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Two-Site Comparison of Broth Microdilution and Semisolid Agar Dilution Methods for Susceptibility Testing of *Cryptococcus neoformans* in Three Media

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This study evaluated the inter- and intralaboratory agreement between results of the semisolid agar dilution and broth microdilution methods of antifungal susceptibility testing of *Cryptococcus neoformans*. Three media were tested in two laboratories. The drugs tested were amphotericin B, flucytosine, itraconazole, fluconazole, and Schering 39304. Analysis by kappa statistics revealed good agreement between the laboratories for the two methods. The highest level of inter- and intralaboratory agreement was observed in RPMI 1640 with L-glutamine followed by Eagle's minimum essential medium and yeast nitrogen broth. The broth microdilution method appears more suitable than the semisolid agar dilution method for testing cryptococci because of its ease in performance, cost, and simplicity.

Infections by *Cryptococcus neoformans* continue to cause significant morbidity and mortality in immunocompromised patients despite the availability of newer agents (1, 2, 5, 6, 12, 14, 15). While antifungal susceptibility testing could potentially be useful for product screening and for guiding therapy, current methods are not standardized (4, 7, 8).

We compared the results of antifungal susceptibility testing of 14 coded isolates of *C. neoformans* by two methods (broth microdilution and semisolid agar dilution) in two laboratories (The University of Texas Medical School—Hermann Hospital [HH] and The University of Texas M. D. Anderson Cancer Center [MDACC]) against five antifungal agents. The data were collected and decoded by an investigator not involved in the performance of the tests.

Five antifungal agents were used: flucytosine (5-FC; Hoffmann-La Roche, Inc., Nutley, N.J.), amphotericin B (AMB; Squibb, Princeton, N.J.), fluconazole (FLU; Pfizer, Sandwich, United Kingdom), Schering 39304 (SCH; Schering-Plough Corp., Bloomfield, N.J.), and itraconazole (ITZ; Janssen Pharmaceuticals, Inc., Piscataway, N.J.). Antifungal powders were dissolved in dimethyl sulfoxide, which was limited to 4% at the highest drug concentration tested. The concentration range for all drugs was 64 to 0.0625 μg/ml except for AMB (8 to 0.03125 μg/ml).

Eagle's minimum essential medium (EMEM; Sigma Chemical Company, St. Louis, Mo.), yeast nitrogen base (YNB; Difco), and RPMI 1640 with L-glutamine (Sigma) were prepared at 1.3× concentration, buffered with morpholinopropanesulfonic acid (MOPS; Sigma) at 0.165 M, and adjusted to pH 7.0. Two percent agar was also prepared with MOPS-buffered water as solvent and sterilized by autoclaving.

Ninety-six-well round-bottom microtiter plates were used for the broth microdilution method. Each plate, which represented eight test panels, was prepared as previously described (16). The plates were stored at −70°C, except for those containing the YNB-AMB combination, which were stored at 4°C to prevent the drug from precipitating. Twenty-four-well flat-bottom trays were used for the semisolid agar dilution method. Dilution was performed according to the method of Gordon et al. (10). The completed trays, which

TABLE 1. Interlaboratory agreement of MIC results[a]

Antifungal agent(s) and method	Kappa value[b]			
	YNB	EMEM	RPMI	Mean
AMB				
Broth	1.0	1.0	1.0	1.0
Agar	1.0	1.0	1.0	1.0
Mean	1.0	1.0	1.0	1.0
5-FC				
Broth	0.72	0.89	0.72	0.78
Agar	0.25	0.80	0.80	0.61
Mean	0.48	0.84	0.76	0.69
FLU				
Broth	0.62	0.91	1.0	0.84
Agar	1.0	0.91	0.81	0.91
Mean	0.81	0.91	0.91	0.87
ITZ				
Broth	0.62	0.62	0.90	0.71
Agar	1.0	0.90	1.0	0.97
Mean	0.81	0.76	0.95	0.84
SCH				
Broth	0.72	0.72	1.0	0.81
Agar	0.80	1.0	0.89	0.90
Mean	0.76	0.86	0.95	0.85
All agents				
Broth	0.74	0.83	0.92	0.83
Agar	0.81	0.92	0.89	0.88
Mean	0.77	0.87	0.91	0.85

[a] AMB, amphotericin B; 5-FC, flucytosine; FLU, fluconazole; ITZ, itraconazole; SCH, Schering 39304; YNB, yeast nitrogen base; EMEM, Eagle's minimum essential medium.

