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New In Vitro Assay Based on Glucose Consumption for Determining Itraconazole and Amphotericin B Activities against *Aspergillus fumigatus*

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We developed a new in vitro method of evaluating antifungal molecules. Fungal growth was determined by measuring glucose consumption, the only carbon source in a synthetic medium. First, the growth of 12 *Aspergillus fumigatus* strains was studied. Glucose consumption was an excellent indicator of fungal growth. Second, the partial inhibition of growth was calculated in terms of the 90% or 50% inhibitory concentration for the 12 strains after treatment with itraconazole and amphotericin B. With a 3-day incubation time, the calculated 90% and 50% inhibitory concentrations agreed with those obtained by a macromethod and with those reported in previous publications. In each case the high degrees of efficacy of itraconazole and amphotericin B against *A. fumigatus* were confirmed. Partial inhibition induced by low concentrations of antifungal agents was quantifiable by this new method.

Systemic mycosis in infectious pathology is becoming more widespread. Immunodeficient people affected by the human immunodeficiency virus (AIDS), transplants, diabetes, or cystic fibrosis or patients undergoing prolonged antibiotic treatment or corticotherapy have conditions that favor the development of infections caused by filamentous fungi (*Aspergillus* species) or opportunistic yeasts. These types of infections are usually fatal if an antifungal treatment is not prescribed.

The emergence of resistance, especially to some azole products, demands a precise analysis of the susceptibilities of the isolated strains to different drugs (21, 29). The MIC, which measures antimicrobial activity, is the method most commonly used for this susceptibility analysis.

However, new concepts for the precise determination of in vitro activity are now available: relative inhibition factor, elaborated by Odds and Abbott (24), and partial inhibitory concentrations which restrain growth by 50% (IC_{50}) or 90% (IC_{90}).

Therefore, it is in this direction that we developed a new in vitro evaluation test in which partial inhibition was easily quantifiable for the filamentous fungi. In the presence of two antifungal agents, itraconazole and amphotericin B, growth inhibition corresponded to a decrease in glucose consumption. Fungal growth was evaluated in a synthetic medium by measuring the kinetics of glucose degradation.

MATERIALS AND METHODS

Strains. Twelve strains of *Aspergillus fumigatus* were tested: 11 were isolated from patients and 1 was isolated from an aeromycological survey.

The origins of the pathogenic strains were diverse: patients with hepatic transplants (strains 8, 11), lung transplants (strains 6 and 10), cystic fibrosis (strains 4, 5, and 7), lung aspergilloma with antecedent tuberculosis (strain 3), and colonization (strains 1 and 2) and patients receiving corticotherapy (strain 12). Strain 9 was isolated during an aeromycological survey.

The strains were conserved at −70°C in a solution of glycerol and distilled water 1:9 (vol/vol). They were maintained by serial passage on a Sabouraud agar medium (Sanofi Diagnostics Pasteur, Marnes la Coquettes, France) containing chloramphenicol and gentamicin as antibacterial agents.

Inoculum preparation. A suspension from 5-day precultures on a Sabouraud agar medium was calibrated with a hemacytometer at 10^6 spores per ml in sterile distilled water. Then, 2 ml was added to 18 ml of sterile Yeast Nitrogen Base with 2% glucose (YNBG) medium. The final inoculum was 10^5 spores per ml.

Culture medium. Strain development was carried out in liquid YNBG medium containing Yeast Nitrogen Base (6.7 g; Difco, Detroit, Mich.), monohydrated glucose (20 g; Merck, Darmstadt, Germany), and distilled water to 1 liter. Aliquots of 18 ml were also prepared.

Study of strain growth. A total of 900 μl of inoculated YNBG medium and 100 μl of sterile water were placed in sterile 1.5-ml microtubes (1.5-ml polypropylene microcentrifuge test tubes; Treff AG, Degershein, Switzerland). Incubation was performed at 25°C, and the glucose concentration in the medium was determined daily.

