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Susceptibility Testing of *Candida albicans* and *Aspergillus* Species by a Simple Microtiter Menadione-Augmented 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2H-Tetrazolium Bromide Assay

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We describe a simple microtiter method for determining the susceptibility of *Candida albicans* and hyphal forms of *Aspergillus fumigatus* against antifungal agents. The assay measures mitochondrial respiration by determining reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan, a process that is enhanced in the presence of menadione. *C. albicans* or conidial suspensions of *A. fumigatus* are seeded into microtiter plates. Hyphal outgrowth of *Aspergillus* spp. was achieved by a 12 to 14-h culture at 30°C. Antifungal agents (amphotericin B, fluconazole, itraconazole) were added to the cultures for 24 h. Thereafter, incubations were continued for 3 h in the presence of MTT plus 0.1 mM menadione. Formazan formation was quantified photometrically after extraction of the formazan with acid isopropanol. Well-defined dose-response curves reflecting impairment of mitochondrial function by the antifungal agents were obtained. With *C. albicans*, the results correlated excellently with the MIC determinations performed according to the standard macrodilution procedure. In confirmation of a recent report, it was found that fluconazole was unable to exert its fungistatic action on a sensitive *C. albicans* strain in the presence of serum. The presented method can easily be integrated in the standard repertoire of a diagnostic microbiology laboratory and should prove useful as a means to assess the antifungal action of various agents on yeasts and filamentous fungi in the presence and absence of serum proteins or body fluids.

Susceptibility testing of fungi is impeded by an array of technical obstacles, and great efforts have been undertaken to establish standard conditions at least for *Candida* organisms (11, 12, 29, 31, 37). Apart from the necessity of employing meticulously controlled experimental protocols, a major and, in the case of hyphal fungi, virtually unresolved problem is the question of inoculum preparation and the readout system (9). Colony countings are useless whenever cell aggregates are present, e.g., after germ tube formation by *Candida albicans* or with hyphal forms of filamentous fungi. Turbidity measurements are also of limited use in such cases (7, 8, 40). Microscopic appraisal, sometimes used to assess growth of hyphal fungi, is cumbersome and lacks quantitative precision (17). Because of these imponderables and low cost-efficiency, few diagnostic laboratories include fungal susceptibility testing in their routine repertoire.

The demand for a simple, cost-effective, and reliable method for susceptibility testing of fungi is growing, however. The incidence of life-threatening fungal infections is increasing with the number of patients suffering from severe neutropenia and general immunosuppression or immunodeficiency (3, 24, 25, 27, 41). Amphotericin B, the classic but toxic drug for treating deep-seated mycoses, is receiving competition from less toxic agents, in particular the azole derivatives fluconazole and itraconazole (10, 15, 18, 23). Liposomal, less toxic formulations of amphotericin B are being considered as another alternative to the conventional amphotericin B regimens (14, 33, 43, 44, 46). Resistance of fungi to amphotericin B has been rare in the past, but exceptions are known (1, 5, 32). Resistance to azole derivatives arises much more frequently, particularly with the use of azole drugs in prophylaxis regimens in immunocompromised hosts and prolonged azole therapy (2, 6, 45). Under the continued pressure of life-threatening infections, new drugs and drug formulations are bound to be sought and found (16, 47). In all probability, the necessity for sometimes assaying fungal susceptibility against these various agents will become a pressing and unavoidable issue (4, 34).

Logically, the obstacles that preclude the use of turbidity measurements and colony countings should be overcome by an assay that is based on measurement of a metabolic activity. In this case, it would be irrelevant whether cells were growing in unicellular or multicellular form. One principle that has been exploited successfully for assaying viability of eukaryotic cells is the measurement of mitochondrial respiration by quantification of formazan that is generated from 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (26). Somewhat surprisingly, these assays have not been widely adapted for antifungal susceptibility testing, although their general utility for assessing the viability of filamentous fungi has been demonstrated (19). Furthermore, this assay has been used for functional studies on hyphal damage of *Aspergillus* spp. (35, 36, 38, 39).

Only recently Tellier et al. showed the applicability of this approach to *Candida* susceptibility testing against amphotericin B, fluconazole, and flucytosine in a macrodilution system (42). A similar method, also for *Candida* species, has been developed by Pfaller and colleagues (28). They employed the colorimetric oxidation reduction indicator Alamar blue for susceptibility testing of a variety of *Candida* species and demonstrated a good correlation with the conventional microdilution method (30).

