotericin B and fluconazole were performed according to the NCCLS M27-A guidelines (11). These validations were done twice before specimen distribution to the participating laboratories. After validation of the susceptibility patterns, yeast colonies were transferred onto 17 sets of Sabouraud's dextrose agar tubes and incubated at 35°C for 48 h. A set of tubes with visible growth was sent to each of the 17 participating laboratories via overnight delivery on December 5, 2001. A set of test specimens was mailed back to the NYSDOH diagnostic mycology laboratory (one of the participating laboratories) to ascertain the effect of the mailing conditions.

PT. Laboratories were reminded that they must follow routine procedures for testing of the PT specimens. They were also instructed to accurately record all information, as they processed the PT specimens, on the answer sheet provided with the test samples. A 3-week response period was set as the deadline for return of results, although the laboratories were expected to report results sooner than the deadline if they indeed handled specimens according to their routine procedures. For each antifungal agent, the results provided by a particular laboratory were independently analyzed and were then compared with total responses. Acceptable results were MICs within ±2 dilutions of the reference range for a particular yeast, as described in NCCLS document M27-A (12). These acceptable results were established from empirical rules that required results to be within ±2 standard deviations of the mean value (3, 7). For *C. lusitaniae* ATCC 200950, the MIC range from an earlier publication was used (1, 10, 19). As per CLIA 1988 guidelines, two out of five results being reported outside the expected range led to a laboratory's receiving an unsatisfactory evaluation (7).

RESULTS AND DISCUSSION

The results of the October 2000 questionnaire revealed that 17 laboratories performed antifungal susceptibility testing. These included 15 microbiology laboratories within the United States and one reference laboratory each from Canada and the United Kingdom. The United States laboratories were located in New York (eight), California (three), and one each in Texas, Virginia, Minnesota, and Utah. These laboratories received the pilot antifungal PT specimens on December 6, 2001. Susceptibility test systems used by the participating laboratories included broth microdilution plates made in-house according to NCCLS M27-A guidelines (eight laboratories), the Sensititre YeastOne colorimetric test (TREK Diagnostics Systems) (seven laboratories), and Etest (AB BIODISK North America) (three laboratories). The quality control for susceptibility test systems was performed as per either the recommendation of the NCCLS antifungal subcommittee or the manufacturers' protocol. This pattern is similar to that of the participating laboratories in the CAP survey, in which 71% of laboratories used broth microdilution and 35% of the laboratories used the YeastOne colorimetric method (3, 13). The supplementary information on the performance of antifungal susceptibility test is summarized in Table 1. MacFarland standard was most commonly used for inoculum preparation, and 0.5×10^3 to 2.5×10^3 CFU/ml was the preferred inoculum size. This method of inoculum preparation and the inoculum size are recommended in the NCCLS M27-A protocol (11). A majority of laboratories used 35°C as the incubation temperature. Similarly, this is the recommended temperature of incubation in the NCCLS M27-A protocol (11). Eighty-eight percent of the participating laboratories used RPMI 1640 to perform antifungal susceptibility testing, as recommended in the NCCLS M27-A document (11). One laboratory used antibiotic medium 3 instead of RPMI 1640 because the in-house protocol speci-

TABLE 1. Summary of supplementary information on antifungal susceptibility testing by participating laboratories

Criterion	No. of participant laboratories
Test method	
NCCLS broth microdilution	7
Sensititre YeastOne colorimetric	7
Etest	3
Media employed	
RPMI 1640	11
RPMI 1640 with Alamar Blue	5
Casitone agar	1
Antibiotic medium 3	1[a]
Inoculum preparation	
MacFarland	9
Spectrophotometric	8
Inoculum size	
0.5×10^3 to 2.5×10^3	9
1.5×10^3 to 8×10^3	8
Incubation temperature (°C)	
30	1
35	13
37	3
Incubation duration (h)	
24	8
48	9
Endpoint reading	
Visual	11
Colorimetric	5
Spectrophotometric	1
Quality control organism	
NCCLS recommended strains	16
Unknown	1

[a] One laboratory used antibiotic medium 3 for amphotericin B and RPMI 1640 for fluconazole susceptibility testing.

TABLE 2. Methods for antifungal susceptibility testing for amphotericin B and comparison of results with the NCCLS reference range

Organism	Reference range (μg/ml)	NCCLS broth microdilution results (μg/ml)	% Agreement	Sensititre YeastOne results (μg/ml)	% Agreement	Etest results (μg/ml)	% Agreement	Overall agreement (%)
C. albicans ATCC 24433	0.25–1.0	0.12–1.0	86	0.12–0.5	86	0.12	0	57
C. krusei ATCC 6258	0.25–1.0	0.5–2.0	100	0.25–1.0	86	0.5–1.5	100	95
C. lusitaniae ATCC 200950	0.25–1.0	0.12–1.0	86	0.25–1.0	100	0.3–0.5	100	95
C. parapsilosis ATCC 22019	0.25–1.0	0.25–1.0	100	0.12–1.0	86	0.25–0.5	100	95
C. tropicalis ATCC 750	0.5–2.0	0.12–1.0	72	0.5–1.0	100	0.12–0.5	67	46
% Overall agreement			89		92		73	85

fied this medium for testing amphotericin B. Incidentally, some published reports suggested that antibiotic medium 3 is more effective for detection of amphotericin B resistance than RPMI 1640 (10, 19). Most of the laboratories obtained MIC results at 48 h by visual endpoint reading. The key features, such as inoculum preparation and size, medium composition, duration and temperature of incubation, and endpoint determination, were the essential component when an effort was being made to develop a standardized antifungal susceptibility testing (13). Adherence to the NCCLS M27-A method provided a greater percentage of agreement with the reference MICs than was found when the NCCLS procedure was not followed (3).