Antifungal agents. An amphotericin B (graciously donated by Bristol-Myers Squibb, Paris, France) stock solution of 462 μg/ml was prepared by dissolving the powdered drug in a mixture containing dimethyl sulfoxide (Merck)–5% Tween 80 (Sigma, St. Louis, Mo.) in distilled water, 1:3 (vol/vol). A range of dilutions between 462 and 2.31 μg/ml was prepared from this stock solution. All dilutions were filtered on 0.22-μm-pore-size Millex-GV filters (Millipore, Molshein, France) and were conserved at −20°C for no longer than 3 months before use as described previously (23, 26, 39).

An itraconazole (graciously donated by the Janssen Research Foundation, Beerse, Belgium) stock solution of 100 μg/ml was prepared in the same way as described above for

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amphotericin B. A range of dilutions from 100 to 0.10 μg/ml was prepared from this stock solution. All dilutions were filtered and conserved as described above for amphotericin B.

Microdilutions. A total of 100 μl of antifungal solution was placed into 1.5-ml sterile microtubes (1.5-ml polypropylene microcentrifuge test tubes; Treff AG). A total of 900 μl of inoculated medium was added to this solution. Quintuplicate determinations were performed for each concentration. The ranges of final concentrations were from 46.2 to 0.23 μg/ml for amphotericin B and from 10 to 0.01 μg/ml for itraconazole. A drug-free control was prepared for each strain by replacing 100 μl of antifungal solution with 100 μl of sterile water.

Incubation. All microtubes were placed in an incubator at 25°C during 11 days for the first protocol and 3 days for the second protocol. The microtubes were agitated and ventilated daily.

Reading of results. After incubation, the microtubes were centrifuged (10,500 × *g*; TDX centrifuge; Abbott). A total of 100 μl of the supernatant and 400 μl of water were poured into vials for analysis with a Kodak Ektachem 700 automatic analyzer. This one-fifth dilution was determined by the range of concentrations read correctly by the analyzer, i.e., from 1.11 to 34.72 mM.

Determining the IC_{90} and IC_{50}. The inhibition of glucose consumption induced by an antifungal concentration was represented by percent efficacy. Percent efficacy was calculated by the following formula:

$$\text{percent efficacy} = \frac{\text{glucose consumption in drug-free control} - \text{glucose consumption in trial}}{\text{glucose consumption in drug-free control}}$$

in which glucose consumption in the drug-free control = the glucose initial concentration − glucose concentration after incubation without the antifungal agent and glucose consumption in the trial = the glucose initial concentration − glucose concentration after incubation with a dilution of the antifungal agent.

The different percent efficacies were transformed into probits by using the Fischer formula or tables (4). In this formula, x is a variable of normal distribution, if probability $(x \leq a) = p$ (probit); thus, $p = a + 5$. For example, p (17.5%) = 4.07. The linear regression was traced by representing the probit in relation to the logarithm of the antifungal concentration. This probit transformation allows the best correlation for linear regression to be obtained (4, 33). From this equation, the IC_{90} or IC_{50} for glucose consumption was calculated.

Macrodilution technique in agar medium. A total of 1.5 ml of each itraconazole dilution was added to 13.5 ml of brain heart infusion agar medium (Diagnostic Pasteur). The mixture was poured into 90-mm-diameter petri dishes. A total of 5 μl of inoculated medium was placed onto the surface of the agar medium.

After 3 days of incubation at 25°C, the colony surface area was calculated by measuring the growth diameter. The percent efficacy of each concentration was calculated and transformed into probits. The graph of this probit in relation to the logarithm of antifungal concentration was drawn. The IC_{90}s and IC_{50}s were calculated with the linear regression equation.

RESULTS

Growth study of the 12 strains. The kinetics of glucose degradation in relation to time was observed for the 12 *A. fumigatus* strains. Figure 1 shows the results for strains 1 and 2.