Recently, it was reported that presence of menadione in an

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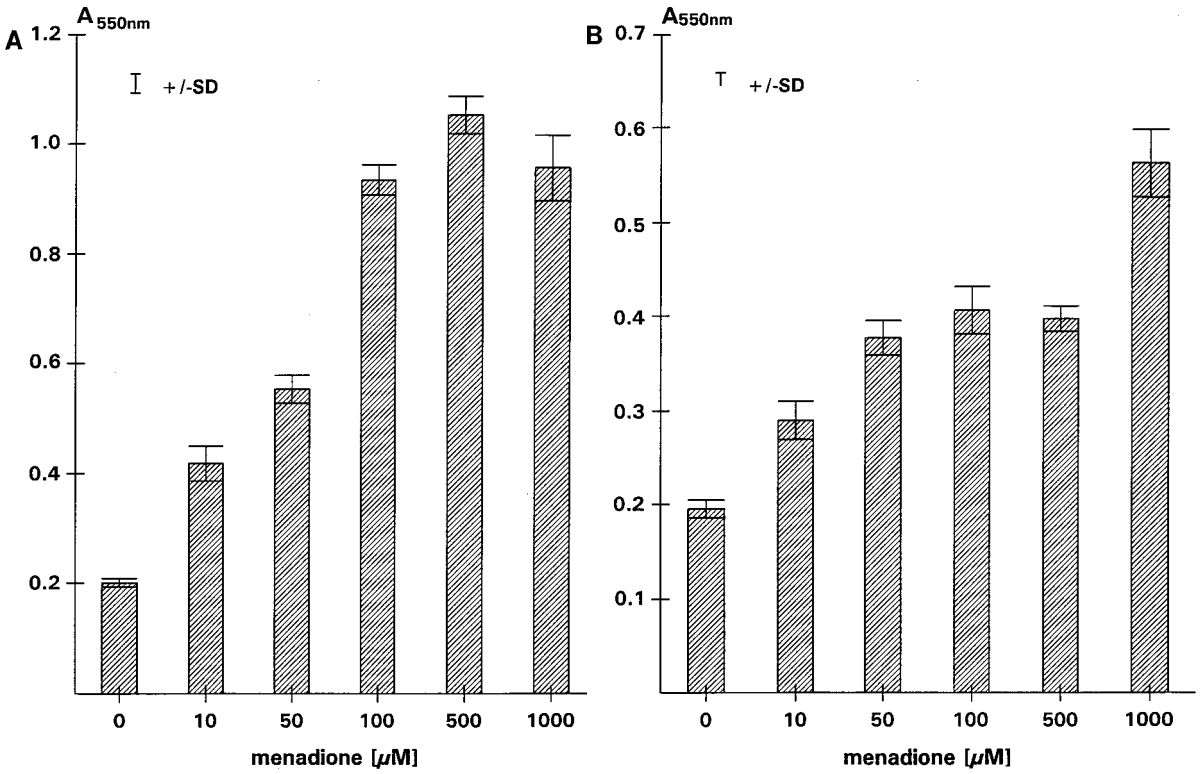

FIG. 1. Augmentation of formazan formation by menadione. (A) *C. albicans* cells (10^5 per well were incubated with MTT in the presence of menadione at the given concentrations. After 3 h, formazan was extracted with acid isopropanol and determined as A_{550} ± standard deviation (SD). An approximately four- to fivefold increase was seen with menadione concentrations of 100 to 1,000 μM. All assays were run as triplicates ($n = 3$). (B) *A. fumigatus* hyphae (5×10^4 per well) were incubated with MTT in the presence of menadione. After 3 h, formazan was extracted with acid isopropanol. An approximately two- to threefold increase in formazan formation was seen with menadione concentrations of 100 to 1,000 μM. All assays were run as triplicates ($n = 3$).

MTT assay markedly enhanced the formation of formazan, thus amplifying the color signal that could easily be measured photometrically (13). We have now applied this principle to devise a simple micromethod for susceptibility testing of both *C. albicans* and *Aspergillus fumigatus* against amphotericin B and two azole derivatives. The results are encouraging, and we believe that this test should easily be extendable to other fungi and antifungal agents.

