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# Colorimetric Assay for Antifungal Susceptibility Testing of *Aspergillus* Species

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**A colorimetric assay for antifungal susceptibility testing of *Aspergillus* species (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus nidulans*, and *Aspergillus ustus*) is described based on the reduction of the tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT) in the presence of menadione as an electron-coupling agent. The combination of 200 μg of XTT/ml with 25 μM menadione resulted in a high production of formazan within 2 h of exposure, allowing the detection of hyphae formed by low inocula of $10^2$ CFU/ml after 24 h of incubation. Under these settings, the formazan production correlated linearly with the fungal biomass and less-variable concentration effect curves for amphotericin B and itraconazole were obtained.**

Tetrazolium salts are heterocyclic organic compounds that substitute the natural final acceptor (oxygen) in the biological redox process and are reduced to formazan derivatives by receiving electrons enzymically from substances of the hydrogen transport system or nonenzymically from artificial electron transporters (phenazine methosulfate and menadione) which enhance the reaction. Tetrazolium salts can penetrate rapidly into intact cells and directly into subcellular membranes with dehydrogenase activity, where they are converted to colored formazan derivatives (1, 14). Therefore, they were used as indicators of reducing systems. The tetrazolium salt MTT has been used for antifungal susceptibility testing of various yeasts and filamentous fungi, and testing was in agreement with the corresponding standard methods recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (2, 7). The disadvantage of MTT, however, is that the process includes the solubilization of formazan derivatives. As an alternative, a new tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT) (10), has been employed for antifungal susceptibility testing of yeasts and resulted in clear-cut endpoints for various antifungal agents (4, 15). XTT is converted into a water-soluble formazan, thereby avoiding the additional steps for the solubilization of formazan derivatives (8, 12), but needs the presence of an electron-coupling agent. The nature and the concentration of this agent are critical in order to obtain a good correlation between the formazan production and the number of viable fungi and less-variable concentration effect curves (1, 15).

We developed a colorimetric assay for the quantification of fungal growth of five different *Aspergillus* species based on the tetrazolium salt XTT by standardizing various factors that influence XTT conversion. This assay was also tested in the presence of antifungal drugs in order to ascertain its potential for antifungal susceptibility testing of filamentous fungi.

**Isolates.** Two clinical isolates of *Aspergillus fumigatus* [AZN5161 (S) and AZN 5241 (R)] and one each of *Aspergillus flavus* (AZN 510), *Aspergillus terreus* (AZN 7320), *Aspergillus nidulans* (AZN 8933), and *Aspergillus ustus* (AZN 9420) from our private collection was selected, and *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control. Isolates were revived by subculturing them twice onto Sabouraud glucose agar (SAB) plates with chloramphenicol first at 30°C and then at 37°C for 5 to 7 days. Conidia were collected with a swab and suspended in sterile saline containing 0.05% Tween 20. After the heavy particles were allowed to settle for 5 to 10 min, the turbidity of the supernatants were measured spectrophotometrically (Spectronic 20D; Milton Roy, Rochester, N.Y.) at 530 nm and transmission was adjusted to 80 to 82%, corresponding to an inoculum size of $1 \times 10^6$ to $5 \times 10^6$ CFU/ml. The inoculum size was confirmed by plating serial dilutions of conidia suspensions on SAB plates.

**Medium.** RPMI 1640 medium (with L-glutamine; without bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 M 3-*N*-morpholinopropanesulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used throughout.

**XTT.** XTT (Sigma Chemical, St. Louis, Mo.) was dissolved in saline at a final concentration of 1 mg/ml. The solution was filtered through a 0.22 μm-pore-size filter.

