Effect of test medium on *in vitro* susceptibility testing results for *Aspergillus fumigatus*

Elias K. Manavathu, Udayashree Nune, Karunasree Kanuri, Pranatharthi H. Chandrasekar and Ooriapadickal C. Abraham

Division of Infectious Diseases, Department of Medicine, Wayne State University School of Medicine, Detroit, USA

Summary

We examined the effect of peptone yeast extract glucose broth (PYG), RPMI 1640 and Antibiotic Medium 3 (M3) on the *in vitro* susceptibility of clinical isolates (n = 200) of *Aspergillus fumigatus* to amphotericin B (AMB), itraconazole (ITZ) and voriconazole (VCZ). The MICs (µg/mL) of various antifungal agents (geometric mean ± standard deviation) obtained in PYG, RPMI 1640 and M3, respectively, were as follows; AMB: 1.64 ± 0.92, 0.42 ± 0.21, 0.33 ± 0.16; ITZ: 0.44 ± 0.54, 0.40 ± 0.65, 0.17 ± 0.32; VCZ: 0.70 ± 0.58, 0.29 ± 0.22, 0.36 ± 0.21. Pair-wise comparisons of the MICs of AMB, ITZ and VCZ obtained in PYG, RPMI 1640 and M3 showed no significant differences except for AMB in PYG broth compared to those obtained in RPMI 1640 and M3 ($p \leq 0.05$). These results suggest that the susceptibility of *A. fumigatus* to AMB, ITZ and VCZ is similar in growth media currently used for susceptibility testing.

Key words Antifungal, Broth microdilution, Susceptibility testing, *Aspergillus fumigatus*, Filamentous fungi

Efecto del medio sobre los resultados de las pruebas de sensibilidad *in vitro* de *Aspergillus fumigatus*

Resumen

Hemos examinado el efecto del caldo de extracto de levadura-peptona-glucosa (PYG), RPMI 1640 y Antibiotic Medium 3 (M3) sobre la sensibilidad *in vitro* de aislamientos clínicos (n = 200) de *Aspergillus fumigatus* a anfotericina B (AMB), itraconazol (ITZ) y voriconazol (VCZ). Las CMIs (µg/ml) de varios agentes antifúngicos (media geométrica ± desviación estándar) obtenidas en PYG, RPMI 1640 y M3, respectivamente, fueron: AMB: 1,64 ± 0,92, 0,42 ± 0,21, 0,33 ± 0,16; ITZ: 0,44 ± 0,54, 0,40 ± 0,65, 0,17 ± 0,32; VCZ: 0,70 ± 0,58, 0,29 ± 0,22, 0,36 ± 0,21. Las comparaciones por pares de AMB, ITZ y VCZ obtenidas en PYG, RPMI 1640 y M3 mostraron diferencias no significativas, salvo para la AMB en caldo PYG respecto a las obtenidas en RPMI 1640 y M3 ($p \leq 0,05$). Estos resultados sugieren que la sensibilidad obtenida para *A. fumigatus* a AMB, ITZ y VCZ es similar en los medios de crecimiento utilizados actualmente en las pruebas de sensibilidad.

Palabras clave Antifungal, Broth microdilution, Susceptibility testing, *Aspergillus fumigatus*, Filamentous fungi

Aspergillus fumigatus is a filamentous fungus that causes severe respiratory and systemic infections in immunocompromised individuals. The compromised hosts often affected are cancer patients undergoing cytotoxic chemotherapy [1-3], recipients of solid organ and bone marrow transplantations [4-6] and AIDS patients [7 8]. *Aspergillus* is acquired via inhalation of conidia and it can colonize the respiratory tract without causing apparent disease. With immunosuppression, the organism causes respiratory disease and may spread through the circulatory system resulting in dissemination. Systemic aspergillosis among immunologically compromised patients is associated with high mortality. In vitro susceptibility testing of clinical isolates from patients is helpful to understand the susceptibility of the organism to antifungal agents. Although no good correlation has been demonstrated often between in vitro susceptibility data and the clinical outcome of antifungal treatment in aspergillosis, determination of the minimum inhibitory concentrations (MICs) of the antifungal agents may be useful as a guideline for choosing the correct drug.

The *in vitro* susceptibility of fungi to antifungal agents as determined by the MICs of drugs may depend on the inoculum size, composition of the medium used for the susceptibility testing, the nature of the biological materials used as inoculum, and the temperature and dura-

Dirección para correspondencia:
Dr. Elias K. Manavathu
Department of Medicine, 427 Lande Building
550 E. Canfield, Detroit, MI 48201, USA
Tel.: +1 313 577 1931. Fax: +1 313 993 0302
E-mail: aa1388@wayne.edu

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Table 1. Susceptibility of *Aspergillus fumigatus* isolates (n = 200) to amphotericin B, itraconazole and voriconazole in PYG broth, RPMI 1640 and Antibiotic Medium 3.