MATERIALS AND METHODS

Microorganisms. *C. albicans* 0815 and 8166 were obtained from our diagnostic laboratory. Strain 0815 has previously been described for flow cytometric studies (20–22). Strain R64, exhibiting a relative amphotericin B resistance, was kindly provided by H. Dermoumi, Institute of Medical Microbiology, Essen, Germany. The fluconazole-resistant strain 3059 was obtained from E. Liehl, Sandoz Forschungsinstitut, Vienna, Austria. Stock cultures were kept in tryptic soy broth (TSB; Difco), containing 17% glycerol at −20°C. Subcultures were prepared on Columbia blood agar. Suspension cultures were prepared by inoculation of single colonies in 4 ml of TSB and 14 to 18 h of growth at 37°C. Prior to preparation of susceptibility assays yeast cells were washed twice in normal saline and resuspended in RPMI (RPMI R6504; Sigma, Deisenhofen, Germany) at a concentration of 10^6/ml.

A. fumigatus strains were obtained as clinical isolates. After culture on Sabouraud agar, conidial suspensions were prepared as described by Roilides (39). In brief, plates were washed with normal saline and the conidial suspension were filtered through a fourfold layer of sterile gauze. Then, 100 U of penicillin per ml and 100 μg of streptomycin per ml (Antibiotic Mix; Gibco, Karlsruhe, Germany) were added, and suspensions were stored at 4°C. In preparation for susceptibility testing, 5×10^4 conidia per well were seeded in a flat-bottom microtiter plate (Greiner, Nürtingen, Germany) in 200 μl of TSB and incubated for 12 to 14 h at 30°C. This allowed hyphal outgrowth of more than 95% of conidia with a hyphal length varying from 50 to 100 μm, as checked by phase-contrast microscopy. Wells were washed twice with normal saline, and then 100 μl of RPMI were added.

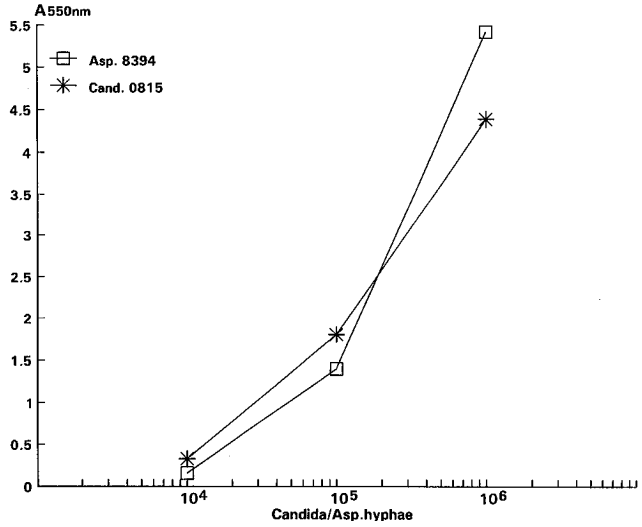

FIG. 2. Correlation between number of viable fungal organisms and formazan formation. Various numbers of *A. fumigatus* hyphae or *C. albicans* blastospores were incubated with MTT-menadione. After 3 h, formazan was extracted and measured photometrically. Samples were diluted so that the measured absorbances remained in the appropriate range. The absorbance values were multiplied with the respective dilution factors, and these extrapolated values are depicted. A good correlation was seen between absorption and the number of fungal organisms. All assays were run as triplicates.