**Electron-coupling agents.** Two electron acceptors were evaluated, menadione (Sigma-Aldrich Chemie GmbH) and phena-

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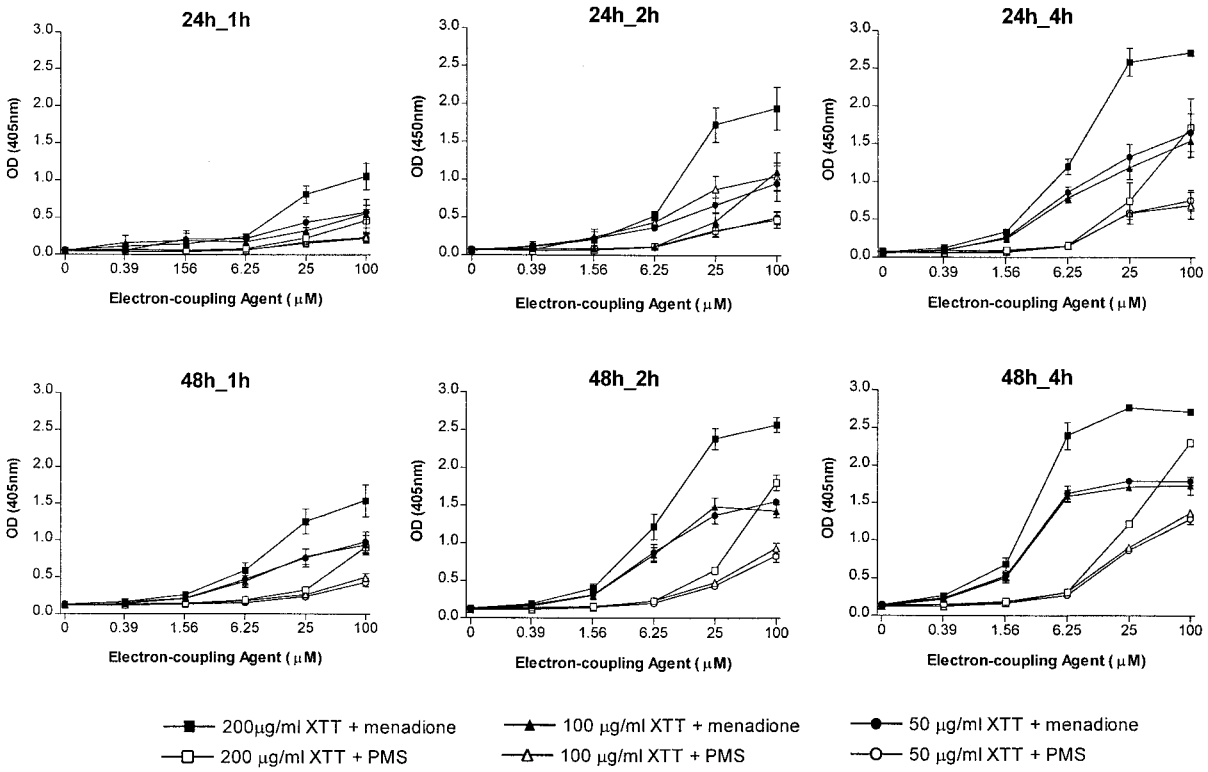


FIG. 1. XTT reduction by *Aspergillus* species. The *Aspergillus* strains were incubated for 24 or 48 h and were then exposed for 1, 2, and 4 h to various concentrations of XTT, 200 (squares), 100 (triangles) and 50 (circles) μg/ml, combined with menadione (close symbols) or PMS (open symbols) at various concentrations (0.39, 1.56, 6.25, 25, and 100 μM). Each data point represents the average of all strains tested in triplicate.

zine methosulfate (PMS) (Sigma-Aldrich Chemie GmbH). Menadione was first dissolved in acetone at a concentration of 10 mM and was then diluted 1:10 in saline. PMS was directly dissolved in saline at a final concentration of 1 mM. Further dilutions of both agents were made in saline.

**Antifungal drugs.** Itraconazole (Janssen-Cilag, Beerse, Belgium) and amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands) were dissolved in dimethyl sulfoxide (DMSO) at final concentrations of 3,200 and 1,600 mg/liter, respectively.

**XTT assay.** Conidia suspensions of each species were diluted 1:100 in the medium, and 200 μl was inoculated in 96-well flat-bottom microtitration plates (Costar, Corning, N.Y.). After 24 or 48 h of incubation at 37°C, 50-μl aliquots of various concentrations of XTT with either menadione or PMS were added to the wells in order to obtain final concentrations of 200, 100, and 50 μg of XTT/ml and 100, 25, 6.25, 1.56, and 0.39 μM menadione or PMS. The microtitration plates were incubated further for 6 h at 37°C and the optical density at 450 nm ($OD_{450}$) was measured at hourly intervals by spectrophotometer (Rosys Anthos ht3; Anthos Labtec Instruments GmbH, Salzburg, Austria). The XTT assay was performed for each species in triplicate and the ODs after 1, 2, and 4 h of exposure to various concentrations of XTT and the two electron coupling agents of fungi incubated for 24 and 48 h were plotted.