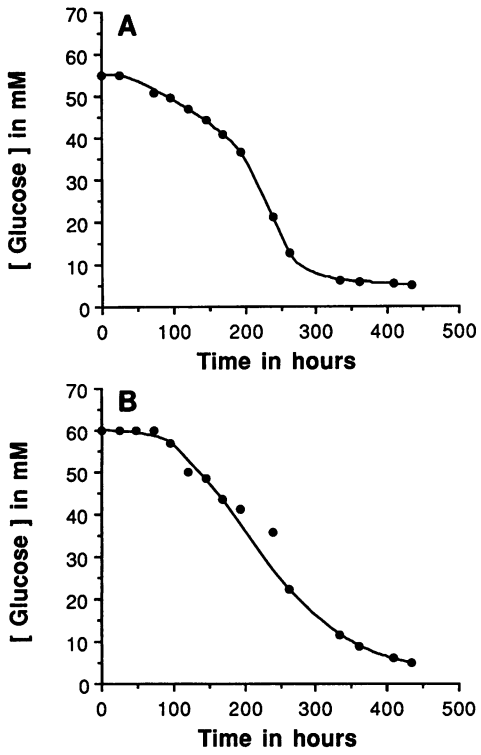

FIG. 1. Kinetics of glucose degradation in YNBG medium at 25°C for strains 1 (A) and 2 (B). The glucose concentration was determined daily.

The data in Fig. 1 are representative of those for all other isolates. The latent phase, exponential growth phase, and stationary phase were observed. The time needed from the beginning of the latent phase to the beginning of the exponential phase ranged from 1 to 3 days. The time necessary for the end of the exponential phase ranged from 10 to 11 days. At the end of the exponential phase, growth was visible in the microtubes. Microscopic observation confirmed the absence of contamination in the 12 strains.

Two incubation times were chosen. In the first protocol it was 11 days, in which the end of the exponential growth phase was attained with maximal glucose consumption. In the second protocol it was 3 days, in which the beginning of the exponential growth phase was attained with measurable glucose consumption.

Determining IC_{90}s and IC_{50}s of itraconazole and amphotericin B by measuring the kinetics of glucose degradation. In the automatic analyzer, the glucose concentration was determined by an enzymatic system. Tests showed that amphotericin B and itraconazole did not interfere with this enzymatic dosage, so it was possible to evaluate the glucose consumption for each antifungal concentration. After 11 or 3 days of incubation at 25°C, the glucose concentration was measured in each microtube. The coefficient of variation was calculated for the quintuplicate replicates and for each strain; if it exceeded 20%, the assay was repeated. The percent efficacy was transformed into probits, and the graph was traced for this probit in relation to the logarithm of the antifungal concentration.

Figure 2 presents the graph for strain 11 after an 11-day incubation at 25°C in a YNBG medium. Results for an incubation time of 3 days at 25°C in a YNBG medium are shown in Fig. 3.