Antifungal agent	Medium	MIC (µg/ml) ± SD	MIC (µg/mL) Range	MIC_{50}	MIC_{90}
Amphotericin B	PYG	1.64 ± 0.92	0.5 - 4	2	4
	RPMI	0.42 ± 0.21	0.25 - 1	0.5	0.5
	M3	0.33 ± 0.16	0.125 - 1	0.5	0.5
Itraconazole	PYG	0.44 ± 0.54	0.25 - 4	0.5	1
	RPMI	0.40 ± 0.65	0.125 - 4	0.25	1
	M3	0.17 ± 0.32	0.0625 - 4	0.125	0.25
Voriconazole	PYG	0.70 ± 0.58	0.25 - 4	0.5	1
	RPMI	0.29 ± 0.22	0.125 - 1	0.25	0.5
	M3	0.36 ± 0.20	0.125 - 1	0.5	0.5

Note: Pair-wise comparisons of geometric mean MIC values obtained for a given antifungal agent in PYG broth, RPMI 1640 and Antibiotic Medium 3 by two-tailed Studentís *t-test* for equal variance showed no significant differences except for amphotericin B in PYG broth vs. RPMI 1640 and Antibiotic Medium 3 (p 0.05).

tion of growth. Among these parameters, the role of the growth medium used for the in vitro susceptibility testing may be important for two reasons. First, the growth rates of fungal isolates may vary with media, which in turn will impact the MIC values by virtue of the operational definition of MIC of antifungal agents. Second, possible interaction of the antifungal agent with one or more components of the medium may interfere with its antifungal activity. RPMI 1640 medium is recommended for the in vitro susceptibility testing of pathogenic yeasts [9] and conidium-forming filamentous fungi [10]. The same medium was used previously for the susceptibility testing of filamentous fungi, including *A. fumigatus* [11-15]. Although RPMI 1640 supports adequate growth of *A. fumigatus* for MIC determination, it grows better in other commonly used fungal growth media such as Sabouraud Dextrose (SD) broth and peptone yeast extract glucose (PYG) broth (Manavathu EK, unpublished, 1998). In addition to RPMI 1640, Antibiotic Medium 3 [16,17] and PYG broth [18] had occasionally been used for fungal susceptibility testing. The selection of a suitable test medium that adequately supports the growth of the organism without drug interaction is essential for reproducible MIC results. Our previous study with a single clinical isolate of *A. fumigatus* showed no significant difference in amphotericin B MICs in PYG broth and RPMI 1640 [18]. We further investigated the susceptibility of 200 clinical isolates of *A. fumigatus* to amphotericin B, itraconazole and voriconazole in RPMI 1640 and compared the results with those obtained in PYG broth and Antibiotic Medium 3.

A. fumigatus isolates (n = 200) used in this study were obtained from the Microbiology Laboratory of the Detroit Medical Center for a period of 4 years (January 1994 to December 1997). Majority of the isolates were from immunocompromised hosts, namely, cancer patients receiving chemotherapy, bone marrow transplant recipients and AIDS patients. The primary cultures obtained on SD agar were subcultured on the same medium to assure the purity of the culture, and then maintained on SD agar slants at 4°C as working cultures. Long term storage of the cultures was done as conidial suspension in 25 % glycerol at -70°C.

PYG [peptone 1 g (Difco Laboratories), yeast extract 1 g (Becton Dickinson, Lincoln Park, NJ, USA), glucose 3 g, per liter of distilled water] medium was prepared and sterilized by autoclaving. Antibiotic Medium 3 was obtained from Difco Laboratories (Detroit, Michigan, USA), and prepared as described by the manufacturer. RPMI 1640 containing MOPS (165 mM final concentration) buffer was purchased from American Bioorganics, Niagara Falls, New York, USA. The medium was prepared in double distilled water and sterilized by filtration through a Millipore filter (0.22 µm) and stored at 4°C until use.

