

Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection

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Given the increased choice of therapeutic agents and the rising incidence of serious invasive disease, it is important that reliable in-vitro methods for detecting antifungal drug resistance in *Aspergillus* spp. are developed. Six clinical isolates of *Aspergillus fumigatus*, obtained from patients in whom the clinical outcome was known, were selected for study. Each was used to examine a range of parameters affecting agar dilution and broth microdilution susceptibility test results. The in-vitro results were compared with outcome in a neutropenic mouse model of invasive aspergillosis. Groups of animals were treated with itraconazole at 25 mg/kg and 75 mg/kg and survival rates and organ burdens were determined. Itraconazole was efficacious against four isolates (susceptible) but failed for two (resistant) in the animal model of infection. Both the resistant isolates had been obtained from patients receiving itraconazole treatment with good serum concentrations of the drug. Conditions for the agar dilution test which produced results that correlated best with our in-vivo observations included the use of RPMI agar with L-glutamine buffered to pH 7 with MOPS, inoculated with 10⁶–10⁷ conidia/mL and incubated for 48–72 h at 28 or 35°C with a no-growth endpoint. Optimal conditions for the broth microdilution method included the use of RPMI medium with L-glutamine and 2% glucose buffered to pH 7 with MOPS, an inoculum of 2 x 10⁵ conidia in 200 µL incubated for 48 h at 35°C with a growth (or trace) endpoint. The MICs for the susceptible isolates were 0.12–1.0 mg/L and ≥ 16 mg/L for the resistant isolates. With careful selection and standardization of test conditions it is possible to generate reproducible in-vitro susceptibility data for *Aspergillus* spp. that will predict clinical outcome.

Introduction

Invasive aspergillosis affects between 10 and 20% of patients with leukaemia and between 5 and 25% of patients following heart or lung transplantation.¹ Until the 1990s amphotericin B was the only drug useful for the treatment of aspergillosis. It has to be given intravenously, is relatively ineffective, and has a number of serious toxicities.^{2–4} Recent lipid-based preparations have ameliorated the toxicity but not materially improved the efficacy of amphotericin B.^{5–8} In 1990 itraconazole became available for the treatment of aspergillus infection and has been successfully used in many hundreds of patients.^{9–18}

Most isolates of *Aspergillus fumigatus* are susceptible *in vitro* to both itraconazole and amphotericin B although many different methods of determining in-vitro susceptibility have been used.^{19–22} However, a recent report of resistance to itraconazole in two isolates of *A. fumigatus*²³ has highlighted the need for reliable methods of in-vitro testing. Standardized methods have been developed for *Candida* spp. and *Cryptococcus neoformans*²⁴ and there is now good evidence of correlation of MIC with clinical outcome in patients and in animal models of yeast infection.^{25–31} To date, however, studies with moulds have concentrated on the selection of optimal in-vitro conditions and little attention has been paid to correlation with

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clinical outcome.^{21,22,32} In this investigation we selected a range of *A. fumigatus* isolates from cases in which the therapeutic outcome had been documented and attempted to determine the in-vitro test conditions which best discriminated between isolates from clinical failures and those from cases that responded to itraconazole.

Methods

Strains

Six clinical isolates of *A. fumigatus* were studied: 1, AF 65; 2, AF 71; 3, AF 210; 4, AF 294; 5, AF 72; 6, AF 91. These strains have been deposited with the National Collection of Pathogenic Fungi (PHLS Mycology Reference Laboratory, Bristol) as NCPF 7097, 7098, 7101, 7102, 7099 and 7100, respectively. These isolates were selected because they had been isolated from patients who had responded to or failed itraconazole or amphotericin B therapy (Table I). The strains were maintained on slopes of Oxoid Sabouraud Dextrose agar (10 g/L mycological peptone, 40 g/L glucose and 15 g/L agar) (Unipath Ltd, Basingstoke, UK) with 0.5% (w/v) chloramphenicol (SAB agar) at -20°C and in vials of sterile water at room temperature.

In-vitro susceptibility testing procedures

Inoculum preparation. When required, strains were sub-cultured on to SAB agar slopes and incubated for 3 days at 28°C with the caps loosened to encourage sporulation. Spores were harvested in 3 mL of sterile water for the agar incorporation method, or in 3 mL of the growth medium under test for the broth microdilution method. The spore

suspensions were vortexed for 10 s to break up clumps of cells and counted using a Modified Fuchs Rosenthal haemocytometer.

Drugs and solutions. Itraconazole (Janssen Research Foundation, Beerse, Belgium) was dissolved at a concentration of 10,000 mg/L in a 50:50 solution of 20% (v/v) acetone in water and 20% (v/v) hydrochloric acid (Fisons, Loughborough; specific gravity 1.18) in water. The drug solution was diluted further to 1000 mg/L with either sterile water or with the growth medium under test for the agar incorporation and broth microdilution methods, respectively.

Determination of MICs by the agar incorporation method. Doubling dilutions of itraconazole from 64 to 0.015 mg/L were prepared in 2 mL volumes of sterile water. Volumes (18 mL) of molten agar (see below) were added, mixed, poured into Petri dishes and allowed to set before drying. Spore suspensions were adjusted to the appropriate density (10^6 or 10^7 conidia/mL) with sterile water. Plates were inoculated with the spore suspensions using a multi-point inoculator (Denley Instruments Ltd, Bootle, UK) and incubated at 28°C and 35°C. Minimum inhibitory concentrations (MICs) were read at 48 and 72 h. The MIC was defined as the lowest concentration of drug at which there was no visible growth. This procedure was repeated three times for each set of growth conditions.

Five media were tested: (a) Bacto Antibiotic Medium 3 (Difco Laboratories, Detroit, MI, USA) with 1.5% (w/v) Oxoid Agar Technical-agar No. 3 (M3 agar); (b) Bacto Antibiotic Medium 3 (Difco) with 1.5% (w/v) Oxoid Agar Technical-agar No. 3 and 2% (w/v) D-glucose (M3G); (c)

Table I. *A. fumigatus* strains selected for study

Strain no.	NCPF no.	Patient details; source of strain	Therapeutic response data
1, AF65	7097	Acute leukaemia; lung biopsy	Intermediate response to high dose amphotericin B despite neutrophil recovery; subsequent relapse
2, AF71	7098	Allogenic BMT; lung aspirate	Good response to itraconazole ³³
3, AF210	7101	Laparostomy for Crohn's disease; liver surface	Excellent response (cure) to amphotericin B 1 mg/kg ³⁴
4, AF294	7102	Multiple myeloma and profound neutropenia; bronchoalveolar	Failed amphotericin B 1 mg/kg over 12 days despite neutrophil recovery
5, AF72	7099	Cured Hodgkin's disease; sputum	Isolated during itraconazole therapy with good serum concentrations ²³
6, AF91	7100	AIDS patient; sputum	Isolated during itraconazole therapy with good serum concentrations ²³

NCPF, National Collection of Pathogenic Fungi.

In-vitro susceptibility testing of *Aspergillus fumigatus*

SAB agar (Unipath Ltd); (d) RPMI Agar in MOPS (Difco, code: 1901-17) with 0.03% (w/v) L-glutamine added (RPMI agar); (e) Yeast nitrogen base with glucose: Bacto Yeast nitrogen base (Difco) and 10% (w/v) D-glucose and 1.5% (w/v) Oxoid Agar Technical-agar No. 3 (YNBG agar).