**Quantitative assay of fungal viability.** The relationship between the number of viable fungi and the amount of XTT reduction was tested by incubating various inocula of *Aspergillus* conidia ($10^2$ to $10^6$ CFU/ml) with XTT and various concentrations of menadione. Conidia suspensions were diluted 1:10 serially in the medium to up to $10^2$ CFU/ml. Then, 96-well flat-bottom microtitration plates were inoculated with 200 μl of each conidia dilution. After 24 h of incubation at 37°C, 50-μl aliquots of XTT and various concentrations of menadione were added to each well in order to obtain final concentrations of 200 μg/ml for XTT and 100, 25, 6.25, 1.56, and 0.39 μM for menadione. The microtitration plates were then incubated for another 2 h, after which 100 μl of the supernatant of each well was transferred in clean wells. The $OD_{450}$ was measured spectrophotometrically. This experiment was performed in triplicate for each species, and the data were analyzed by linear regression analysis. Regression lines were plotted for each concentration of menadione and for each species, together with the 95% confidence intervals. The slopes and the $r^2$ of each regression line were reported as an estimation of steepness of the line and the goodness of fit, respectively. An $r^2$ value of 1 indicates perfect correlation. The nonlinearity of the curves was checked by a runs test following the linear regression.

**Antifungal susceptibility assay.** Stock solutions of antifungal drugs were serially diluted twofold in DMSO, and then each drug concentration was diluted 1:50 in the medium in order to obtain twofold final concentrations which ranged from 0.015 to 16 mg of amphotericin B/liter and 0.03 to 32 mg of itraconazole/liter. Wells of 96-well flat-bottom microtitration plates were filled with 100 μl of each drug concentration. A drug-free well containing 2% DMSO in the medium served as the growth control. Each well was inoculated with 100 μl of conidia suspensions diluted 1:50 in medium in order to obtain a final

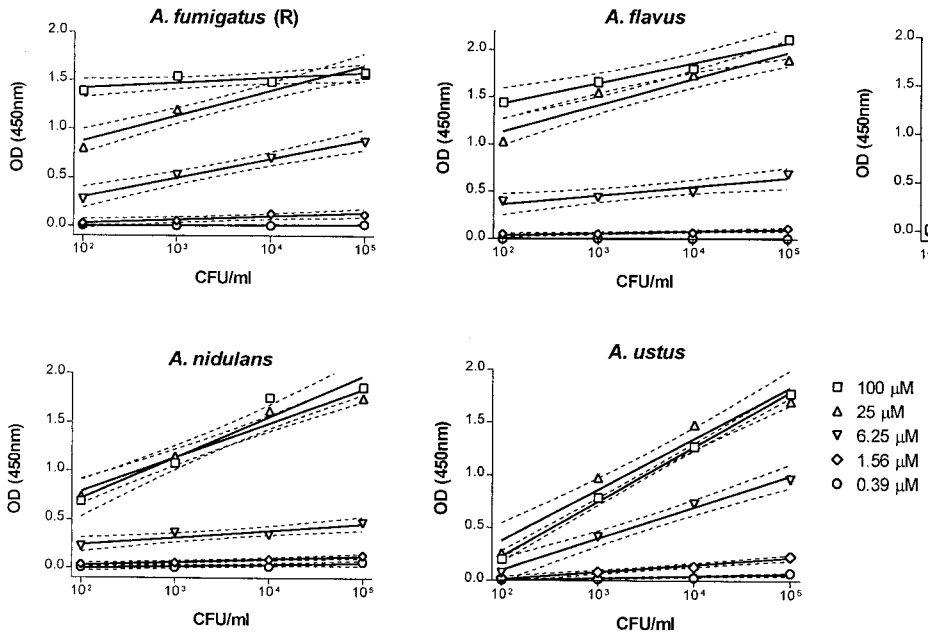


FIG. 2. Relationship between the amount of XTT and increasing inocula of each *Aspergillus* species. The reduction of 200 μg of XTT/ml in the presence of various concentrations of menadione (0.39, 1.56, 6.25, 25, and 100 μM) was tested in triplicate for each strain. The symbols are the means of the triplicates, and the lines were generated by linear regression analysis. Dotted lines represent the 95% confidence intervals of the regression lines.