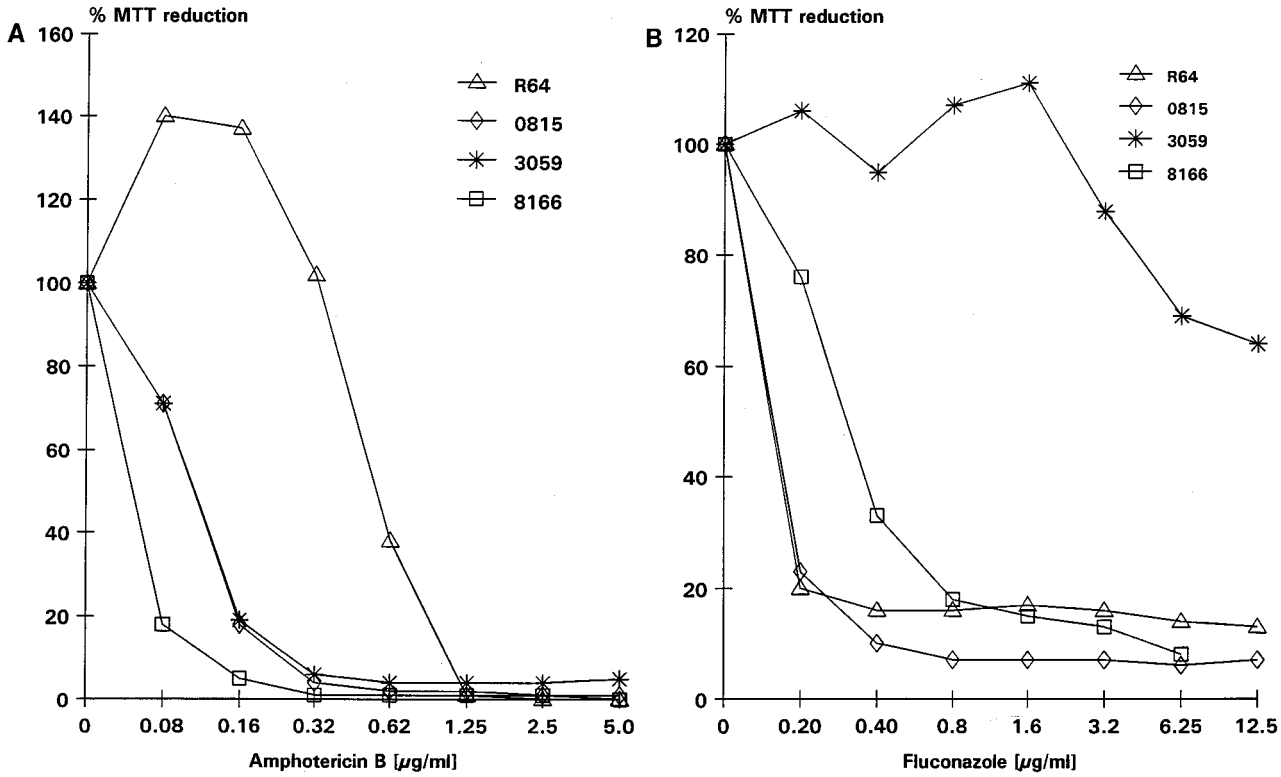

FIG. 3. Susceptibility testing of *C. albicans* with the MTT assay. (A) *C. albicans* cells (10^5) were incubated in RPMI in the presence of amphotericin B for 24 h. MTT and menadione were added for 3 h, and formazan formation was determined. The MTT reduction is expressed as a percentage of growth control. Three of the four strains tested showed a MIC_{90} of 0.32 μg/ml. With R64, a strain relatively resistant to amphotericin B, concentrations of 1.25 μg/ml were required to cause 90% growth inhibition. (B) *C. albicans* cells (10^5) were incubated in RPMI in the presence of fluconazole for 24 h. MTT and menadione were added for 3 h, and formazan formation was determined. Three of the four strains tested showed a MIC_{80} of 3.2 μg/ml. With 3059, a strain resistant to fluconazole, concentrations up to 12.5 μg/ml led to no significant growth inhibition.

Antifungal agents. Amphotericin B was purchased from Squibbs Pharma, Vienna, Austria, and was kept as a 5-mg/ml stock in distilled water at −20°C. Working solutions were prepared in water.

Fluconazole (Diflucan) was obtained from Pfizer, Karlsruhe, Germany, and kept at room temperature; working solutions were prepared in normal saline.

Itraconazole was purchased from Biotrend, Neuss, Germany. Stock solutions (5 mg/ml) and working dilutions were prepared by polyethylene glycol (PEG 400 [PEG]; Aldrich, Steinheim, Germany) and stored at room temperature. Working dilutions were prepared in PEG. The final concentration of PEG in susceptibility assays was 1%.

Susceptibility testing. (i) Macrodilution susceptibility test. Macrodilutions for control experiments with *Candida* spp. were performed according to standard National Committee for Clinical Laboratory Standards procedures as described by Galgiani (12). Dilutions were prepared in 1 ml of RPMI; the inoculum was 10^3 *C. albicans* cells. The tubes were incubated for 48 h at 37°C, and turbidity was read visually.

TABLE 1. MICs for *C. albicans* strains (n = 3)

Strain	MIC (μg/ml)			
	Amphotericin B[a]		Fluconazole[b]	
	MTT test	Macrodilution	MTT test	Macrodilution
0815	0.32	0.156	0.8–1.6	0.8
R64	1.24–2.5	4.0[c]	0.4–1.6	0.4
3059	0.16–0.32	0.625	>50	>50
8116	0.16	0.32	1.6–3.2	0.8

[a] MIC defined as ≥90% inhibition compared to growth control.
[b] MIC defined as ≥80% inhibition compared to growth control.
[c] As determined by H. Dermoumi.

MICs were calculated in comparison to growth control as 90% inhibition (MIC_{90}) for amphotericin B and 80% inhibition (MIC_{80}) for azole derivatives.