Determination of MICs by the broth microdilution method. Doubling dilutions of itraconazole were prepared in 100 μ L volumes of broth in the wells of a microtitre plate, giving final drug concentrations from 16 to 0.06 mg/L. Spore suspensions were adjusted to 2×10^6 conidia/mL in the growth medium under test. Volumes of 100 μ L of the spore suspension were dispensed into the wells containing the drug dilutions giving a final concentration of 10^6 conidia/mL. Plates were incubated at 28°C or 35°C and the

MICs were assessed visually after 24 and 48 h incubation. The MIC was taken as the lowest drug concentration at which there was no growth or virtually no growth (trace). Each experiment was repeated three times.

Five media were tested: (a) Bacto Sabouraud Dextrose Broth (Difco) (SAB broth), typical formula; 10 g/L Difco neopeptone, 20 g/L Bacto dextrose; (b) Bacto Antibiotic Medium 3 (Difco) with 2% (w/v) D-glucose (M3G broth); (c) RPMI-1640 Broth: 10.4 g/L RPMI-1640 Medium (with L-glutamine, without sodium bicarbonate) (Sigma Chemical Co., Poole, UK, code: R6504); 2 g/L sodium bicarbonate; 34.53 g/L 3-(*N*-morpholino) propane-sulphonic acid (MOPS) (RPMI broth); (d) RPMI-1640 broth with glucose: 1 L RPMI-1640 liquid medium with L-glutamine and sodium bicarbonate (Sigma, code: R8758); 20 g/L D-glucose, 34.5 g/L MOPS, pH 7.0. (RPMIG broth); (e) Yeast nitrogen base with glucose: Bacto Yeast nitrogen

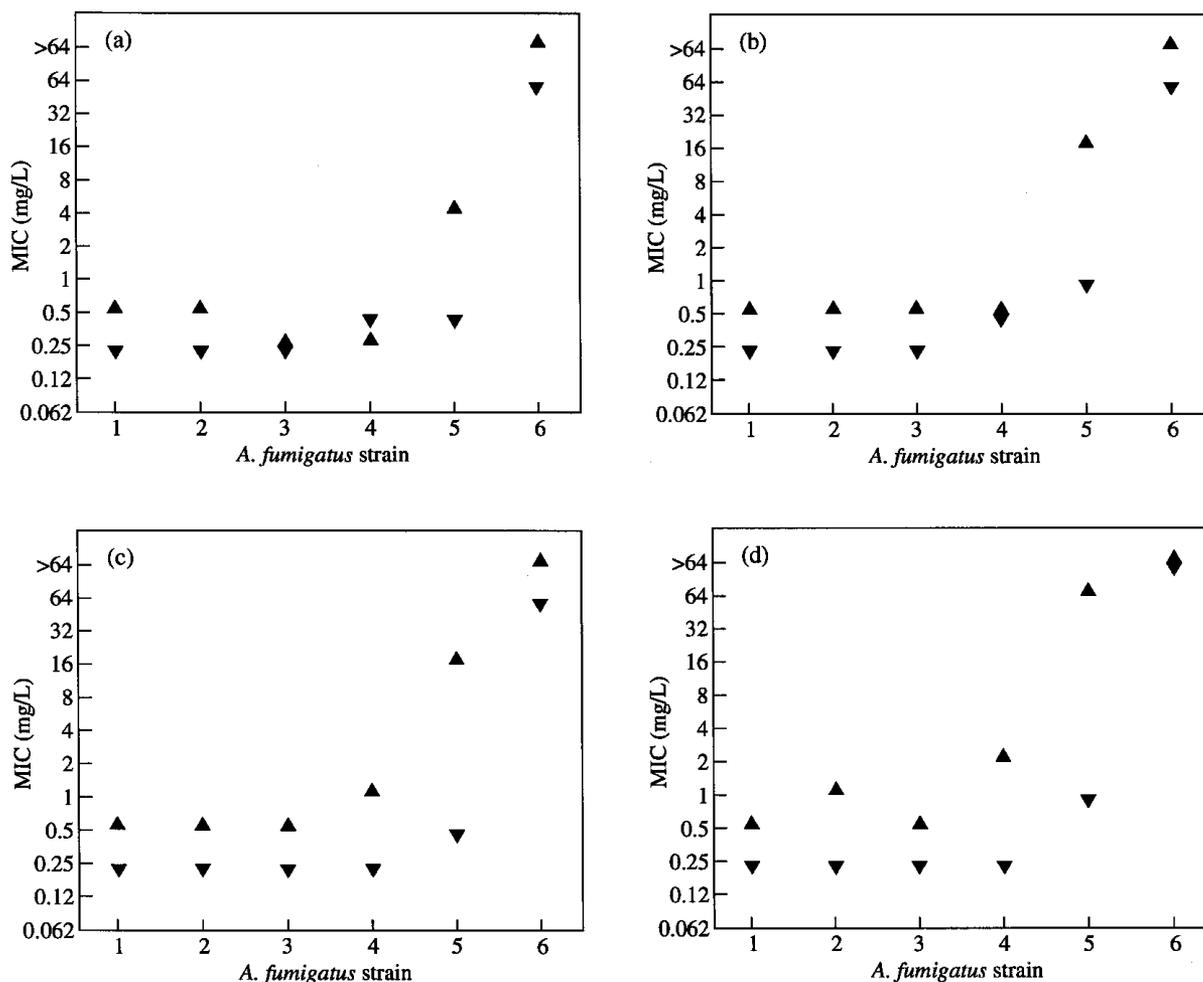


Figure 1. Variation of itraconazole MICs for six strains of *A. fumigatus* with inoculum concentration, incubation temperature and duration of incubation. Agar incorporation method with SAB agar. (a) 28°C, 48 h; (b) 28°C, 72 h; (c) 35°C, 48 h; (d) 35°C, 72 h. ▼, 10^6 conidia/mL; ▲, 10^7 conidia/mL. Each point is the mean of three values. Asterisk denotes mean MIC values where one of the three values is \geq four-fold doubling dilution higher than the other two values.

base (Difco) and 10% (w/v) D-glucose (YNBG broth).

Data analysis. The mean MIC of the triplicate values was calculated for each set of conditions. MICs of >64 and <0.015 mg/L or >16 and <0.06 mg/L for the agar incorporation and broth microdilution methods, respectively, were assigned to the next highest or lowest concentration.

In-vivo experiments

Animals. Virus-free, male CD-1 mice 4–5 weeks of age, weighing 18–20 g, were purchased from Charles River UK Ltd., Margate. Mice were reassorted after weighing, housed in groups of ten, and allowed food and water ad libitum. Mice were immunocompromised with a single dose of cyclophosphamide (200 mg/kg) which was administered intravenously via the lateral tail vein on day –3. This yielded profound neutropenia which began 4 days after immunosuppression and lasted for 4 days.³⁵

Inoculum. The inoculum was prepared by culturing the organisms on potato dextrose agar (Oxoid) for 10 days at 37°C. Conidia were collected in sterile 0.9% phosphate-buffered saline containing 0.05% Tween 80 (PBS–Tween) and stored at 4°C. The viability of the conidial suspension was determined by plating serial dilutions prepared in PBS–Tween on blood agar (41 g/L) Columbia agar base, (Lab M, Bury, UK) and 50 mL sterile horse blood (TCS microbiology, Botolph Claydon, UK). The inoculum was then stored at 4°C until the day of infection. Preliminary studies were performed to determine the LD₉₀ (an inoculum lethal for 90% of untreated mice) for each of the six *Aspergillus* strains. Mice were infected via the lateral tail vein with 0.15 mL of the inoculum on day 0. Treatment was commenced 18 h after infection and continued from day 1 to day 10.