inoculum of $1 \times 10^4$ to $5 \times 10^4$ CFU/ml. The plates were incubated for 24 or 48 h at 37°C, and the MIC-0 was determined visually by four different observers to be the lowest drug concentration showing no visible growth (NCCLS) (9). Afterwards, the OD of each well at 405 nm was measured in order to quantify the biomass of fungal growth (spectrophotometric method). Then, 50 μl of XTT-menadione was added to each well in order to obtain final concentrations of 200 μg/ml and 25 μM, respectively. After 2 h of further incubation, the OD of each well at 450 nm was measured in order to quantify the formazan production (colorimetric method). Background OD was obtained by spectrophotometric measurements of noninoculated wells processed in the same way as the inoculated wells. The relative ODs for each well based on both measurements at 405 and 450 nm were calculated (in percent) based on the following equation: [(OD of drug containing well − background OD)/(OD of drug-free well − background OD of drug-free well)] × 100%. The tests were carried out in triplicate in three independent experiments for each strain. Results from each experiment were analyzed by nonlinear regression analysis by using a four parameter logistic model (sigmoid curve with variable slope) known as the $E_{max}$ model, which is described by the following equation: $E = E_{max} \times (D/EC_{50})^m/[1 + (D/EC_{50})^m]$, where $E$ is the relative OD (dependent variable), $E_{max}$ is the maximum relative OD, $D$ is the drug concentration (independent variable), $EC_{50}$ is the drug concentration producing 50% of the $E_{max}$, and $m$ is the slope that describes the steepness of the curve (3). Since data were normalized by using the relative ODs, the top and the bottom of the $E_{max}$ model corresponded to 100 and 0%, respectively. Analysis was carried out using the GraphPad Prism Software (San Diego, Calif.). Deviation from the model was tested by the runs test, and goodness of fit was checked by the $r^2$ values. In order to compare the concentration effect curves generated by the spectrophotometric and colorimetric methods, the best-fit values of $EC_{50}$ and slope ($m$) obtained by the regression analysis were used. The differences between the best-fit values of the concentration effect curves for all species were analyzed by anal-

TABLE 1. Results of linear regression analysis of the relationship between the amount of XTT reduction in the presence of different concentrations of menadione and increasing inocula of each *Aspergillus* species after 24 h of incubation[a]

| Species | Relationship between menadione concn and *Aspergillus* inoculum (log CFU/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 100 μM | | 25 μM | | 6.25 μM | | 1.563 μM | | 0.39 μM | |
| | Slope | $r^2$ | Slope | $r^2$ | Slope | $r^2$ | Slope | $r^2$ | Slope | $r^2$ |
| *A. fumigatus* (R) | 0.05 | 0.368 | 0.26 | 0.884 | 0.20 | 0.853 | 0.04 | 0.524 | 0.01 | 0.359 |
| *A. flavus* | 0.22 | 0.756 | 0.28 | 0.873 | 0.09 | 0.549 | 0.02 | 0.671 | 0.01 | 0.529 |
| *A. terreus* | 0.65 | 0.869 | 0.57 | 0.878 | 0.38 | 0.934 | 0.13 | 0.862 | 0.02 | 0.745 |
| *A. nidulans* | 0.42 | 0.894 | 0.35 | 0.929 | 0.07 | 0.635 | 0.03 | 0.787 | 0.02 | 0.462 |
| *A. ustus* | 0.52 | 0.994 | 0.49 | 0.936 | 0.30 | 0.926 | 0.08 | 0.908 | 0.02 | 0.802 |

[a] Each replicate was analyzed as a separate point. Data used for calculations were OD and log CFU.

TABLE 2. Best-fit values and the 95% confidence intervals obtained by nonlinear regression analysis[a]