(ii) Menadione-augmented MTT test. Determinations of MIC were performed in RPMI with glutamine (Sigma). Microdilutions for assessment by the menadione-augmented MTT test were prepared in flat-bottom microtiter plates (Greiner, Nürtingen, Germany); 10^5 yeast cells per well were seeded in a volume of 100 μl of RPMI. The respective antifungal agent was added in 100 μl of RPMI for 24 h at 37°C.

For testing of *Aspergillus* spp., hypha-containing wells were washed twice in saline and refilled with 100 μl of RPMI. The antifungal agent was added in a volume of 100 μl, and the plates were incubated for 24 h at 30°C.

After 24 h of incubation with the respective agent, 25 μl of RPMI containing 5 mg of MTT (Serva, Heidelberg, Germany) and 1 mM menadione (Sigma) was added. Incubations were continued for another 3 h at 37°C. For *C. albicans*, plates were spun at 1,000 × *g* for 10 min and the supernatants were carefully aspirated. Centrifugation was not required in the case of *Aspergillus* spp. because of its adherence to the plates. After addition of 0.1 ml of acid isopropanol (95 ml of isopropanol, 5 ml of 1 N HCl), the plates were placed on a shaker for 5 min to dissolve formazan crystals. Measurements were done with a microplate reader (Titertek Multiskan) at 550 nm. Wells containing fungi and 0.1 ml of acid propanol without menadione-formazan served as background controls. Samples exceeding the linear range of photometric measurements were diluted with isopropanol and reevaluated. All assays were run as triplicates.

The antifungal activities of amphotericin B and fluconazole were additionally assessed in the presence of 50% human serum. In these experiments, *C. albicans* was incubated with the respective agents overnight. The supernatants were then removed and replaced with RPMI-MTT-menadione as described above. Formazan formation was measured after 3 h at 37°C.

(iii) XTT assay. In the experiments described above, MTT was replaced by its analog [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]) (XTT) (Sigma). This replacement leads to a formation of a water-soluble formazan salt. After 90 to 180 min, formazan formation by 1×10^5 *C. albicans* cells or 5×10^4 outgrown *A. fumigatus* hyphae was assessed directly by measurement of A_{492}.