[b] Kappa values of observer agreement based on end point readings (0 for AMB, +1 for all other agents) that were identical or varied by twofold dilution. Calculations based on percentage of isolates for which MICs were similar by both test methods.

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TABLE 2. Intralaboratory agreement of MIC results obtained by broth microdilution and semisolid agar dilution methods[a]

Antifungal agent(s) and laboratory	Kappa values[b]			
	YNB	EMEM	RPMI	Mean
AMB				
HH	−0.45	1.0	1.0	0.52
MDACC	−0.45	0.90	0.89	0.45
Mean	−0.45	0.95	0.94	0.48
5-FC				
HH	0.25	0.39	0.49	0.38
MDACC	0.62	0.89	0.62	0.71
Mean	0.43	0.64	0.56	0.54
FLU				
HH	0.62	−0.13	0.72	0.40
MDACC	0.62	−0.04	0.72	0.43
Mean	0.62	−0.09	0.72	0.42
ITZ				
HH	−0.32	−0.32	−0.32	−0.32
MDACC	−0.32	−0.23	−0.23	−0.26
Mean	−0.32	−0.27	−0.27	−0.29
SCH				
HH	0.59	0.29	0.56	0.48
MDACC	0.81	0.67	0.81	0.76
Mean	0.70	0.48	0.69	0.62
All agents				
HH	0.14	0.25	0.49	0.29
MDACC	0.26	0.44	0.56	0.42
Mean	0.20	0.34	0.53	0.36

[a] HH, Hermann Hospital; MDACC, M. D. Anderson Cancer Center. Other abbreviations are defined in Table 1, footnote *a*.

[b] Kappa values of observer agreement based on end point readings (0 for AMB, +1 for all other agents) that were identical or varied by twofold dilution.

represented two test panels each, were stored at 4°C until use.

Test strains included HH2, 3, 4, 6, 7, and 8; MDACC 307; ATCC 7472, 34544, 28958, 4189, and 34875; and NIH B-3501 and B-3502. Inocula were prepared from a 48-h culture on Sabouraud's dextrose agar plates incubated at 35°C as previously described (16, 20). Ten microliters of the resulting inocula was dispensed in each of the wells. The plates were incubated at 35°C and read for growth at 24 and 48 h by using end point criteria as defined by the National Committee for Clinical Laboratory Standards (17). The MIC was defined as the lowest concentration at which no growth was seen (for AMB) or that showed no more than 25% of the growth in the drug-free control (for the other agents).

Statistical analysis was performed by the Department of Biomathematics at MDACC. For each lab-method-drug-medium combination, the isolates were scored, and statistics were computed to evaluate the agreement in MIC end points between laboratories and between methods. The MICs were assumed to be independent of one another and distributed randomly over the standard MIC range of the given drug, i.e., 0.03 to 8.0 (2^{-5} to 2^{+3}) for AMB and 0.06 to 64+ (2^{-4} to 2^{+6}) for 5-FC, SCH, FLU, and ITZ, resulting in expected agreement levels of 31% for AMB and 24% for the other agents. Kappa values indicate the strength of the observed agreement compared to the expected level, with $\kappa = 1.0$ indicating perfect agreement, $\kappa = 0$ signifying no agreement, and negative values representing disagreement (21). Range and median MICs were also tabulated for each combination.

The percentage of agreement between laboratories ranged from 43 to 100%, with the kappa values ranging from 0.245 to 1.00 (Table 1). The analyses indicated that the observed

TABLE 3. Range and median MICs for 14 clinical isolates of *C. neoformans* in three media[a]