Preparation of drugs. Itraconazole was solubilized in hydroxypropyl-β-cyclodextrin to produce a stock of 25,000

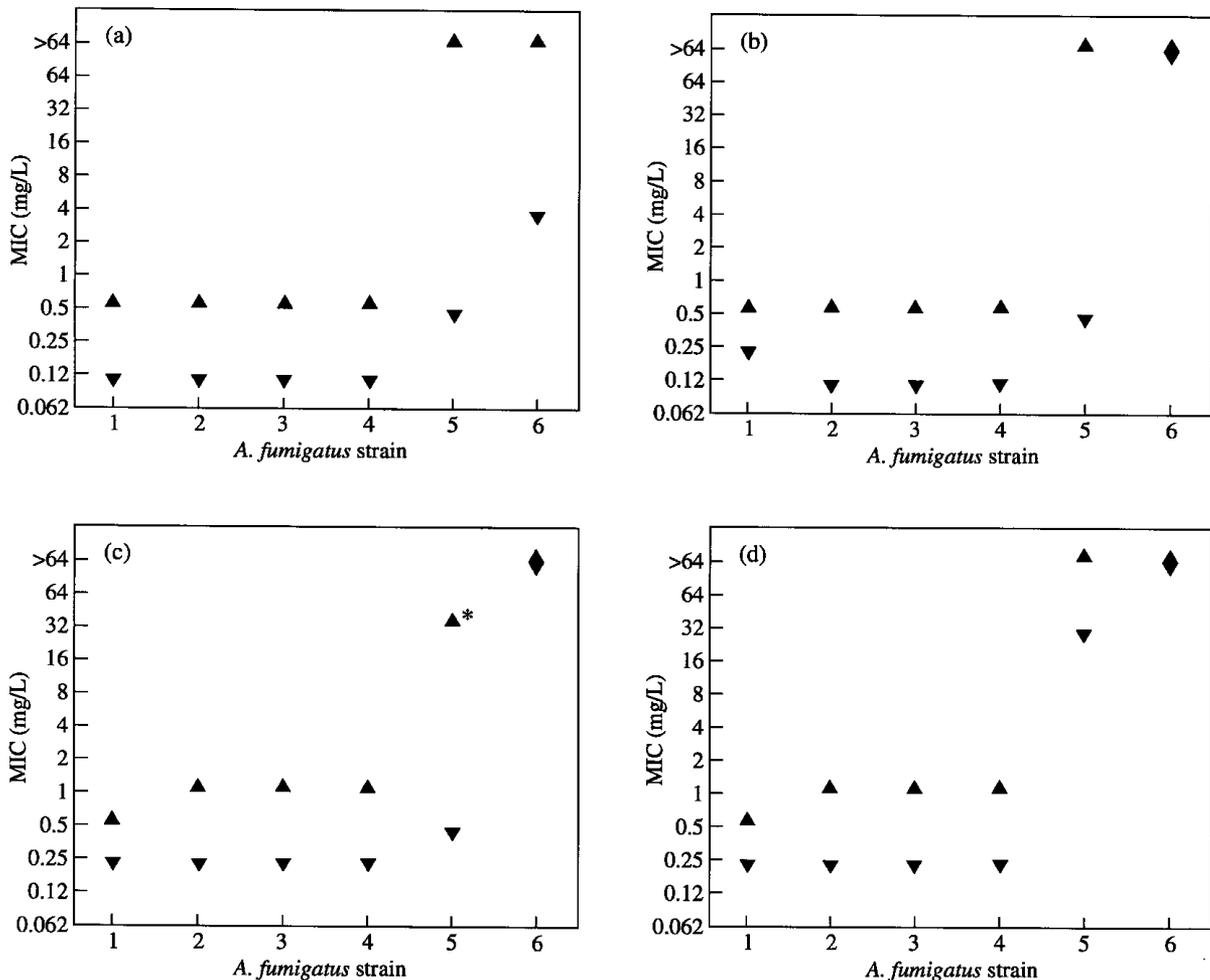


Figure 2. Variation of itraconazole MICs for six strains of *A. fumigatus* with inoculum concentration, incubation temperature and duration of incubation. Agar incorporation method with M3 agar. Legend as in Figure 1.

In-vitro susceptibility testing of *Aspergillus fumigatus*

mg/L which was further diluted in water and stored at 4°C. Amphotericin B with sodium desoxycholate (Fungizone Squibb, Hounslow, UK) was diluted in 5% dextrose in water to produce a stock solution of 5000 mg/L. Dilutions of this stock were prepared in water and stored protected from the light at 4°C.

Dosing regimens. Itraconazole was administered in 0.25 mL doses by gavage at concentrations of 25 or 75 mg/kg. Three doses were given on days 1 and 2 and two doses on days 3–10. Amphotericin B was administered in 0.1 mL doses by ip injection at a concentration of 5.0 mg/kg. The drug was given once daily on days 1, 2, 4 and 7. This is the optimal regimen for this model. One control group of mice were given 5% dextrose by gavage and another by ip injection.

Kinetics. Itraconazole serum concentrations were measured in uninfected cyclophosphamide-treated mice, housed in

groups of three. The mice were treated according to the itraconazole dosing regimen and bled by cardiac puncture 6 h after dosing on day 7. Serum concentrations were measured by bioassay⁹ and results are expressed as the mean of three.

Cultures. Surviving mice were killed on day 11 by cervical dislocation. Lungs and kidneys were removed, placed into 5 mL aliquots of sterile PBS-Tween containing penicillin 100 IU/mL and streptomycin 100 µg/mL and were homogenized in a tissue grinder for 15–30 s. Three serial 10-fold dilutions were made and 0.5 mL of each dilution plated on to SDA. The plates were incubated at 37°C and colonies counted daily for 5–6 days.

Statistics. Mortality and quantitative culture data were compared by the Mann-Whitney ranked sum test or the Kruskal-Wallis test if the Mann-Whitney was not possible (i.e. all values identical for one group). Mice that died