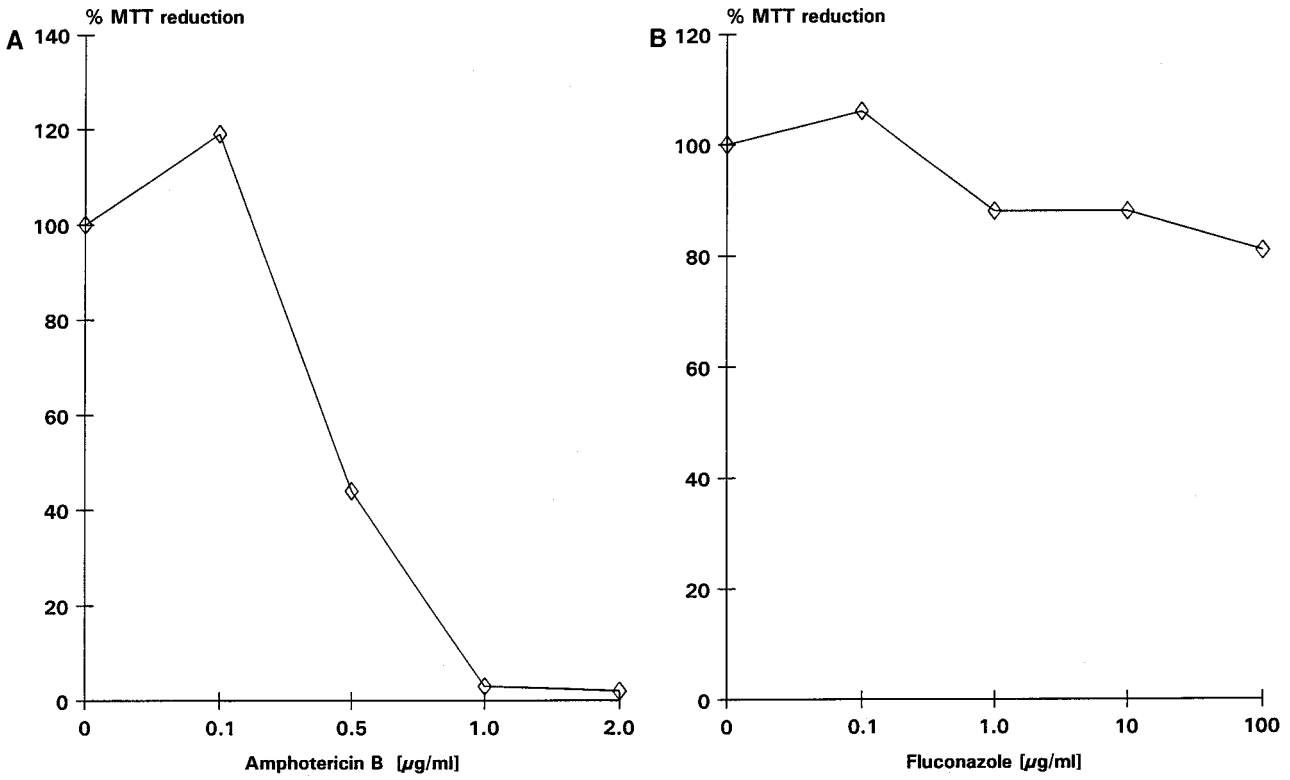

FIG. 4. Action of amphotericin B and fluconazole on the sensitive *C. albicans* strain 0815 in the presence of 50% human serum. After 24 h of incubation with the antifungal agents, the yeasts were pelleted by centrifugation and the supernatants were removed. The MTT test was performed in the absence of serum for 3 h. (A) Dose-response curve obtained with amphotericin B. Note the antifungal action observed with 0.5 μg/ml of this agent. (B) Analogous dose-response curve obtained with fluconazole, showing essential lack of fungistatic activity of this agent in the presence of serum.

RESULTS

Effect of menadione on formazan formation. To determine the effect of menadione on reduction of MTT by *Candida* spp. and *Aspergillus* spp., the amount of formazan obtained with 1×10^5 *C. albicans* cells and 5×10^4 outgrown *A. fumigatus* hyphae was assessed. As shown in Fig. 1, addition of menadione resulted in an augmentation of formazan formation. With *Candida* spp. an approximately 4.5-fold increase was seen compared with the amounts of formazan formed in the absence of menadione (Fig. 1A). When aspergilli were tested, an approximately 2.5-fold increase was seen in the presence of menadione (Fig. 1B). With both organisms, substantial increases were detected at menadione concentrations of 0.1 mM, and this concentration was employed in further studies.

Correlation between number of viable fungal organisms and MTT test. The correlation between amounts of formazan generated and numbers of viable fungal organisms was determined in a range from 10^4 to 10^6 *C. albicans* cells. As shown in Fig. 2, a good correlation existed between the number of yeast cells seeded into the wells and the amounts of formazan detected. In parallel experiments, a similar correlation between numbers of *A. fumigatus* hyphae and formazan formation was noted in a range of 10^4 to 10^6 hyphae.

When XTT was used, a less well defined correlation between number of organisms and absorption values was observed, particularly with *Aspergillus* spp. (data not shown). For this reason all further experiments were carried out with MTT.

Susceptibility testing of *C. albicans*. Four different *C. albicans* strains were tested: two wild-type strains (0815 and 8166), a relatively amphotericin B-resistant strain (R64), and one strain exhibiting fluconazole resistance (3059). When amphotericin B susceptibility was determined, the 90% inhibition values ranged from 0.16 to 0.32 μg/ml for strains 0815, 3059, and 8166. The 90% inhibition value for the amphotericin B-resistant strain R64 was 1.25 to 2.5 μg/ml (Fig. 3 and Table 1). When assayed in a macrodilution system, the MIC_{90} for this strain was 4 μg/ml (Table 1), whereas other strains exhibited MIC_{90} values of 0.16 to 0.32 μg/ml.

When fluconazole was employed, values ranged from 0.4 to 3.2 μg/ml for the fluconazole-susceptible strains R64, 0815, and 8166. Metabolic activity of the fluconazole-resistant strain 3059 remained unaltered at fluconazole concentrations up to 50 μg/ml (Fig. 3 and Table 1). In the macrodilution assays performed according to National Committee for Clinical Laboratory Standards guidelines, fluconazole MICs of 0.4 to 0.8 μg/ml were found for strains R64, 0815, and 8166. The fluconazole-resistant strain 3059 exhibited no growth inhibition at fluconazole concentrations of 50 μg/ml (Table 1).