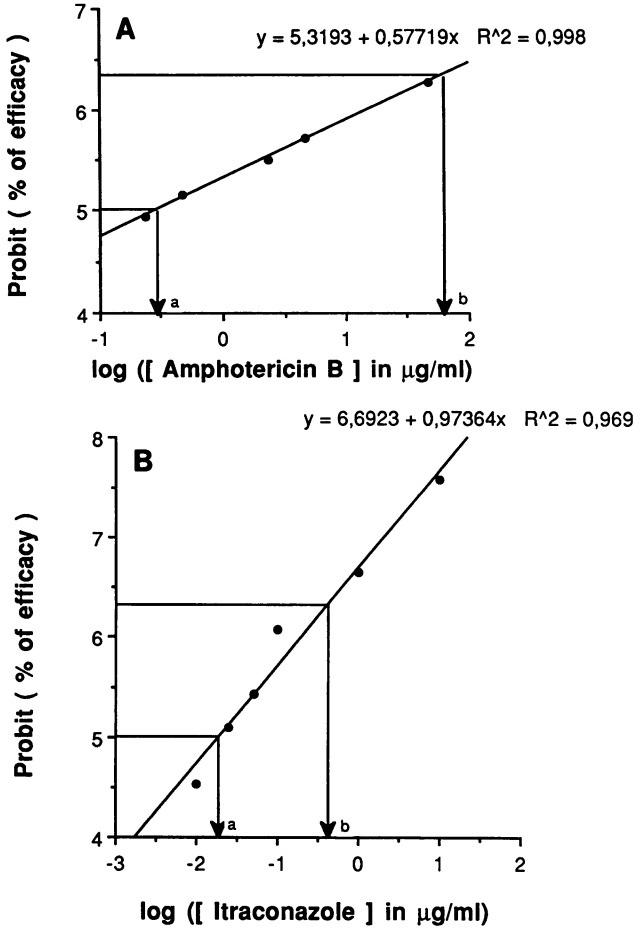

FIG. 2. Linear regression representing the probit of percent efficacy in relation to the logarithm of the amphotericin B (A) and itraconazole (B) concentrations for strain 11. The incubation time was 11 days at 25°C in YNBG medium. The logarithms of IC_{50} (A) and IC_{90} (B) were calculated with the linear regression equation. Percent efficacies were determined by measuring the kinetics of glucose degradation.

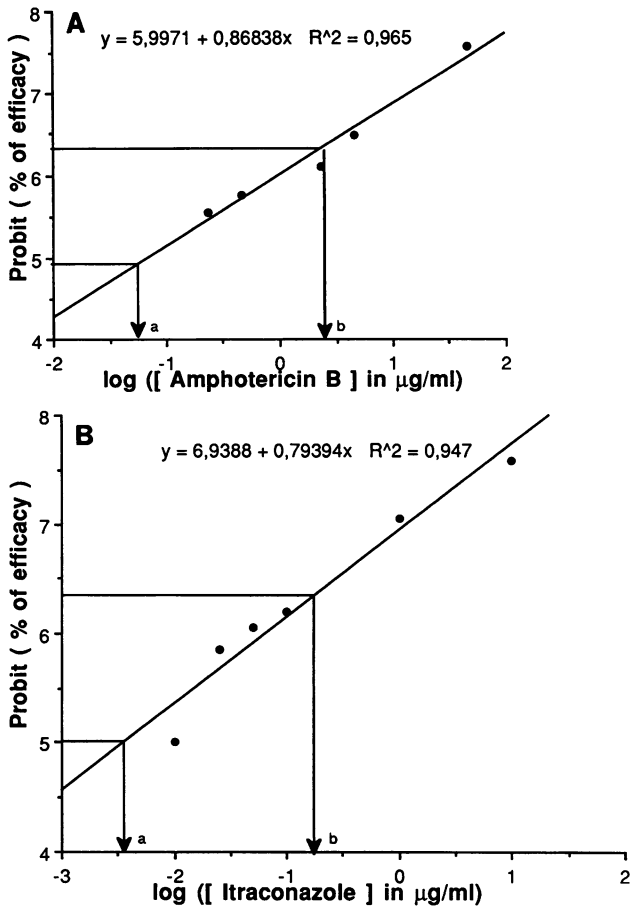

FIG. 3. Linear regression representing the probit of percent efficacy in relation to the logarithm of the amphotericin B (A) and itraconazole (B) concentrations for strain 11. The incubation time was 3 days at 25°C in YNBG medium. The logarithms of IC_{50} (A) and IC_{90} (B) were calculated with the linear regression equation. Percent efficacies were determined by measuring the kinetics of glucose degradation.

IC_{90}s and IC_{50}s were calculated with the linear regression equation. Table 1 regroups the IC_{90}s and IC_{50}s of itraconazole and amphotericin B for the 12 *A. fumigatus* strains after an 11-day incubation at 25°C in YNBG medium.

Table 2 presents the IC_{90}s and IC_{50}s of itraconazole and amphotericin B for the 12 *A. fumigatus* strains after a 3-day incubation at 25°C in YNBG medium.