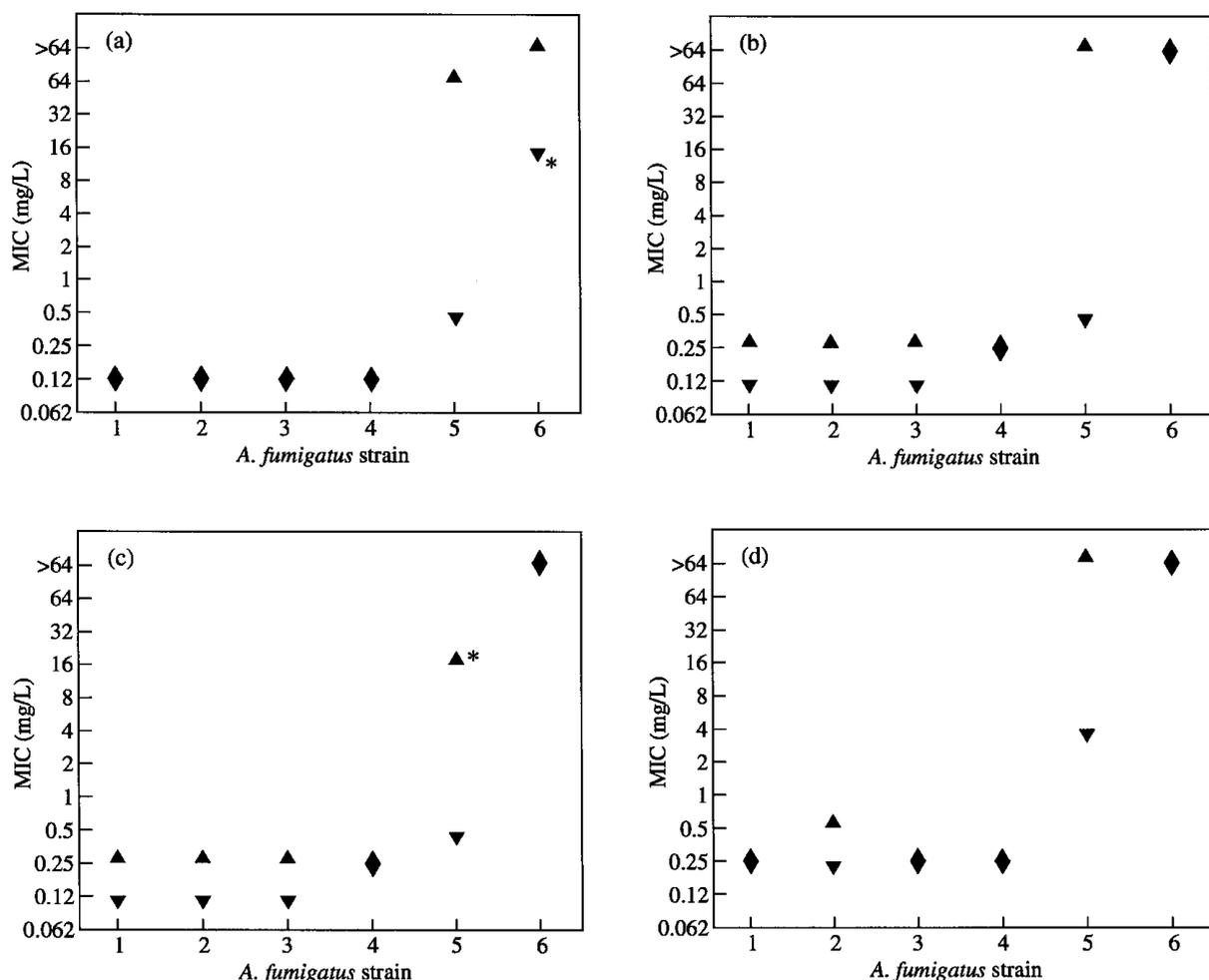


Figure 3. Variation of itraconazole MICs for six strains of *A. fumigatus* with inoculum concentration, incubation temperature and duration of incubation. Agar incorporation method with M3G agar. Legend as in Figure 1.

before day 10 were assumed to have organ quantitative cultures at least as high as the highest counts in surviving mice in calculation of the culture result statistics. All control group mice were placed in a single group for analysis with a given experiment. All analyses were done with the Minitab computer package (Minitab Data Analysis Software, Philadelphia, PA, USA).

Results

Agar incorporation method

In initial experiments, the effect of four different inocula of 10^4 , 10^5 , 10^6 and 10^7 conidia/mL on the MICs for the six *A. fumigatus* strains were compared. There was poor growth of these strains with the low inoculum concentrations of 10^4 and 10^5 conidia/mL and the MICs were not reproducible. Inocula of 10^6 and 10^7 conidia/mL were, therefore, used in subsequent experiments. Initial experiments also showed that reading MICs after 24 h incubation did not give reproducible results. MICs were, therefore,

read after 48 and 72 h incubation. YNBG agar was found to give high MIC readings with all the six isolates tested and there were no differences in MICs between the putative sensitive and resistant strains (data not shown). This growth medium was therefore not used in further studies.

The mean MICs (mg/L) for each of the six strains under the different growth conditions are shown in Figures 1–4. In most cases, the triplicate MICs for the six strains agreed to within two doubling dilutions with the four growth media. Where variations of ≥ 4 doubling dilutions occurred, this has been noted in the figures.

Marked differences were seen in the MICs for strains 5 and 6 compared with the lower MICs for strains 1, 2, 3, and 4; the latter ranged from 0.12 to 1 mg/L under all sets of conditions. The MIC for strain 6 was high (4 to >64 mg/L) in all the experiments. Two growth media, M3G agar and RPMI agar, gave the greatest differentiation between the MIC for strain 6 and the MICs for strains 1–4 (Figures 3 and 4).

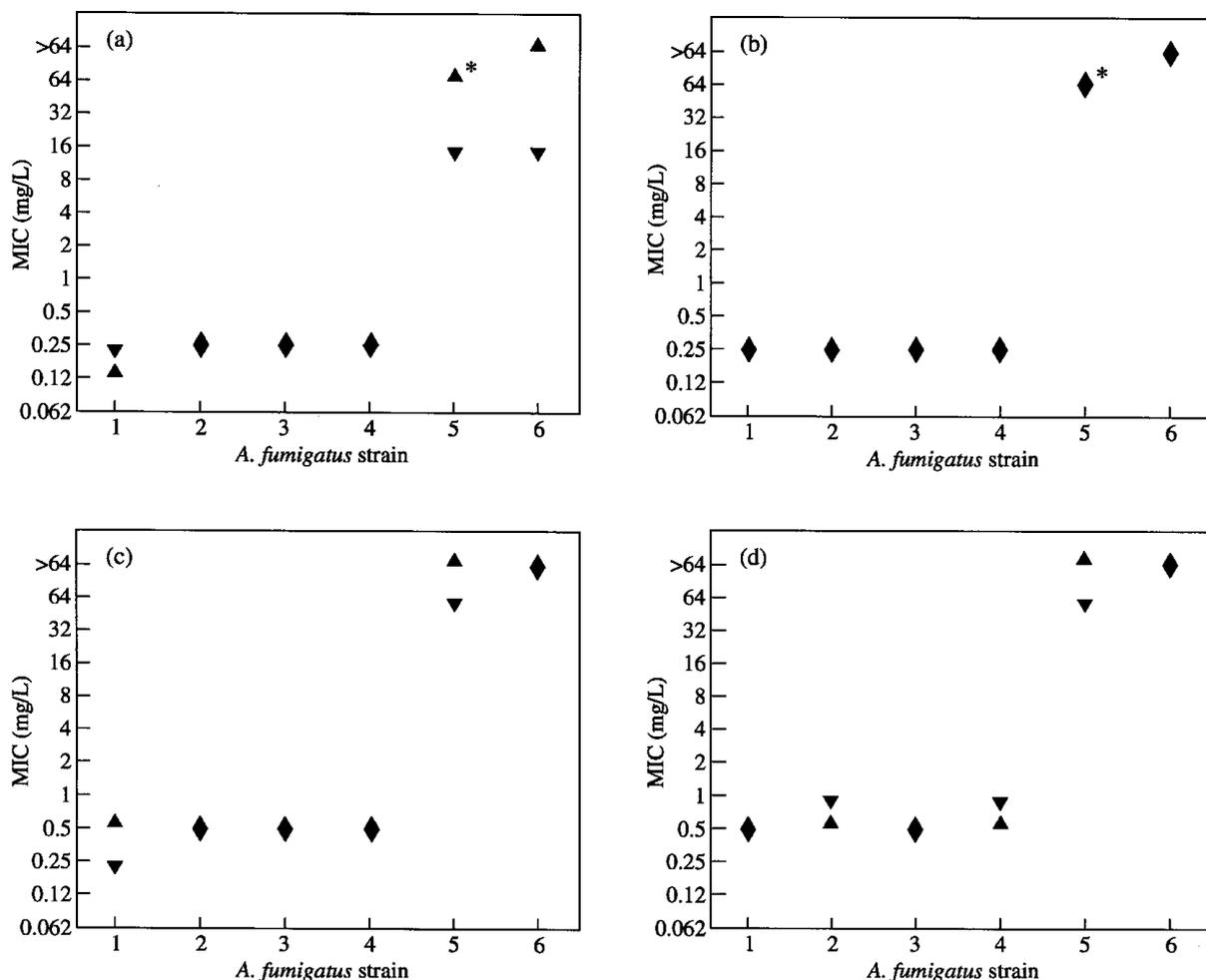


Figure 4. Itraconazole MICs for six strains of *A. fumigatus* with inoculum concentration, incubation temperature and duration of incubation. Agar incorporation method with RPMI agar. Legend as in Figure 1.