Antifungal agent, method, and lab	Range (median) of MIC (μg/ml)[b]		
	YNB	EMEM	RPMI
AMB			
Broth			
HH	2.0–4.0 (2.0)	0.06–0.25 (0.06)	0.03–0.25 (0.06)
MDACC	2.0–4.0 (2.0)	0.03–0.125 (0.125)	0.03–0.25 (0.125)
Agar			
HH	0.25–0.5 (0.5)	0.06–0.25 (0.125)	0.06–0.25 (0.06)
MDACC	0.5 (0.5)	0.015–0.25 (0.125)	0.015–0.25 (0.125)
5-FC			
Broth			
HH	0.5–64.0 (2.0)	0.5–64.0 (2.0)	0.5–>64 (2.0)
MDACC	0.5–64.0 (8.0)	0.5–64.0 (4.0)	0.5–64 (4.0)
Agar			
HH	0.125–32.0 (1.0)	0.25–16.0 (2.0)	0.25–2.0 (1.0)
MDACC	0.25–>64.0 (4.0)	0.25–>64 (2.0)	0.25–>64 (2.0)
FLU			
Broth			
HH	0.25–8.0 (2.0)	0.25–8.0 (0.5)	0.25–8.0 (2.0)
MDACC	0.5–8.0 (4.0)	0.5–4.0 (2.0)	0.5–8.0 (2.0)
Agar			
HH	0.5–8.0 (2.0)	0.06–16.0 (2.0)	1.0–8.0 (2.0)
MDACC	0.25–8.0 (4.0)	1.0–16.0 (8.0)	1.0–16.0 (4.0)
ITZ			
Broth			
HH	1.0–16.0 (4.0)	0.5–8.0 (2.0)	0.5–32.0 (16.0)
MDACC	2.0–16.0 (4.0)	0.125–32.0 (8.0)	0.125–64.0 (8.0)
Agar			
HH	0.06 (0.06)	0.06 (0.06)	0.06 (0.06)
MDACC	0.06 (0.06)	0.06–64.0 (0.06)	0.06 (0.06)
SCH			
Broth			
HH	0.25–16.0 (2.0)	0.5–8.0 (2.0)	0.25–8.0 (2.0)
MDACC	0.5–16.0 (8.0)	1.0–64.0 (4.0)	0.5–8.0 (2.0)
Agar			
HH	0.5–16.0 (4.0)	1.0–16.0 (4.0)	1.0–16.0 (4.0)
MDACC	0.5–16.0 (4.0)	0.5–16.0 (8.0)	1.0–16.0 (4.0)

[a] Abbreviations are defined in Tables 1 and 2, footnotes *a*.

[b] Kappa values of observer agreement based on end point readings (0 for AMB, +1 for all other agents) that were identical or varied by twofold dilution.

agreement was always greater than that expected by chance alone. Among the drugs (Table 1), AMB showed the highest agreement (mean $\kappa = 1.0$), and 5-FC showed the lowest (mean $\kappa = 0.69$). By medium, the agreement for EMEM and RPMI was higher than that for YNB (mean $\kappa = 0.87$ and 0.91 versus 0.77, respectively), and this generally held true for all drugs. Overall agreement by method between laboratories was comparable (mean $\kappa = 0.88$ for semisolid versus 0.83 for broth microdilution).

There was generally less correspondence between readings when intralaboratory comparisons were made between methods. Agreement ranged from 0 to 100%, with the kappa statistics ranging from −0.45 to 1.00 (Table 2). Of the drugs, the best agreement between methods was observed for SCH (mean $\kappa = 0.62$), while the highest overall disagreement occurred with ITZ (mean $\kappa = -0.29$). The highest agreement for all drugs (mean $\kappa = 0.53$) was observed in the RPMI medium, while the lowest occurred in YNB (mean $\kappa = 0.20$). The agreement between methods was greater at one laboratory than at the other (mean $\kappa = 0.42$ versus mean $\kappa = 0.29$), and this trend generally held true when examined within results for each drug or each medium.

Table 3 gives the range and median MICs for each drug-method-lab-medium combination. The median MICs were equivalent or within a twofold to fourfold dilution between methods for all drugs except ITZ. Equivalent values were obtained for all drugs between laboratories. MICs of AMB in YNB were severalfold higher than in other media. Major differences in median MICs of ITZ were seen between methods, with the semisolid agar dilution method giving consistently lower values.

The most prominent finding in this study was that RPMI 1640 with L-glutamine gave more consistently reproducible results for the in vitro susceptibility testing of *C. neoformans* regardless of method or laboratory. We also found this to be true when testing *Candida albicans* (20). Other investigators have shown similar findings when testing *Candida* spp. and two strains of *C. neoformans* (16, 17). A literature review revealed that YNB was the most frequently tested medium (3, 4, 9, 10, 13, 17, 18, 19). YNB was affected by pH, and the doubling times of some organisms were prolonged when buffer was added (3, 11). In our study, YNB and EMEM showed consistently less agreement between methods than did RPMI.

Another encouraging finding was the good agreement between laboratories as indicated by the overall positive kappa values. This result, together with the consistent values obtained with RPMI, suggest that standardization of susceptibility testing of *C. neoformans* may be a reachable goal. It is important to add, however, that only 14 strains were tested. Additional studies with a larger number of strains and species would be useful.

In conclusion, our study suggests that RPMI is a suitable medium for testing cryptococci and that the broth microdilution and the semisolid agar dilution methods of testing give comparable but not identical results.

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