We previously noted that fluconazole apparently was unable to exert its fungistatic action on the sensitive *C. albicans* strain 0815 when applied in the presence of serum (20). The conclusion was derived from data obtained with a novel flow cytometric assay. In the present work, additional experiments were conducted wherein fluconazole and amphotericin B were applied to *C. albicans* 0815 in the presence of 50% serum overnight and MTT reduction was assessed on the following day. It was important to remove the serum during the MTT test, since unspecific formation of formazan otherwise occurred. With the given protocol, it was possible to demonstrate that amphotericin B, but not fluconazole, exerted an antifungal effect on *C.*

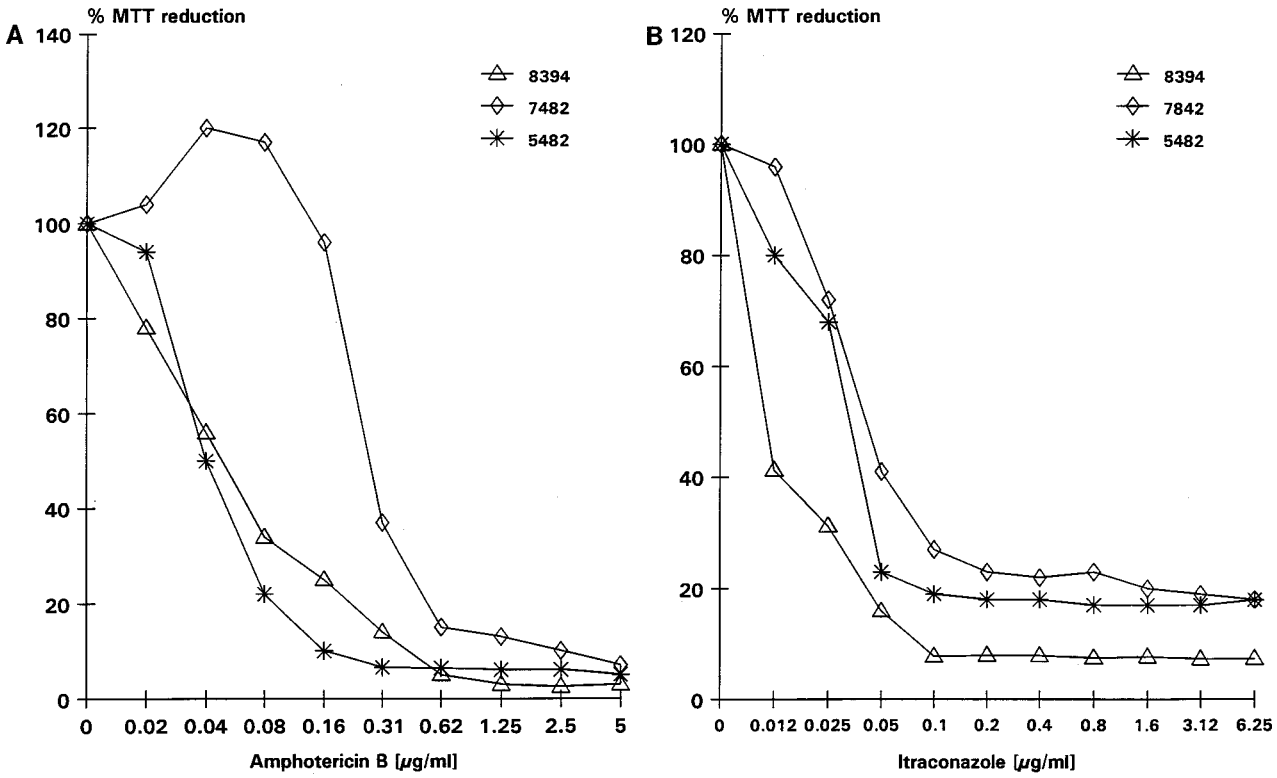

FIG. 5. Susceptibility testing of *A. fumigatus* with the MTT assay. (A) *Aspergillus* hyphae (5×10^4) were incubated in RPMI in the presence of amphotericin B for 24 h. MTT and menadione were added for 3 h, and formazan was determined. An MIC_{90} of 1.25 μg/ml was found for all three tested strains. (B) Experiments similar to those for panel A, but conducted with itraconazole. All strains exhibited an MIC_{80} of ~0.4 μg/ml.

albicans in the presence of serum (Fig. 4). The dose-response curve obtained with amphotericin B correlated excellently with that previously obtained with the flow cytometric method (20–22).

Susceptibility testing of *Aspergillus* spp. For *Aspergillus* spp., the MTT test was performed with two antifungal agents, amphotericin B and itraconazole. Clearly interpretable dose-response curves were obtained with both agents (Fig. 5). With amphotericin B, the MIC_{90} for *A. fumigatus* 8394 ranged between 0.32 and 0.64 μg/ml and 0.62 and 1.25 μg/ml for strain 7842 (Table 2). An MIC_{90} of 0.64 μg/ml was determined for strain 6912 in all three measurements. MIC_{80} values for itraconazole were 0.05 to 0.1 μg/ml for strain 8394, 0.05 μg/ml for strain 7842, and 0.1 to 0.2 μg/ml for strain 6912.