Macrodilution technique in agar medium. In order to evaluate our new technique, the IC_{90} and IC_{50} of itraconazole were determined by a macrodilution technique in a brain heart medium. Growth was evaluated by measuring the colony surface area. The percent efficacies were calculated and were transformed into probits. Figure 4 shows the graph for strain 11 after a 3-day incubation at 25°C in a brain heart agar medium. Table 3 regroups the IC_{90}s and IC_{50}s obtained by this traditional technique.

The geometric mean IC_{90}s and IC_{50}s as well as the extreme values in relation to the incubation time obtained by our new technique and the traditional macromethod are presented in Table 4.

DISCUSSION

The development of the new evaluation procedure described here was motivated by the difficulties of using the traditional methods with filamentous fungi. Some of these difficulties are described in the following paragraphs.

In antifungal test diffusion (6), a disc is impregnated with an active substance and is placed on the surface of an agar medium. MIC determination is difficult by this method and is applicable only to yeasts or highly sporulating filamentous fungi.

Dilution in a liquid medium is the most commonly used method at present. However, for MIC determinations, a problem arises for the growth evaluation of the different strains. Dilutions in microplates are also widely used (8, 22, 25, 30); however, the last concentration at which no growth was visible must be located. Turbidimetric procedures have been proposed (2, 22, 25, 30), but they require a large range of dilutions in order to obtain the most correct MIC.

It is necessary today to be able to quantify growth in order to study the partial inhibition induced by low antifungal concentrations. As Odds (23) indicates, the notion of MIC has been defined by standardization test efforts for traditional antifungal

TABLE 1. IC_{90}s and IC_{50}s of itraconazole and amphotericin B for 12 strains of *A. fumigatus* in YNBG medium at 25°C[a]

Strain	Itraconazole		Amphotericin B	
	IC_{90} (μg/ml)	IC_{50} (μg/ml)	IC_{90} (μg/ml)	IC_{50} (μg/ml)
1	0.069	0.022	8.07	0.901
2	0.107	0.015	3.95	0.269
3	0.452	0.034	5.18	0.458
4	0.136	0.027	4.82	0.403
5	0.133	0.021	8.08	0.811
6	0.098	0.012	>46.2	0.012
7	0.045	0.020	>46.2	0.156
8	0.121	0.016	4.01	0.173
9	0.162	0.018	16	0.595
10	>10	0.192	>46.2	>46.2
11	0.377	0.018	>46.2	0.280
12	0.401	0.017	2.60	0.154

[a] The incubation time was 11 days. The values were obtained by the new method by measuring the kinetics of glucose degradation.

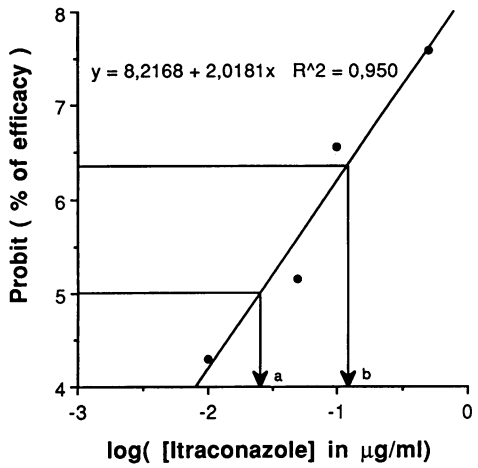

FIG. 4. Linear regression representing the probit of percent efficacy in relation to the logarithm of the itraconazole concentration for strain 11. The incubation time was 3 days at 25°C on brain heart agar medium. The logarithms of IC_{50} (A) and IC_{90} (B) were calculated with the linear regression equation. The percent efficacies were determined on solid medium by measuring the colony surface area.

agents (polyenes, flucytosine). Many investigators do not consider the MIC to be representative of azole activity, and in order to better correlate in vitro and in vivo efficacy, new analytical methods based on partial inhibition studies must be applied (20, 23, 30).