| Drug and incubation period | Species | Results of antifungal susceptibility assay | | | | |
|---|---|---|---|---|---|---|
| | | MICs-0 (NCCLS)[b] | $EC_{50}$ ± 95% CI (mg/liter) | | Slope ($m$) ± 95% CI | |
| | | | SP | XTT | SP | XTT |
| AB, 24 h | *A. fumigatus* (S) | 0.5 (0.125–0.5) | 0.20 ± 0.05 | 0.22 ± 0.04 | −1.65 ± 0.63 | −2.18 ± 0.78 |
| | *A. fumigatus* (R) | 2 (1–4) | 0.51 ± 0.06 | 0.38 ± 0.11 | −7.04 ± 7.04 | −1.33 ± 0.44 |
| | *A. flavus* | 1 (0.5–1) | 0.42 ± 0.19 | 0.65 ± 0.08 | −1.27 ± 0.61 | −7.33 ± 3.18 |
| | *A. nidulans* | 1 (1–4) | 0.21 ± 0.09 | 0.33 ± 0.03 | −1.47 ± 0.72 | −4.11 ± 1.09 |
| | *A. terreus* | 1 (1–2) | 0.27 ± 0.12 | 0.66 ± 0.10 | −1.00 ± 0.41 | −8.69 ± 4.71 |
| | *A. ustus* | 2 (1–2) | 0.47 ± 0.09 | 0.35 ± 0.12 | −3.58 ± 2.56 | −1.11 ± 0.34 |
| AB, 48 h | *A. fumigatus* (S) | 1 | 0.41 ± 0.07 | 0.37 ± 0.05 | −2.88 ± 1.25 | −5.09 ± 2.14 |
| | *A. fumigatus* (R) | 4 (2–4) | 1.13 ± 0.93 | 1.00 ± 0.68 | −6.42 ± 6.42 | −1.22 ± 0.51 |
| | *A. flavus* | 1 (1–2) | 0.73 ± 0.18 | 1.23 ± 0.14 | −2.94 ± 1.79 | −8.41 ± 4.29 |
| | *A. nidulans* | 2 (2–4) | 0.25 ± 0.10 | 1.09 ± 0.12 | −1.24 ± 0.51 | −5.10 ± 3.98 |
| | *A. terreus* | 4 (2–4) | 0.63 ± 0.16 | 0.96 ± 0.09 | −2.67 ± 1.54 | −7.00 ± 7.00 |
| | *A. ustus* | 2 (1–2) | 0.27 ± −0.09 | 0.45 ± 0.13 | −1.08 ± 0.37 | −1.14 ± 0.31 |
| ICZ, 24 h | *A. fumigatus* (S) | 0.5 (0.5–1) | 0.15 ± 0.02 | 0.16 ± 0.04 | −1.26 ± 0.23 | −1.39 ± 0.42 |
| | *A. fumigatus* (R) | >32 | 6.27 ± 5.80 | >32 | −0.42 ± 0.17 | −0.24 ± 0.12 |
| | *A. flavus* | 0.25 (0.25–0.5) | 0.07 ± 0.01 | 0.09 ± 0.02 | −1.42 ± 0.25 | −1.64 ± 0.54 |
| | *A. nidulans* | 0.5 (0.25–0.5) | 0.07 ± 0.01 | 0.10 ± 0.01 | −2.54 ± 0.86 | −1.86 ± 0.41 |
| | *A. terreus* | 0.5 (0.25–0.5) | 0.07 ± 0.02 | 0.05 ± 0.01 | −1.46 ± 0.44 | −1.32 ± 0.21 |
| | *A. ustus* | 2 (0.5–>32) | 0.20 ± 0.08 | 0.37 ± 0.08 | −0.89 ± 0.26 | −1.83 ± 0.61 |
| ICZ, 48 h | *A. fumigatus* (S) | 1 (0.5–1) | 0.15 ± 0.03 | 0.17 ± 0.05 | −1.26 ± 0.27 | −1.33 ± 0.40 |
| | *A. fumigatus* (R) | >32 | >32 | >32 | −0.04 ± 0.04 | 0.00 ± 0.00 |
| | *A. flavus* | 0.5 | 0.07 ± 0.01 | 0.21 ± 0.01 | −1.17 ± 0.18 | −6.06 ± 1.92 |
| | *A. nidulans* | 0.5 (0.25–0.5) | 0.08 ± 0.01 | 0.17 ± 0.02 | −3.44 ± 1.44 | −1.87 ± 0.43 |
| | *A. terreus* | 0.5 (0.5–1) | 0.11 ± 0.02 | 0.09 ± 0.02 | −1.63 ± 0.53 | −1.36 ± 0.33 |
| | *A. ustus* | >32 | 0.48 ± 0.22 | 3.34 ± 1.41 | −0.56 ± 0.16 | −0.53 ± 0.12 |

[a] Data were obtained by nonlinear regression analysis using the $E_{max}$ model with variable slopes for the concentration effect curves obtained by spectrophotometric (SP) and colorimetric (XTT) methods after 24 and 48 h of incubation with serial twofold dilutions of amphotericin B (AB) and itraconazole (ICZ). 95% CI, 95% confidence intervals.