In-vitro susceptibility testing of *Aspergillus fumigatus*

The MIC for strain 5 was found to vary from 0.5 to >64 mg/L, depending on the growth conditions. It was a relatively slow growing organism, which showed marked variation in MIC depending on the initial inoculum concentration used in tests on SAB, M3 or M3G agar (Figures 1–3). With these media, the MICs for strain 5 were generally low with an inoculum of 10^6 and high with an inoculum of 10^7 mg/L. With RPMI agar (Figure 4), there was not such a marked inoculum effect and the MICs for this strain were high (≥ 16 mg/L) with both inoculum concentrations.

The duration of incubation either had no effect on MICs for the strains or resulted in a slight increase in MIC at 72 h compared with 48 h. Similarly, a rise in the incubation temperature from 28 to 35°C resulted in either no change or a slight increase in MIC.

Broth microdilution method

YNBG broth was found to be unsuitable as a growth medium for the microdilution method as the MICs for all the six strains of *A. fumigatus* were high (data not shown). This medium was therefore not used in further investigations. Initial experiments with other media showed that MICs were not reproducible with inocula of 10^4 or 10^5 conidia/mL (data not shown). An inoculum concentration of 10^6 conidia/mL was therefore selected for further tests.

The mean MICs for the six strains, read after 24 and 48 h, for the different growth media, are presented in Figure 5. The triplicate MICs showed marked variation (≥ 4 doubling dilutions) with SAB broth as the growth medium (Figure 5a and b). With M3G, RPMI and RPMIG broth triplicate MICs were within two doubling dilutions (Figure 5c–h). MICs were easier to read with SAB, M3G and RPMIG broth than with RPMI broth. This was because there was less growth of the *A. fumigatus* strains in tests with RPMI broth than with the other three media. Inclusion of glucose in the RPMI broth

enhanced growth and facilitated the determination of endpoints.

It was possible to detect differences between the MICs for the six strains with the microdilution method. Strain 6 had high MICs, whereas strains 1–4 had lower MICs. The MICs recorded for strains 1–4 with the broth microdilution method, when the growth media were SAB, M3G or RPMI broth, were overall higher than those recorded with the agar incorporation method. There was poor differentiation between the MICs for the six strains in SAB broth (Figure 5a and b). The clearest differentiation between the MICs for the sensitive strains 1–4 and the high MIC for strain 6 was recorded with RPMIG broth (Figure 5g and h). The MICs for strain 5 were again found to vary depending on the growth conditions. The MICs for this strain ranged from 2 to >16 mg/L. MICs of >16 mg/L were recorded with RPMI or RPMIG broth after 48 h incubation at 28 or 35°C (Figure 5e–h).

An increase in incubation temperature from 28 to 35°C resulted in either no change in MICs or a slight increase in MICs. MICs were clearer and easier to read after 48 h than after 24 h incubation.

In-vivo results

Each of the six strains caused a lethal infection in mice as demonstrated by the mortality curves shown in Figures 6–11. The inocula varied by 12-fold from the lowest of 1.0×10^6 conidia/mL for strain 4 to 1.2×10^7 conidia/mL for strain 6.

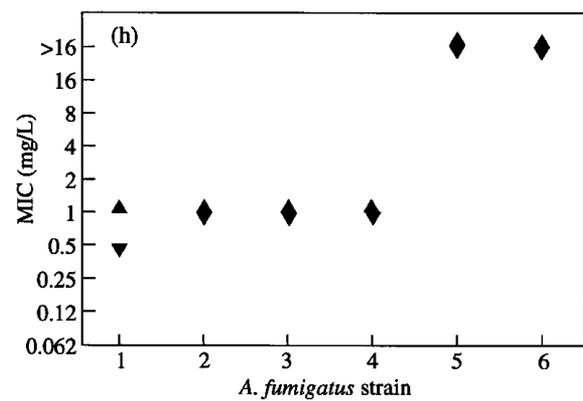
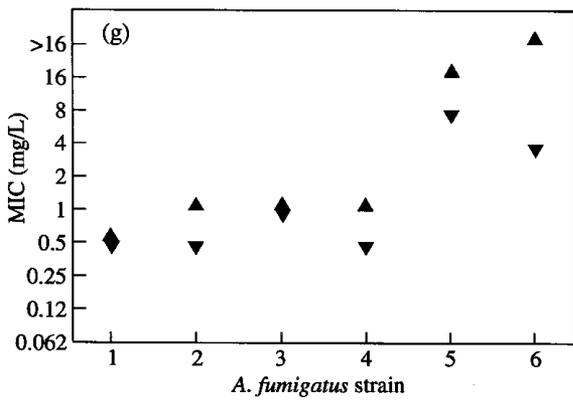
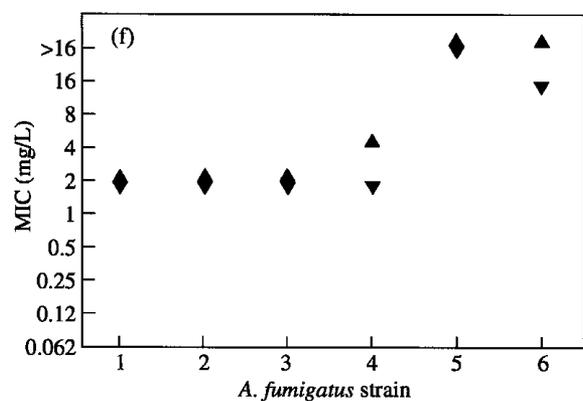
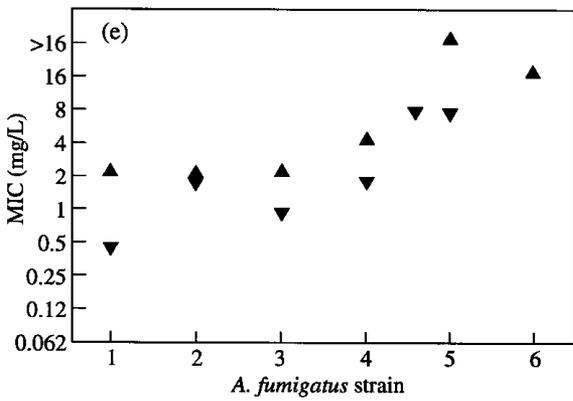
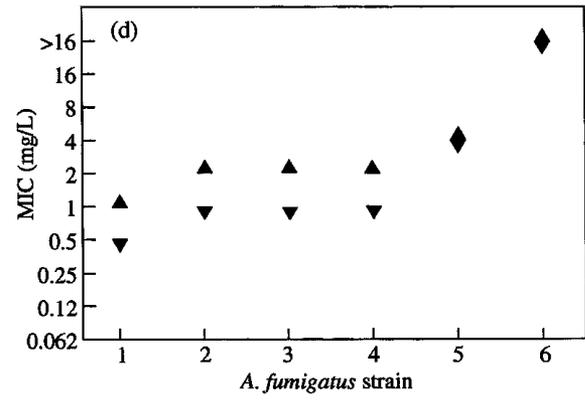
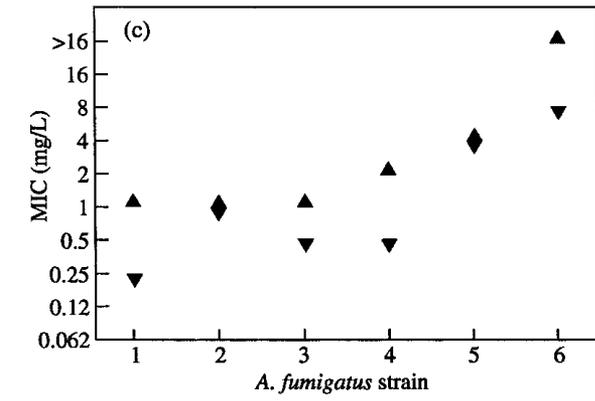
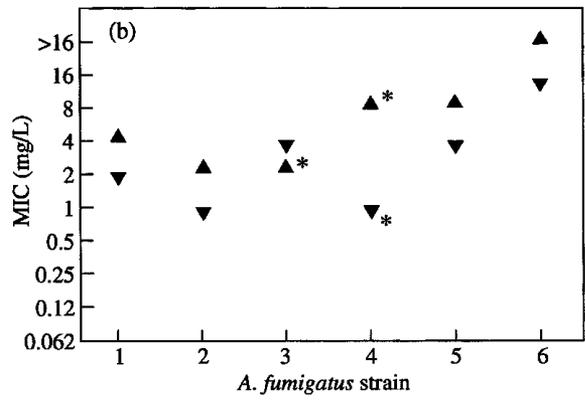
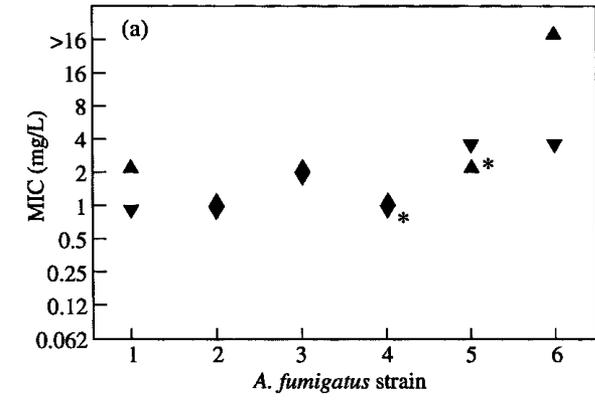
Strain 1, AF65. All control mice infected with strain 1 died (Figure 6). In the treatment groups 70% of mice given itraconazole at 25 mg/kg and 80% of mice given itraconazole at 75 mg/kg survived. Both doses of itraconazole significantly prolonged survival compared with the control mice ($P \leq 0.005$). Treatment with amphotericin B resulted in an 80% survival, significantly better than for mice receiving no treatment ($P < 0.005$).