Many solutions to the question of how partial inhibition should be measured have been proposed. In the assay described here we used a macromethod in an agar medium. Partial inhibition was measurable; however, other problems appeared. At certain concentrations we observed colony decoloration or the absence of spores. It was not possible to quantify these phenomena by measuring the surface area, so it was difficult to evaluate weak, partial inhibition of growth. Turbidimetric methods in microplates have also been proposed (2, 23), but these can be used only with yeasts. However, new methods have been appearing for several years. One of these new methods is mycelium elongation (20), which is applicable for *Aspergillus* spp.; however, a complex image analysis system is necessary. Another method is determination of intracellular ATP dosage (13), which allows fungal biomass quantification. A combination of different techniques is also very efficient; drug evaluation is possible not only by measuring MICs but also by associating turbidimetric measurements with the number of living cells after treatment (39). Intracellular organelle metabolism can also be used as an indirect method of drug evaluation, and tetrazolium salt reduction by mitochondria allows for a delicate analysis (32). In microplates, yeast growth is seen by adding an acidic indicator such as phenol red (8) to the culture medium. Measurement of the amounts of different extracellular matrix components such as mannan (27) or chitin (28) also allows yeast growth to be analyzed. In recent assays aimed at standardizing evaluation methods (1, 11, 12, 25, 26, 30), it seems that the technique of growth reading chosen also plays a role in limiting the reproducibility of the assay. However, these new methods, which allow for the study of partial inhibition, are not easily applicable to filamentous fungi.

In the assay described here the kinetics of glucose degradation was chosen as the criterion for cell growth evaluation. We determined glucose consumption because glucose is the only carbon source in YNBG medium. Any modification of activity induced by low antifungal concentrations must affect glucose consumption.

The glucose concentration was easy to determine. Numerous inexpensive commercial kits are available. In the Toulouse

TABLE 2. IC_{90}s and IC_{50}s of itraconazole and amphotericin B for 12 strains of *A. fumigatus* in YNBG medium at 25°C[a]

Strain	Itraconazole		Amphotericin B	
	IC_{90} (μg/ml)	IC_{50} (μg/ml)	IC_{90} (μg/ml)	IC_{50} (μg/ml)
1	0.119	0.001	1.262	0.036
2	0.011	0.002	0.002	0.001
3	0.046	0.001	0.202	0.001
4	0.055	0.001	0.369	0.004
5	0.025	0.006	0.127	0.001
6	0.025	0.012	1.356	0.037
7	0.035	0.001	1.671	0.053
8	0.024	0.005	1.348	0.021
9	0.085	0.022	1.251	0.035
10	0.328	0.004	3.054	0.075
11	0.152	0.002	2.117	0.071
12	0.127	0.001	2.642	0.134

[a] The incubation time was 3 days. The values were obtained by the new method by measuring the kinetics of glucose degradation.

TABLE 3. IC_{90}s and IC_{50}s of itraconazole for 12 *A. fumigatus* strains calculated by measuring colony diameter on brain heart agar medium[a]

Strain	IC_{90} (μg/ml)	IC_{50} (μg/ml)
1	0.168	0.037
2	0.120	0.029
3	0.151	0.036
4	0.123	0.035
5	0.055	0.023
6	0.051	0.015
7	0.191	0.044
8	0.060	0.021
9	0.054	0.015
10	0.180	0.050
11	0.108	0.024
12	0.125	0.039

[a] The incubation time was 3 days, and incubation was done at 25°C.

TABLE 4. Average and range for IC_{90}s and IC_{50}s of itraconazole and amphotericin B[a]

Antifungal agent and test	Geometric mean		Range	
	IC_{90} (μg/ml)	IC_{50} (μg/ml)	IC_{90} (μg/ml)	IC_{50} (μg/ml)
Itraconazole				
Glucose consumption at:				
11 days	0.149	0.023	0.045–>10	0.012–0.192
3 days	0.056	0.003	0.011–0.328	0.001–0.012
Diam at 3 days	0.103	0.029	0.051–0.191	0.015–0.050
Amphotericin B				
Glucose consumption at:				
11 days	5.67	0.255	2.60–>46.2	0.012–>46.2
3 days	0.59	0.015	0.002–3.054	0.001–0.134

[a] Values were calculated by the new method of glucose consumption after an 11-day or 3-day incubation at 25°C in YNBG liquid medium. For itraconazole, we also used a traditional method in which we measured the growth diameter on brain heart agar medium after a 3-day incubation at 25°C.