The susceptibility of *A. fumigatus* isolates to various drugs was determined using a broth macrodilution technique [11,12,18]. Briefly, fresh conidia were collected from *A. fumigatus* isolates [13] and resuspended in appropriate medium at a density (determined by haemocytometer count) of 2 x 10^4 conidia/ml. Two times the required concentrations of the drugs were prepared in the same medium (0.5 ml) by serial dilution in sterile 6-mlpolystyrene tubes (Falcon 2054, Becton Dickinson, Lincoln Park, NJ, USA) and inoculated with an equal volume (0.5 ml) of the conidial suspension. The tubes were incubated at 35°C for 48 h and scored for visible growth after vortexing the tubes gently. The MIC was defined as the lowest concentration of the drug which produced no visible growth (i.e. 100% inhibition). Each MIC determination was performed in duplicate and the experiment was repeated once. In all cases, the repeated MIC values for a given isolate were either identical or within ± one dilution. The data reported in this paper were obtained for one of the experiments. All statistical analyses (geometric mean, standard deviation and *p* value) were performed using the Computer Program Quattro Pro 8 (Corel Corporation, Ottawa, Canada).

Amphotericin B (Batch No. 20-914-29670, Squibb Institute for Medical Research, Princeton, NJ), itraconazole (R51 211, Batch No. STAN-9304-005-1, Janssen Pharmaceutica, Beerse, Belgium) and voriconazole (Pfizer Pharmaceuticals, New York, NY) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml and stored as 0.25-ml aliquots at -20°C. The frozen stock was thawed at room temperature and vortexed gently several times to ensure that any remaining crystals were completely dissolved before use. Comparable concentrations of DMSO were tested to examine its effect on the growth of *A. fumigatus*. No detectable inhibition of growth occurred at the concentrations used. Since amphotericin B is light-sensitive, the stock solutions and the MIC tubes were covered with aluminum foil to prevent from light exposure. Drug concentrations ranging from 0.0625 - 4 µg/ml were used.

A summary of the results obtained for amphotericin B, itraconazole and voriconazole in PYG, RPMI 1640 and Antibiotic Medium 3 is shown in the Table 1. The mean MIC value of amphotericin B obtained in PYG was approximately four- and five-fold higher than those obtained for the drug in RPMI 1640 and Antibiotic Medium 3, respectively. In the case of itraconazole, no significant difference in MIC value was obtained in PYG and RPMI 1640, whereas the mean MIC value obtained for this drug in Antibiotic Medium 3 was approximately two-fold lower than those obtained in PYG and RPMI 1640. The mean MIC value obtained for voriconazole in PYG was approximately two-fold higher than those obtained for the drug in RPMI 1640 and Antibiotic Medium 3. The MIC_{50}

and the MIC_{90} values obtained for the same drug in different media also showed similar results (Table 1). In general the MIC_{90} values obtained in PYG medium were two- to four-fold higher than those obtained for the same drug in RPMI 1640 and Antibiotic Medium 3. The range and frequency of MIC values of amphotericin B, itraconazole and voriconazole obtained in various media for 200 clinical isolates of *A. fumigatus* is shown in Figure 1. In general, a wider range of MIC values were obtained for amphotericin B, itraconazole and voriconazole in PYG medium whereas the MIC range was narrow for amphotericin B and voriconazole in both RPMI and Antibiotic Medium 3. These data suggest that the MIC values obtained for commonly used antifungal agents against *A. fumigatus* are similar in RPMI 1640, PYG broth and Antibiotic Medium 3 except for amphotericin B in PYG broth which gave significantly ($p \leq 0.05$) higher geometric mean MIC value than those obtained in RPMI 1640 and Antibiotic Medium 3.

There are several possibilities for the test medium to affect the MIC value of an antifungal agent. It is possible that the drug may interact directly with a component(s) of the medium resulting in significant reduction of the effective concentration of the antifungal agent. Additionally, the growth rates of various isolates of *A. fumigatus* in different media may be different. Since MIC is defined as the concentration of the drug that inhibits the growth completely within a given time interval (usually 48 h), sufficient amount of growth may not have occurred in a certain medium within that time period. The MIC value is thus influenced by the slow growth rate of the organism. A third possibility is that a component(s) of a particular medium may interfere with the uptake of the antifungal agent resulting in altered susceptibility of the organism to the antifungal agent.

A suitable test medium possessing the following characteristics is essential for reproducibility of susceptibility testing of fungi: (1) the test medium should support the growth of the organism within reasonable time period (48-72 h) since MIC is defined as a function of time; (2) it should provide a milieu as closely as possible to that exist in the host where the organism produces the infection; (3) batch to batch variability of the test medium should be minimum; (4) the test medium should be suitable for susceptibility studies of various antifungal agents; (5) the cost of the test medium.