Table II. Survival rates for the six *A. fumigatus* strains in a murine model of infection

Isolate	Controls median survival (days)	Itraconazole 25 mg/kg		Itraconazole 75 mg/kg		Amphotericin B 5.0 mg/kg median survival (days)
		median survival (days)	serum concentration (mg/L)	median survival (days)	serum concentration (mg/L)	
1	3	11 ^a	1.6	11 ^a	17.5	11 ^a
2	4	11 ^a	1.5	11 ^a	12.4	11 ^a
3	5	11 ^a	4.3	11 ^a	–	11 ^a
4	4	10 ^a	2.3	10 ^a	17	11 ^a
5	4	6 ^b	0.9	10.5 ^a	15.0	11 ^a
6	6	4.5	1.5	8.5	12.8	11 ^a

^a $P < 0.005$ (compared with controls).

^b $P < 0.05$ (compared with controls).



In-vitro susceptibility testing of *Aspergillus fumigatus*

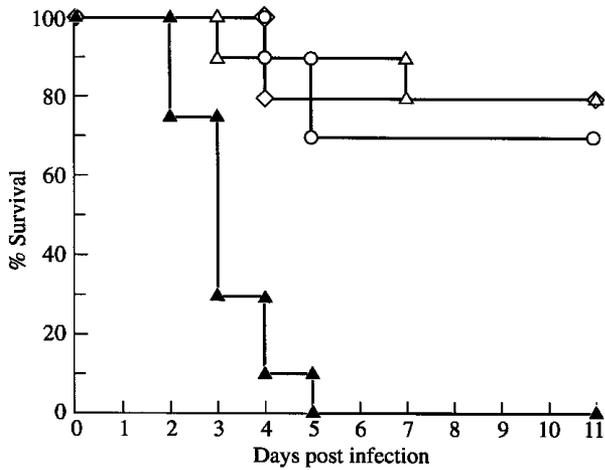


Figure 6. Survival curves for temporarily neutropenic mice infected with strain 1 and treated with itraconazole and amphotericin B. ▲, Dextrose; ○, itraconazole 25 mg/kg; ◇, itraconazole 75 mg/kg; △, amphotericin B 5.0 mg/kg.

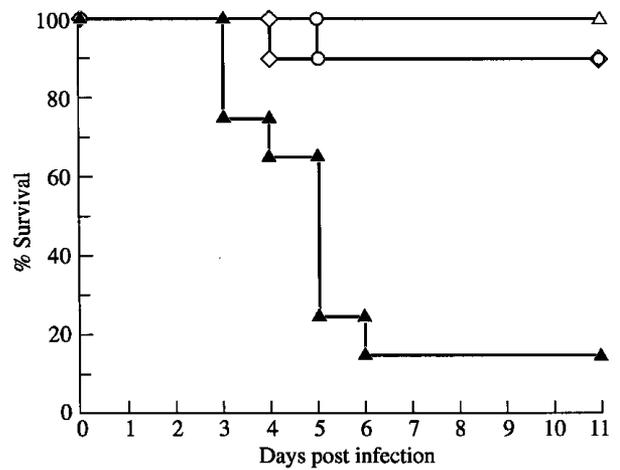


Figure 8. Survival curves for temporarily neutropenic mice infected with strain 3 and treated with itraconazole and amphotericin B. Legend as in Figure 6.

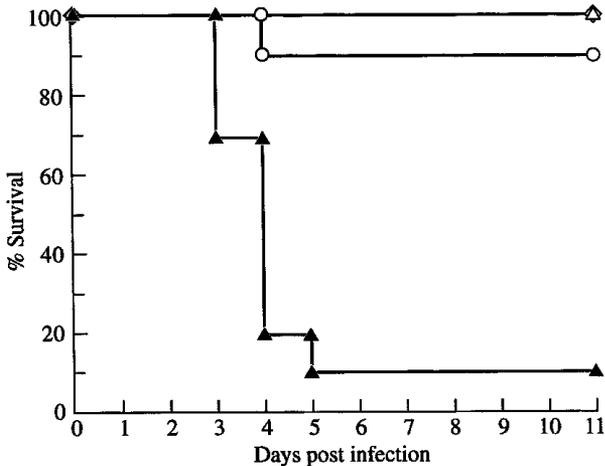


Figure 7. Survival curves for temporarily neutropenic mice infected with strain 2 and treated with itraconazole and amphotericin B. Legend as in Figure 6.

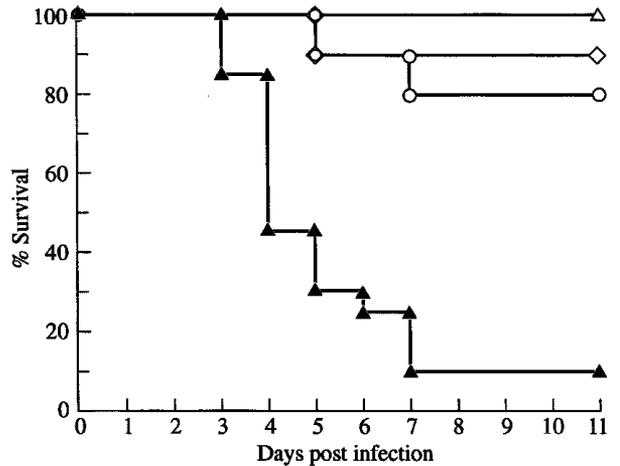


Figure 9. Survival curves for temporarily neutropenic mice infected with strain 4 and treated with itraconazole and amphotericin B. Legend as in Figure 6.