The results obtained in this study demonstrated an agreement between the NCCLS reference range and the reported MICs of amphotericin B and fluconazole of 85 and 74%, respectively. Percentages of agreement, based on methodology, between the NCCLS reference range and the MIC results obtained for amphotericin B against the five isolates are summarized in Table 2. Overall agreement between the NCCLS reference range and Sensititre YeastOne was 92%, while that between the NCCLS reference range and Etest was 73%. Good performance was noted for *C. krusei*, *C. lusitaniae*, and *C. parapsilosis*. The percentage agreement was 95% for each of these isolates. Expansion of the reference range by one dilution improved the performance to 100% for *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and also *C. albicans*. Similar results had been observed with expansion of the reference range by one dilution in the CAP program for antifungal susceptibility testing (3, 13). For *C. tropicalis*, with expansion of the reference range by one dilution, the percentage of agreement increased to 88%. The incorrect MICs reported for any isolate were always lower than the reference MICs. It has been shown that M27 methodology, Etest, and the use of colorimetric Alamar Blue marker all have limited ability to detect high MICs of amphotericin B (4, 6, 19). None of the laboratories was able to detect the amphotericin B resistance of *C. lusitaniae* ATCC 200950, irrespective of the susceptibility method used. The expected amphotericin B MIC ranges for *C. lusitaniae* ATCC 200950 were 0.25 to 1.0 μg/ml with RPMI 1640 broth and 1.0 to 4.0 μg/ml with AM3 medium (2, 11, 20). The observed discrepancies in amphotericin B resistance testing were discussed in the critique of test events provided to the participating laboratories.

All laboratories except one were able to detect fluconazole resistance in *C. krusei* ATCC 6258, irrespective of the susceptibility method used. Similarly, the reported MIC ranges were within the reference range for *C. lusitaniae* ATCC 200950, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750. For *C. albicans* ATCC 24433, a majority of the laboratories reported higher MICs (range, 1.5 to 8.0 μg/ml). The percentages of agreement, based on methodology, for results for five isolates are summarized in Table 3. Overall agreement between the NCCLS reference range and Sensititre YeastOne was 80%, while that between the NCCLS reference range and Etest was 67%. Good performance was noted for *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*. The agreement was 94% for each of the isolates. Expansion of the reference range by one dilution improved the performance to 100% for *C. parapsilosis* and *C. tropicalis*. These results compare favorably with those reported in CAP surveys (3, 13). It is not clear why participating laboratories reported higher MICs for *C. albicans* ATCC 24433. This strain has been included among quality control isolates by the NCCLS subcommittee (12, 15, 16). The reported fluconazole MIC was 0.25 to 4.0 μg/ml (15, 16). This strain is currently under evaluation in our laboratory for its antifungal susceptibility profile. At the end of the test event, the NYSDOH Mycology PT program provided a detailed analysis of the test results and a review of relevant literature.

A PT survey conducted for 48 microbiology laboratories by Project ICARE (Intensive Care Antimicrobial Resistance Ep-

TABLE 3. Methods for antifungal susceptibility testing for fluconazole and comparison of results with the NCCLS reference range

Organism	Reference range (μg/ml)	NCCLS broth microdilution results (μg/ml)	% Agreement	Sensititre YeastOne results (μg/ml)	% Agreement	Etest results (μg/ml)	% Agreement	Overall agreement (%)
C. albicans ATCC 24433	0.25–1.0	0.25–4.0	15	2.0–8.0	0	1.5–6.0	0	5
C. krusei ATCC 6258	16.0–64.0	4–64	86	16.0–32.0	100	32.0–64.0	100	95
C. lusitaniae ATCC 200950	0.25–1.0	0.25–16.0	86	0.25–0.5	100	0.12–1.5	67	84
C. parapsilosis ATCC 22019	2.0–8.0	1.0–8.0	86	2.0–4.0	100	2.0–8.0	100	95
C. tropicalis ATCC 750	0.5–2.0	1.0–4.0	100	1.0–2.0	100	0.5–1.5	67	89
% Overall agreement			75		80		67	74

idemiology) highlighted the need for monitoring of susceptibility testing methods to detect antimicrobial resistance (21). The Bacteriology PT program at the NYSDOH surveyed 320 participating laboratories and found suboptimal compliance with NCCLS guidelines (9). The Centers for Disease Control and Prevention along with the World Health Organization conducted a PT survey of 130 laboratories; their results indicated the need for educational programs that emphasized the proper use of laboratory protocols (21). The CAP survey of 802 laboratories found better reliability of rifampin and isoniazid susceptibility testing than of ethambutol and streptomycin testing (22). Thus, an overview of some published studies suggests that PT for antimicrobial susceptibility testing will be valuable in providing educational materials, training methods, and improvement in quality for the participating laboratories.

In summary, the NCCLS broth dilution method and commercial systems were equally efficacious for susceptibility testing of *Candida* spp. against amphotericin B and fluconazole. However, none of these methods was able to detect amphotericin B resistance in *C. lusitaniae*. The deviations from the NCCLS M-27 protocol reported in our survey did not affect the final MICs for test isolates obtained by various laboratories. It is concluded that a suitably designed PT program could adequately monitor the competence of the clinical laboratories performing antifungal susceptibility testing.

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