Rangueil Hospital Center, we used an automatic analyzer which performed 250 assays per h.

Other techniques, such as the Amperometric method, are available. Those techniques can measure the glucose concentration in a complex medium (19, 31).

To begin our study, we analyzed the growth of 12 *A. fumigatus* strains. The different phases were very visible. A 3-day average was necessary before beginning the exponential growth phase, and 11 days was necessary before ending the exponential growth phase.

In order to validate the technique, we evaluated the activities of two antifungal agents widely used against *Aspergillus* spp. in medical mycology. The first was amphotericin B, a standard in initial treatments and a drug that is now widely used (16, 17). The second was itraconazole, which is the last commercialized triazole. Its physicochemical properties (14), activity in vitro (9, 34, 35) and in vivo (36, 37), and mode of action (3, 18, 38) are now well known. It stands out from the other azole products (5, 7, 10, 40) because of its high degree of efficacy against *Aspergillus* spp.

For itraconazole, the MIC range for *A. fumigatus* given by Espinel-Ingroff et al. (9) varied from 0.063 to 2 μg/ml. The average MIC was 0.12 μg/ml. Numerous investigators define the MIC as the lowest concentration at which growth does not exceed 10 to 25% (23, 30, 34). Therefore, we can compare published MICs with the IC_{90} found by the macromethod or by the kinetics of glucose degradation.

Using the macromethod as our reference for strain susceptibility, we observed that the 12 strains were susceptible to itraconazole. The IC_{90} range varied from 0.051 to 0.191 μg/ml, and the geometric mean IC_{90} was 0.103 μg/ml. These values, in agreement with those of Espinel-Ingroff et al. (9), confirmed the high degree of efficacy of itraconazole.

In order to validate our new method, the 12 strains had to remain susceptible to itraconazole. With the kinetics of glucose degradation, two incubation times were used: 3 and 11 days.

After a 3-day incubation, all of the strains remained susceptible to itraconazole. The IC_{90} range varied from 0.011 to 0.328 μg/ml, and the geometric mean IC_{90} was 0.056 μg/ml. After an 11-day incubation, one strain showed a low level of susceptibility to itraconazole (IC_{90} of >10 μg/ml). The IC_{90} range varied from 0.045 to >10 μg/ml, and the geometric mean IC_{90} was 0.149 μg/ml.

Therefore, the best incubation time for the kinetics of glucose degradation was 3 days since susceptibility was preserved in the 12 strains in accordance with the macromethod.

For amphotericin B, the MIC extremes given by Hughes et al. (15) varied from 1.56 to 3.13 μg/ml. In their studies, all of the *A. fumigatus* strains remained susceptible to amphotericin B. The average MIC was 3.13 μg/ml. With the kinetics of glucose degradation, all of the strains remained susceptible to amphotericin B after a 3-day incubation. IC_{90}s ranged from 0.002 to 3.054 μg/ml. The geometric mean IC_{90} was 0.59 μg/ml.

After an 11-day incubation, four strains showed low degrees of susceptibility to amphotericin B (IC_{90}, >46.2 μg/ml). IC_{90}s ranged from 2.60 to >46.2 μg/ml. The geometric mean IC_{90} was 5.67 μg/ml.

As was observed with itraconazole, the best incubation time for the kinetics of glucose degradation was also 3 days.

Therefore, in conclusion, a 3-day incubation time is ideal for standardizing the results obtained by the macromethod. By using the determined time and an incubation temperature of 25°C in YNBG medium, it was possible to evaluate more precisely the partial inhibition of filamentous fungi induced by low antifungal agent concentrations by determining the kinetics of glucose degradation than by the macromethod or other techniques.

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