Both concentrations of itraconazole significantly reduced lung and kidney burdens compared with controls ($P \leq 0.005$) (Table III). Itraconazole at 75 mg/kg was significantly better than at 25 mg/kg ($P \leq 0.05$) in reducing fungal load in the kidneys. Treatment with amphotericin B at 5.0 mg/kg produced the greatest reduction in lung fungal burden and a significant reduction in kidney fungal burden ($P < 0.005$) compared with untreated mice. Mean serum concentrations of itraconazole were 1.6 mg/L in the low

dose itraconazole group and about ten-fold higher in the high dose itraconazole group (17.5 mg/L) (Table II).

Strain 2, AF71. The survival of mice infected with strain 2 is shown in Figure 7. In the control groups all mice given dextrose intraperitoneally died and only 20% survived in the dextrose gavage group. All mice given itraconazole at 75 mg/kg survived, as did 90% of those given itraconazole 25 mg/kg. Survival rates for treated groups were greater

Figure 5. Variation of itraconazole MICs for six strains of *A. fumigatus* with incubation temperature, duration of incubation and test medium with the microbroth method at an inoculum concentration of 10^6 conidia/mL. (a) SAB broth, 24 h; (b) SAB broth, 48 h; (c) M3G broth, 24 h; (d) M3G broth, 48 h; (e) RPMI broth, 24 h; (f) RPMI broth, 48 h; (g) RPMIG broth, 24 h; (h) RPMIG broth, 48 h. ▼, 28°C; ▲, 35°C. Each point is the mean of three values. Asterisk denotes mean MIC values where one of the three values is \geq four-fold doubling dilution higher than the other two values.

than for control groups ($P < 0.005$) for both doses of itraconazole. Amphotericin B also protected all mice from death and survival was significantly better than that of control mice ($P < 0.005$).

Both concentrations of itraconazole lowered fungal burdens in the lungs and kidneys compared with untreated mice ($P \leq 0.005$) (Table III). Amphotericin B reduced fungal infection in both the lungs and the kidneys in comparison with control mice ($P < 0.005$). Amphotericin B was superior at reducing fungal growth in the lungs (but not kidneys) compared with itraconazole at 25 mg/kg ($P \leq 0.05$), but not itraconazole at 75 mg/kg. Serum concentrations of itraconazole were higher in the itraconazole 75 mg/kg group (12.4 mg/L) compared with the itraconazole 25 mg/kg group (1.5 mg/L) (Table II).

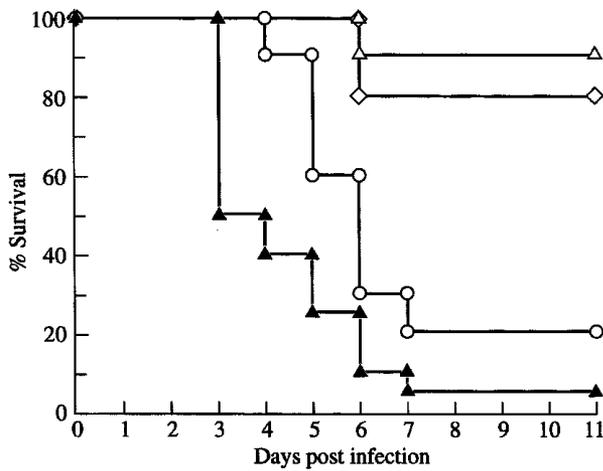


Figure 10. Survival curves for temporarily neutropenic mice infected with strain 5 and treated with itraconazole and amphotericin B. Legend as in Figure 6.

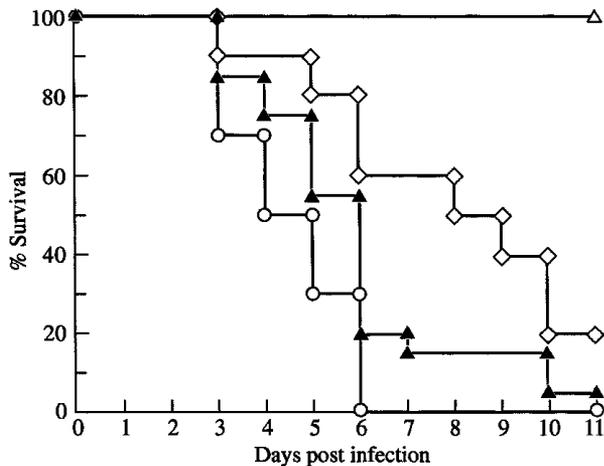


Figure 11. Survival curves for temporarily neutropenic mice infected with strain 6 and treated with itraconazole and amphotericin B. Legend as in Figure 6.

Table III. Quantitative organ culture results from in-vivo experiments with the six *A. fumigatus* strains

Strain	Controls		Itraconazole 25 mg/kg		Itraconazole 75 mg/kg		Amphotericin B 5.0 mg/kg	
	survivors/ no. in group	lungs mean log cfu (s.d.) $\times 10^3$	kidneys mean log cfu (s.d.) $\times 10^3$	lungs mean log cfu (s.d.) $\times 10^3$	kidneys mean log cfu (s.d.) $\times 10^3$	lungs mean log cfu (s.d.) $\times 10^3$	kidneys mean log cfu (s.d.) $\times 10^3$	
1	0/20	100 (0)	114 (0)	23.4 ^a (44.7)	12.6 ^a (46.3)	1.08 ^a (46.7)	0.58 ^a (40.7)	7.4 ^a (50.4)
2	2/20	74.7 (30.1)	81.2 (24.4)	3.66 ^a (50.4)	2.04 ^a (45.4)	3.8 ^a (41.6)	0.06 ^a (31.3)	0.57 ^a (30.6)
3	3/20	44.3 (32.6)	71.4 (34.4)	0.13 ^a (31.4)	0.24 ^a (33.1)	0.56 ^a (32.6)	0.20 ^a (31.1)	0.65 ^a (30.8)
4	2/20	78.0 (28.1)	77.9 (28.0)	12.5 ^a (43.2)	12.1 ^a (38.6)	2.8 ^a (41.1)	5.3 ^a (36.6)	11.8 ^a (42.5)
5	1/20	82.2 (16.9)	46.1 (8.9)	100 (0)	50 (0)	23.8 ^b (19.6)	9.2 ^a (43.0)	5.04 ^a (19.4)
6	1/20	1.1 (0.3)	44.7 (7.8)	1.7 (0)	48.0 (0)	36.6 (14.1)	0.09 ^a (0.5)	0.19 ^a (0.6)

^a $P < 0.005$ (compared with controls).
^b $P < 0.01$ (compared with controls).

Strain 3, AF210. Figure 8 demonstrates the survival of mice infected with strain 3. Overall 85% of control mice died. In comparison, 90% of mice given itraconazole at both doses survived. No mice died if treated with amphotericin B. Both doses of itraconazole therefore significantly prolonged survival of the mice ($P \leq 0.005$) compared with untreated mice, as did amphotericin B ($P < 0.005$).

Mice treated with itraconazole at 25 and 75 mg/kg had significantly lower fungal burdens in both the kidneys and the lungs ($P \leq 0.005$) compared with controls (Table III). The higher dose of itraconazole was inferior to the lower dose in reducing burdens in the lungs ($P < 0.005$). Amphotericin B treatment was superior in reducing fungal infection in both organs compared with controls ($P \leq 0.005$) as well as being significantly better at reducing fungal load in the lungs compared with the highest dose of itraconazole ($P \leq 0.05$). The serum concentration of itraconazole in the 25 mg/kg group was higher than for most other experiments at this dose level (4.3 mg/L) although unfortunately blood was unobtainable from the 75 mg/kg group.