DISCUSSION

The microtiter method for determining susceptibility of *C. albicans* and *A. fumigatus* against amphotericin B, fluconazole, and itraconazole integrates principles that have been expounded by other researchers previously. Similar tests have recently been exploited as methods for susceptibility testing of *C. albicans* and as a method to measure phagocytic killing of *Aspergillus* spp. (28, 30, 35, 36, 38, 39). It is based on the fact that mitochondrial respiratory activity can be quantified with a simple colorimetric procedure and that reduction in mitochondrial activity usually will correlate with the number of viable and metabolically active fungal cells (19, 26). It is realized that a particular agent by inhibiting fungal oxidative mechanisms without killing the fungus itself may also diminish MTT reduction. However, fermentation independent of oxidation is probably not a significant factor in the present system and the correlation therefore will generally apply. The addition of the electron-coupling agent menadione (13) enhances formazan formation, thus rendering the test more sensitive. This was a prerequisite for development of a microtiter format for hyphal susceptibility testing of fungicidal as well as fungistatic agents.

TABLE 2. MICs for *Aspergillus* strains, as determined by menadione-augmented MTT test ($n = 3$)

Strain	MIC (μg/ml)	
	Amphotericin B[a]	Itraconazole[b]
8394	0.32–0.64	0.05–0.1
7842	0.64–1.25	0.05
6912	0.64	0.1–0.2

[a] MIC defined as ≥90% inhibition compared to growth control.
[b] MIC defined as ≥80% inhibition compared to growth control.

Tellier et al. demonstrated the applicability of a similar system to *C. albicans* testing, whereby XTT was employed as a substrate (42). We confirmed their results with *C. albicans*; however, formazan formation by *Aspergillus* spp. correlated less well with the number of organisms than when MTT was employed in the microtiter plate system.

The use of Alamar blue for quantification of fungal organisms as described recently by Pfaller has been used for a variety of *Candida* species (28, 30). The possibility of applying this indicator for metabolic activity assessment of *Aspergillus* spp. has not been studied.

With Pfaller et al. (28–30) and Tellier et al. (42), we believe that a colorimetric assay based on measurement of mitochon-

drial metabolic activity should become useful for susceptibility testing of fungi in the routine microbiological laboratory. A strength of the present method is its applicability to testing of hyphal as well as mold-growing fungal organisms. Preparation of yeast inocula follows simple routine procedures in the case of *Candida* spp. Sample preparations for *Aspergillus* spp. and other filamentous fungi are more demanding but not beyond the capacity of a diagnostic laboratory. Performance of susceptibility testing in microtiter plates renders the assay simple to perform and to evaluate. Comparison of results with those obtained by the cumbersome, conventional macrodilution methods indicates excellent agreement in the case of *C. albicans*. Note that the present colorimetric assay generates easily interpretable dose-response curves in all cases, quite in contrast to conventional assays. Since no standard test is available for testing *Aspergillus* spp., we have only our results to present for these fungi. However, the inhibition values generated for amphotericin B were well in line with published data obtained in different macrodilution systems (7, 8). For itraconazole, inhibition values were lower than those given elsewhere in the literature (7, 8, 17) but were comparable to the results given in one other study (40). It is noteworthy that all the data published to date were obtained with conidial suspensions as inocula. In the present system, hyphal structures were the target of the antifungal agents, which we believe more realistically reflected the in vivo situation.

The MTT test can also be utilized to assay the antifungal action of various agents in the presence of serum or body fluids. The only point to be heeded is that serum must be removed prior to application of MTT, since unspecific formazan formation otherwise occurs. We are presently conducting a systematic study on the effects of serum components on the antifungal action of azoles. The first experiments have confirmed our previous finding that fluconazole is virtually devoid of fungistatic capacity if applied in whole serum. The remarkable extent of the discrepancy between the action of this agent in the absence or presence of serum is apparent from a comparison of the dose-response curves of Fig. 3 and 4. This finding may prove to be clinically relevant, especially in cases of *C. albicans* sepsis.

Conclusion. The menadione-augmented MTT test is a simple and reproducible means to assay the antifungal activity of amphotericin B, fluconazole, and itraconazole against *C. albicans* and *A. fumigatus*. The method should be extendable to other medically important yeasts and antifungal agents.

ACKNOWLEDGMENT

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