Recently, the National Committee for Clinical Laboratory Standards (NCCLS) proposed a broth microdilution protocol [10] for the susceptibility testing of conidium forming fungi, including *A. fumigatus*. The NCCLS M38-P protocol recommends RPMI 1640 as the test medim for the MIC determination of conidium forming fungi. There are two advantages of using RPMI 1640. First, since it is a fully defined commercially available synthetic medium quality control as well as minimum lot-to-lot variations of the medium are easily achieved. Second, RPMI 1640 is often used as a mammalian cell culture medim and thus it provides in vitro a milieu close to what is found in human body fluid(s) where the fungal pathogen produces infection. One of the main drawbacks of using RPMI 1640 for the susceptibilty testing of conidium forming fungi is that it often fails to discriminate amphotericin B resistant isolates from susceptible ones, the exact reason(s) for is not understood.

The NCCLS M38-P protocol calls for using partial inhibition of growth (e.g., 80% inhibition of growth compared to the drug-free control) as end-point to define MICs of various azoles. In our investigation we used 100% inhibition of growth as end-point to define MICs of

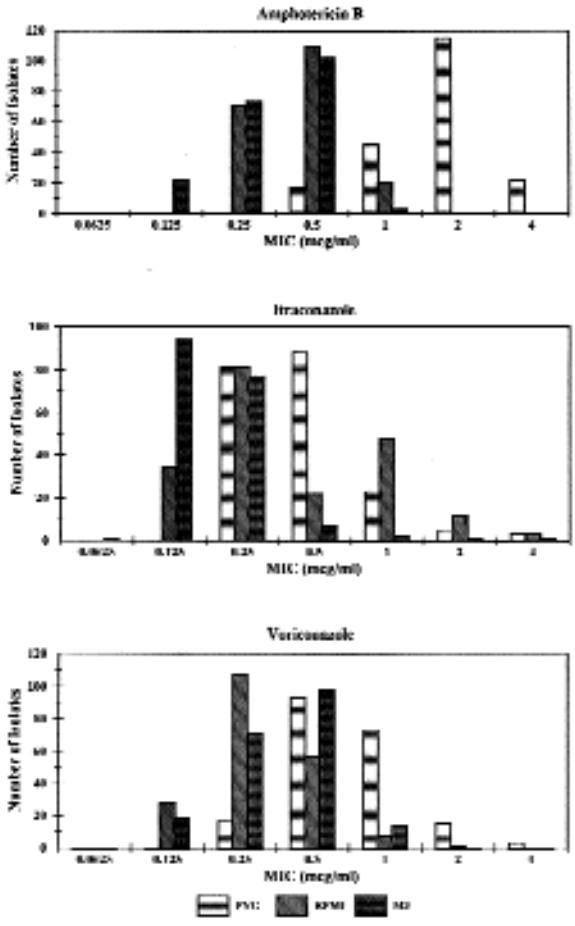

Figure 1. The range and frequency of MIC values of amphotericin B, itraconazole and voriconazole for *A. fumigatus* isolates in peptone yeast extract glucose broth, RPMI 1640 and Antibiotic Medium 3.

azoles such as itraconazole and voriconazole. The partial inhibition of growth as the end-point for MIC determination for azoles is based on the experience with the pathogenic yeasts for which azoles in general are fungistatic agents, and a clear end-point is seldom obtained. But in the case of *Aspergillus* species a definition of MIC based on partial inhibition of growth is not required since azoles are fungicidal agents [19] and a clear end-point without trailing often is obtained after 48 h of incubation at 35°C. There is no significant difference in the MIC values of azoles for *A. fumigatus* after 48 h and 72 h of incubation. In addition, a definition of MICs based on complete inhibition of growth is not subjected to operator dependent error. This is especially important in collaborative studies where interlaboratory agreement of the results is crucial.

The NCCLS proposed M38-P protocol for susceptibility testing of conidium forming fungi recommends a broth microdilution format using microtiter plates (0.2 ml total volume). Previously, most of the susceptibility testing of conidium forming fungi was carried out by the broth macrodilution method using 6-ml polystyrene tube and 1 ml volume of growth medium. Only few comparative studies were undertaken previously for evaluating the suitability of the two methods for the susceptibility testing of conidium forming fungi. Limited amount of data available indicate that the broth microdilution method tends to give 2-4 fold higher MIC values than that obtained by the broth macrodilution method [11,15,18]. This modest increase in MIC value may be a small price to pay for the

convenience and the cost savings that the broth microdilution technique would bring to a clinical laboratory routinely doing antifungal susceptibility testing. In summary, a broth microdilution technique using RPMI 1640 will be suitable for most drug/fungus combination except for amphotericin B where apparent resistance to this drug is not always detected in RPMI 1640. Since the growth medium plays an important role, it is essential to pay attention to the test media used when different sets of results are compared.

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