Strain 4, AF294. Figure 9 illustrates the survival of mice infected with strain 4. Eighty per cent of the dextrose gavage group died as did all mice in the dextrose ip group. In the treatment groups 80% of mice given itraconazole at 25 mg/kg survived as did 90% of mice given 75 mg/kg. Both doses of itraconazole significantly prolonged survival compared with controls ($P \leq 0.005$). No mice died in the amphotericin B group.

Infected mice treated with itraconazole at both doses had significantly reduced fungal burdens in both lungs and kidneys compared with those receiving no therapy ($P < 0.005$), as did those treated with amphotericin B ($P \leq 0.005$ to ≤ 0.01) (Table III). Serum concentrations of itraconazole were 2.3 and 17 mg/L respectively for the low and high dose itraconazole groups.

Strain 5, AF72. Figure 10 shows the survival of mice infected with strain 5. Ninety-five per cent of control mice died. Survival for every treatment group was significantly better than that for mice receiving no treatment; for itraconazole at 25 mg/kg, $P \leq 0.05$, for itraconazole at 75 mg/kg, $P \leq 0.005$, (50% survival) and for amphotericin B, $P \leq 0.005$, (90% survival). The higher dose of itraconazole was significantly better at prolonging survival than the lower dose ($P \leq 0.01$). Only 10% of the mice treated with amphotericin B died, which was significantly better than for the low dose of itraconazole ($P \leq 0.005$). However, the serum concentration of itraconazole for the lower dose group was only 0.9 mg/L compared with 15 mg/L in the higher dose group.

Infected mice treated with itraconazole at 75 mg/kg had a significantly reduced fungal burden in the lungs and kidneys compared with those that were untreated ($P \leq 0.005$) (Table III). Treatment of infection with this particular strain with itraconazole at 25 mg/kg did not significantly

reduce the fungal growth in either the lungs or the kidneys in comparison with the controls. Amphotericin B was significantly better in reducing burdens in both kidneys and lungs compared with controls ($P \leq 0.005$) and the low dose itraconazole group ($P \leq 0.005$).

Strain 6, AF91. Figure 11 shows the survival of mice when infected with strain 6. Ninety-five per cent of the mice receiving no treatment died, as did all mice treated with low dose itraconazole (25 mg/kg) and 80% of those given high dose treatment (75 mg/kg). Itraconazole therapy did not significantly prolong survival over controls. No mice treated with amphotericin B died, which was significantly better than for no therapy and both itraconazole dosage groups ($P < 0.005$). Serum concentrations of itraconazole were 1.5 and 12.8 mg/L for the low and high dose itraconazole groups, respectively.

Mice infected with strain 6 and treated with itraconazole at 25 mg/kg and 75 mg/kg did not have a significant reduction in fungal growth in either the lungs or the kidneys compared with untreated mice (Table III). Treatment with amphotericin B was better at reducing fungal load compared with both doses of itraconazole and the controls ($P \leq 0.005$).

Discussion

The principal objective of this study was to develop one or more standardized methods of in-vitro susceptibility testing of *A. fumigatus*. A set of six strains was selected on the basis of therapeutic outcome in patients; two were judged to be resistant, having been isolated during itraconazole therapy from patients who had 'therapeutic' serum concentrations of the drug. The clinical impression of resistance of these two strains and susceptibility to itraconazole of a further four strains was confirmed in a murine model of invasive aspergillosis. We conducted in-vitro tests with five media each in an agar dilution and broth microdilution format and varied many other parameters including temperature, inoculum concentration and test duration. We were able to identify in-vitro test conditions that consistently differentiated the two resistant strains from the four susceptible ones. This study differs from previous work where there has been no attempt to correlate in-vitro testing with clinical outcome.^{21,22,32}

In this study, strain 6 was shown to be highly resistant to itraconazole. Previous work on this strain indicated that the mechanism of resistance may be a mutation of the target 14 α -demethylase enzyme.²³ Under all in-vitro conditions tested this strain had high MICs either at or approaching the highest drug concentration tested. In the murine model itraconazole, at both concentrations tested, demonstrated no benefit over untreated controls in prolonging survival or reducing aspergillus counts in the lungs and kidneys.

Strain 5 also exhibited a degree of resistance to itraconazole. Markedly lower intracellular itraconazole concentrations have been found in this strain.²³ Overexpression of an efflux pump, as has been described in *Candida* spp. and *Aspergillus nidulans*,^{36,37} may be the mechanism of resistance, although this has yet to be confirmed. It is interesting that the mechanism of resistance of this strain differs from that seen in strain 6, and this could account for differences observed in susceptibility to itraconazole. The results of in-vitro susceptibility tests with strain 5 were profoundly influenced by the test conditions, most notably inoculum concentration and medium. This was probably partly due to the poor growth rate of this strain, which made interpretation of endpoints more difficult. In our murine model of aspergillosis this strain yielded only partially reproducible results, which may be attributable to variable itraconazole blood concentrations. In all experiments itraconazole at either dose was less effective therapeutically than amphotericin B whereas with the four susceptible strains these two drugs were essentially equivalent in efficacy.

There have been few attempts to investigate the effect of test conditions on the results of in-vitro susceptibility tests with moulds. Numerous methods have been applied often with very different results. Most published work has shown itraconazole to be more active *in vitro* than ketoconazole or fluconazole,¹⁹ a finding that correlates with greater efficacy in animal models of infection and clinical data. This alone would suggest that susceptibility testing could yield useful results. However, extrapolating from a population of isolates with greater or lesser MICs to predicting clinical outcome for an individual isolate is fraught with uncertainty.

Many methods of endpoint determination have been utilized in antifungal susceptibility testing, including no growth, reduction to trace growth and marked reduction in growth as visual endpoints.^{38,39} In addition, spectrophotometric measurement of reductions in growth to 20, 50 or 70% of control growth^{28,40,41} have been used. Two colorimetric endpoints have been tried.⁴²

Our observations of patterns of growth in microtitre wells (which include surface growth, growth up the side of the well, small clumps of growth suspended in the medium, and growth adherent to the bottom of the well) lead us to suggest that spectrophotometric endpoints are inaccurate and that a visual endpoint is preferable. As part of our continuing studies of inter-laboratory reproducibility we will examine issues of the optimal endpoint in a large collection of isolates rather than simply the six strains used in this study.

This study suggests that either an agar dilution method with RPMI medium or a broth microdilution method with RPMI medium with added glucose (Table IV) can be used to detect itraconazole resistance in *A. fumigatus*. We anticipate that these test conditions would also be suitable for testing other azole drugs and examining other *Aspergillus* spp. and pathogenic moulds from closely related genera. The inter-laboratory reproducibility is currently being evaluated. We are unable to speculate on breakpoints for clinical reporting; to establish these will require a larger number of clinical isolates with varying degrees of resistance and known clinical outcome (including serum itraconazole concentrations). The data presented here do not establish the frequency of itraconazole resistance in *Aspergillus* spp. and we cannot predict whether this is likely to become a significant clinical problem. Many more isolates will have to be tested to establish the frequency of this phenomenon.

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Table IV. Proposed methods for susceptibility testing of *Aspergillus* spp. to itraconazole

	Agar dilution	Microtitre method
Medium	RPMI agar with 0.03% L-glutamine	RPMI-1640 with L-glutamine, NaHCO ₃ , and 2% glucose buffered with MOPS to pH 7.0
Inoculum	Multipoint inoculation from 10 ⁶ to 10 ⁷ conidia/mL	10 ⁵ conidia (100 µL)
Itraconazole dilution series	0.03–64 mg/L	0.06–16 mg/L
Temperature	28 or 35°C	35°C
Duration	48–72 h	48 h
Endpoint determination	Visual, no growth	Visual, no growth

MOPS, 3-(*N*-morpholino) propanesulphonic acid.

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