[b] Data are the most frequently observed MIC and the range of MICs. MICs were determined visually by four different observers in three independent experiments.

ysis of variance followed by a Bonferroni post test. The significance level of 0.05 was chosen.

**Results of XTT assay.** The tetrazolium salt XTT was not metabolized by any of the *Aspergillus* species until an electron-coupling agent was added. Conversion of XTT by a certain amount of hyphae depended on the concentration of XTT and the exposure time as well as the electron-coupling agent and its concentration (Fig. 1). Between the three concentrations of XTT used, 200 μg/ml resulted in two-times-higher formazan production than 100 and 50 μg/ml, for which the formazan production was similar. Among the two electron-coupling agents, menadione was eight times more potent than PMS for all *Aspergillus* species. The use of PMS resulted in a background absorbance higher than that of menadione, especially at the concentration of 100 μM, where the OD of the blank after 6 h of exposure was 0.805 for PMS and 0.347 for menadione. Relatively lower background absorbances (≈0.2) were observed at maximal concentrations of 25 μM menadione and 1.56 μM PMS (data not shown). Any increase of menadione concentration beyond 25 μM did not have any effect in formazan production when XTT was exposed for longer than 2 h to fungi which were incubated for 48 h.

**Results of quantitative assay of fungal viability.** The relationship between the XTT conversion and increasing inocula of *Aspergillus* species was tested using 200 μg of XTT/ml with 25 μM menadione after 2 h of exposure. The analysis of results showed a linear relationship between the OD and log CFU (Fig. 2). The slopes and the coefficients obtained by the regression analysis depended on the concentration of menadione used for each species. A general pattern of concentration dependence was observed for all five species. At menadione concentrations lower than 1.56 μM, low XTT conversion rates were apparent (slopes ranged between 0.01 and 0.13) for all species. For *A. nidulans* and *A. flavus*, low conversion rates were also observed at a menadione concentration of 6.25 μM (Table 1). The conversion rates increased at higher concentrations of menadione, generating higher slopes, up to 0.65, as in the case of *A. terreus* with 100 μM menadione. However, at this concentration of menadione, larger deviations from linearity were observed for all species (except *A. ustus*) and very low slopes were obtained for *A. fumigatus* (R) and *A. flavus* (0.05 and 0.22, respectively). In addition, at the concentration of 100 μM menadione, formazan production exceeded an OD of 3 after 4 h of exposure of XTT, which was the detection limit of the spectrophotometer, especially when hyphae were exposed to XTT after 48 h of incubation.

Among the different concentrations of menadione that were evaluated, relatively higher conversion rates and more linear relationships between formazan production and viable fungi were generated with the concentration of 25 μM for all species (Table 1). In addition, the formazan production at this concentration was high within 2 h of exposure without exceeding the detection limit of the spectrophotometer (OD of 3) and low background absorbance was obtained.

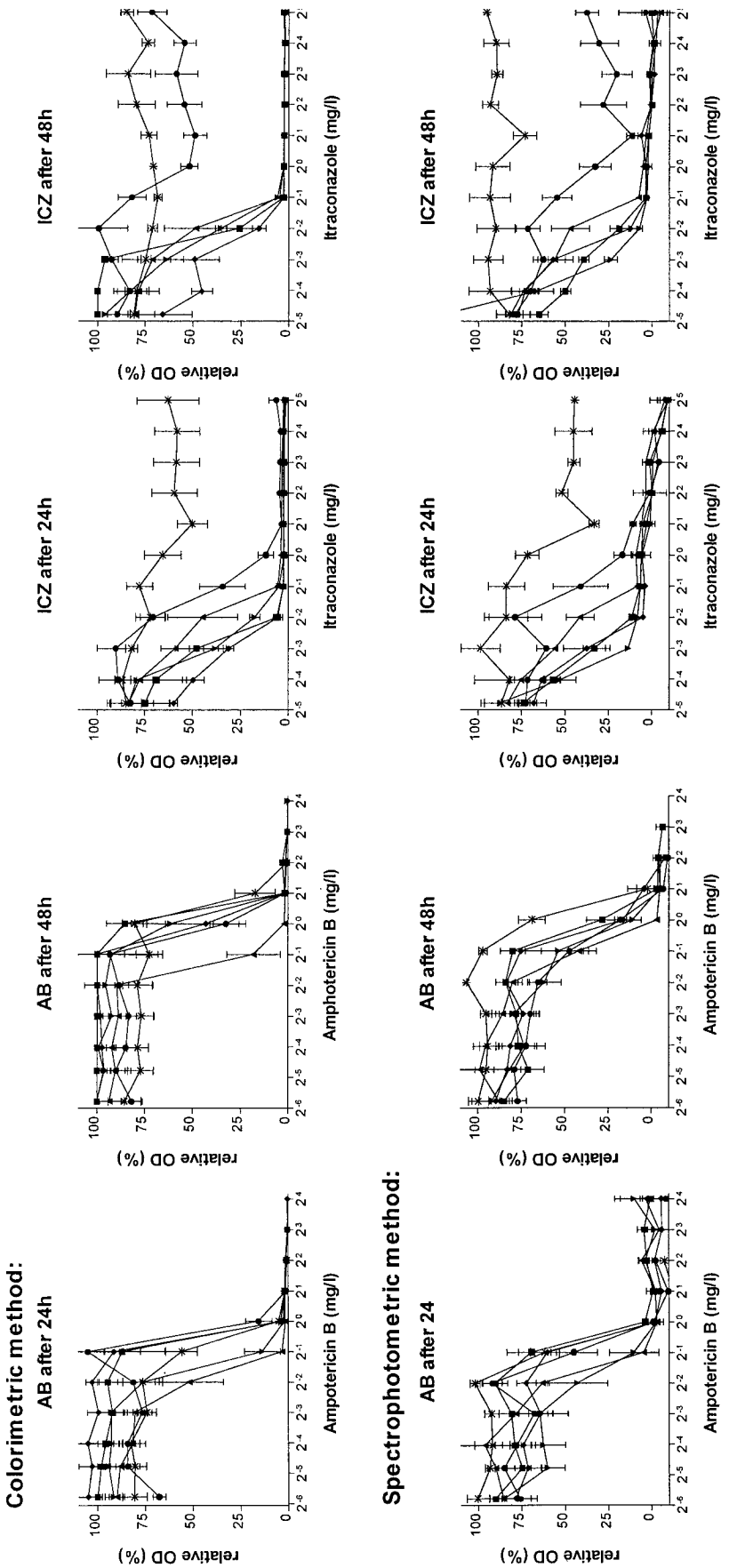


FIG. 3. Concentration effect curves for amphotericin B (AB) and itraconazole (ICZ). Conidia of each *Aspergillus* sp. tested were incubated for 24 and 48 h and the OD of hyphal growth at 405 nm was measured for each well. Then, hyphae were exposed to 200 μg of XTT/ml plus 25 μM of menadione for 2 h and the $OD_{450}$ was measured. Concentration effect curves based on the colorimetric method (top) and spectrophotometric method (bottom) were constructed, and the relative ODs for each drug concentration represent the amount of formazan produced and the fungal biomass, respectively, for each method compared with the growth control. *A. ustus* and *A. fumigatus* (R) showed NCCLS MICs higher than 2 mg of itraconazole/liter. Means with the standard errors of triplicates are represented for each strain.

**Results of antifungal susceptibility assay.** For all *Aspergillus* species, the NCCLS MICs of amphotericin B ranged from 0.5 to 4 mg/liter and those of itraconazole ranged from 0.25 to 0.5 mg/liter except for *A. fumigatus* (R) and for *A. ustus*, for which the MICs were higher than 2 mg/liter (Table 2). Concentration effect curves based on the conversion of XTT by hyphae are shown in Fig. 3 (top). All curves show similar patterns and are characterized by two plateaus connected by a drop in relative OD. In the case of a resistant strain, significant XTT conversion was observed in higher concentrations [*A. ustus* and *A. fumigatus* (R) against itraconazole; Fig 3]. The three phases of XTT conversion were clearly distinguishable for amphotericin B, for which steep concentration effect curves were obtained. In contrast, for itraconazole, more shallow curves were generated.

The concentration effect curves obtained by the colorimetric and the spectrophotometric assessments of fungal growth (Fig. 3) were analyzed by the $E_{max}$ model with variable slope, and the best-fit values of the two variables $EC_{50}$ and slope ($m$) were compared for each species, drug, and incubation period. The model fitted the data very well since the $r^2$ ranged between 0.85 and 0.99 (median, 0.95) and no statistically significant deviations from the model were found ($P > 0.1$). No statistically significant differences were found by analysis of variance between the best-fit values of the two concentration effect curves for each drug and incubation period ($P > 0.05$) with the exception of the concentration effect curves of amphotericin B after 48 h, where statistically significantly steeper curves (higher absolute $m$ values) were obtained by the colorimetric method than by the spectrophotometric method ($P < 0.01$). However, the slopes and the $EC_{50}$s of the fitted model were similar for the concentration effect curves obtained by the two methods (Table 2). Overall, the median coefficient variation of the log $EC_{50}$s and the slope $m$ of the fitted model for the six strains among the replicates was 22 and 25%, respectively, for the colorimetric method and 37 and 38%, respectively, for the spectrophotometric method. Furthermore, the discrimination between the in vitro itraconazole-resistant strains and the other itraconazole-susceptible strains was clearer in concentration effect curves of the colorimetric method than those of spectrophotometric method (Fig. 3).

The tetrazolium salt XTT was converted by *Aspergillus* species only in the presence of an electron-coupling agent, as was found with yeasts (14). As opposed to mammalian cells (12) but similar to yeasts (15), among the two electron-coupling agents tested menadione was more potent than PMS. Since PMS exhibited a background higher than that found in previous studies (12, 13) and inhibitory effects at elevated concentrations were reported previously (1), menadione was chosen as the electron-coupling agent for the XTT assay of *Aspergillus* species.

The concentration of XTT is another important factor since it was found that high concentrations of XTT result in inhibition of formazan production while very low concentrations can result in poor conversion (15). No inhibition was observed among the three concentrations tested in this study. Preliminary studies showed that XTT was not toxic for conidia (unpublished observation). At higher concentrations of XTT (200 μg/ml), the rate of formazan production increased rapidly. Therefore, a concentration of 200 μg of XTT/ml was used for further studies. XTT and menadione at the above concentrations were stable when they were stored separately for up to 3 months at room temperature and 4, −20, and −70°C (unpublished data).

The linear relationship between the formazan production using 200 μg of XTT/ml and menadione with increasing inocula of *Aspergillus* species indicates that the XTT assay is a reliable indicator of fungal biomass. The linearity and the slopes of this relationship depended on the concentration of menadione for each of the *Aspergillus* species. At a concentration of 25 μM, high coefficients and slopes for all *Aspergillus* species tested were found, unlike with yeasts, for which lower concentrations of menadione (1 μM) were required to generate a linear relation between XTT conversion and CFU (15). For *Aspergillus* species, menadione concentrations higher than 25 μM generated a less linear CFU-OD relationship, which explains the conclusions of Jahn et al., who found that when XTT was used with 1,000 μM menadione, a less-well-defined correlation was observed (5).

The XTT assay was applied in the presence of antifungal drugs generating less-variable concentration effect curves. For amphotericin B, clear-cut endpoints were obtained since XTT conversion was absent once the MIC was exceeded, as was found previously (15). For itraconazole, shallow concentration effect curves were obtained due to a partial inhibition of growth and possible interference of the drug in the metabolic status of fungi. Furthermore, as was found in a previous study (14), discrimination between susceptible and resistant strains may be facilitated by using the XTT assay since resistant strains converted XTT even at a high concentration of antifungal drugs.

The need for the use of menadione in the XTT assay described here offers the possibility of increasing the sensitivity of this assay by adjusting the concentration of menadione. In this way, it would be possible to detect the metabolic capacity of slow-growing fungi. Also, incubation periods proposed by the NCCLS to determine the MICs may be shortened if conversion of XTT of the growth control is sufficient. However, the development of a tetrazolium salt which might not require the addition of an electron-coupling agent would be a favorable step since the need of an electron-coupling agent complicates the assay more and may increase variability (13). Variability may also be increased by the lack of a step for the termination of XTT conversion since small variation in incubation time would influence the formazan production.

In conclusion, the XTT-menadione system described in this study provides an assay which enables the quantification of metabolic activity of *Aspergillus* species and which could be applied for other filamentous fungi like it was shown with MTT (7).

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