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Effect of pH on the In Vitro Activities of Amphotericin B, Itraconazole, and Flucytosine against *Aspergillus* Isolates

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The in vitro susceptibilities of 21 *Aspergillus* isolates were tested against three antifungal agents in RPMI 1640 and yeast nitrogen base at pH 5.0 and 7.0 by a broth microdilution format of the NCCLS method. The MICs of amphotericin B and itraconazole were higher, while those of flucytosine were lower, at pH 5.0 than at pH 7.0. The poor correlation between in vitro results and clinical outcome could be due to a difference in pH between the in vitro susceptibility test and at the site of infection.

In vitro susceptibility testing of filamentous fungi has become increasingly important. A good standard in vitro susceptibility test must give reproducible results, predict the resistance of molds, and correlate with clinical outcome (5).

In 1998 the National Committee for Clinical Laboratory Standards (NCCLS) proposed a standard method for the determination of the in vitro antifungal susceptibility of conidium-forming filamentous fungi. This document is now approved (3). Although the standardized NCCLS method has been found to give better inter- and intracenter reproducibility, in vitro antifungal susceptibility testing of filamentous fungi is still faced with several problems such as the correlation of in vitro results with clinical outcome.

Clinical outcome may be affected by various factors related to the host, the drugs, the fungus, and their interactions (9). These factors are not taken into account in the in vitro tests, and it may be for this reason that the prediction of antifungal efficacy or failure from in vitro susceptibility tests remains difficult. One of the host-related-factors is the pH at the site of infection. In the human body the pH is carefully regulated at 7.4, but it may be lower at the site of infection due to necrosis (7), the production of organic acids by fungi (6), or lysosome activity of granulocytes and macrophages (1).

The aim of this study was to investigate the effect of pH on the in vitro activities of three different antifungal agents against 21 *Aspergillus* isolates in two different media.

Twenty-one clinical *Aspergillus* isolates were tested: five itraconazole (ITZ)-susceptible and five ITZ-resistant *Aspergillus fumigatus* isolates, five *A. flavus* isolates, and six *A. terreus* isolates. Isolates had been frozen in glycerol broth at −80°C; they were revived by subculturing twice on Sabouraud glucose agar tubes supplemented with 0.5% chloramphenicol and were incubated for 5 to 7 days at 35°C. All isolates were tested in duplicate on different days.

Candida parapsilosis ATCC 22019 and *C. krusei* ATCC 6258 were used for quality control. Amphotericin B (AMB; Bristol-Myers Squibb, Woerden, The Netherlands), ITZ (Janssen Pharmaceutica B.V., Tilburg, The Netherlands), and flucytosine (5FC; ICN Pharmaceuticals, Zoetermeer, The Netherlands) were obtained as powders. AMB and ITZ were dissolved in dimethyl sulfoxide, and 5FC was dissolved in distilled water. The final concentrations of the antifungal agents at pH 5.0 ranged from 256 to 0.25 μg/ml for AMB, from 16 to 0.016 μg/ml for ITZ, and from 1,024 to 0.001 μg/ml for 5FC in both yeast nitrogen base (YNB) and RPMI 1640. At pH 7.0 the final concentrations ranged from 16 to 0.016 μg/ml for AMB and ITZ and from 1,024 to 0.001 μg/ml for 5FC in YNB, and in RPMI 1640 they ranged from 16 to 0.016 μg/ml for AMB and ITZ and from 256 to 0.25 μg/ml for 5FC.

Drug dilutions were made in RPMI 1640 medium with L-glutamine but without bicarbonate (GIBCO BRL, Life Technologies, Woerden, The Netherlands) and in YNB (DIFCO Laboratories, Sparks, Md.). Both media were prepared according to the manufacturers' instructions and buffered to pH 5.0 with 10 mM citrate buffer or to pH 7.0 with 0.165 M 3-[N-morpholino]propanesulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Susceptibility was tested by a broth microdilution method according to NCCLS guidelines (M38-A) (3). Aliquots (100 μl) of the drugs at twice the targeted final concentration were dispensed in the wells of flat-bottom 96-well microtiter plates (Costar, Corning, N.Y.). Conidial suspensions were prepared spectrophotometrically and were further diluted in medium. In order to obtain a final inoculum concentration of 0.4×10^4 to 5×10^4 CFU/ml, 100 μl of the inoculum was added to the wells. The microtiter plates were incubated at 35°C for 48 h.

After 48 h, growth was assessed spectrophotometrically with a microplate reader (Anthos htIII; Anthos Labtec Instruments, Salzburg, Austria) at 405 nm. For AMB and ITZ the lowest

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concentration that showed no growth in comparison to that of the growth control (MIC-0), and for 5FC the lowest concentration that showed no more than 50% growth in comparison with that of the growth control (MIC-2), was taken as the MIC end point (3).

Results were analyzed by the Mann-Whitney test, and differences were considered statistically significant at a P value of <0.01.

At pH 7.0 the susceptibilities of the two quality control strains against the three tested antifungal agents were within the recommended limits of the NCCLS in both media. At pH 5.0, however, the MICs of both AMB and ITZ were higher than those at pH 7.0, whereas the MICs of 5FC were lower. This result was independent of the medium used.

Table 1 shows the MICs of all three antifungal agents in both media at pH 5.0 and 7.0. There was a significant difference between the MICs found in RPMI 1640 versus YNB when testing was conducted at either pH 5.0 or 7.0. In most cases the MICs found in YNB were higher than those in RPMI 1640. There was also a significant difference between the MICs found at pH 5.0 versus 7.0 for both AMB and 5FC for all species tested. For ITZ there was a significant difference for some data sets of the ITZ-susceptible *A. fumigatus* isolates, the *A. flavus* isolates, and the *A. terreus* isolates. No significant difference was found for the ITZ-resistant *A. fumigatus* isolates. The difference was independent of the medium used but dependent on the antifungal agent tested. Figure 1 shows the effect of pH on the in vitro activities of AMB, ITZ, and 5FC against an *A. flavus* isolate in both media. In general, the in vitro activities of both AMB and ITZ decreased when the medium pH was lowered, while for 5FC the in vitro activity increased when the medium pH was lowered. Dramatic changes in the MIC were observed for both AMB and 5FC. For AMB, MICs below 1 μg/ml at pH 7.0 increased to >8 μg/ml at pH 5.0, above the concentrations achievable in vivo. The reverse was found for 5FC, where for most isolates high MICs at pH 7.0 converted to very low MICs at pH 5.0, below concentrations achievable in vivo.
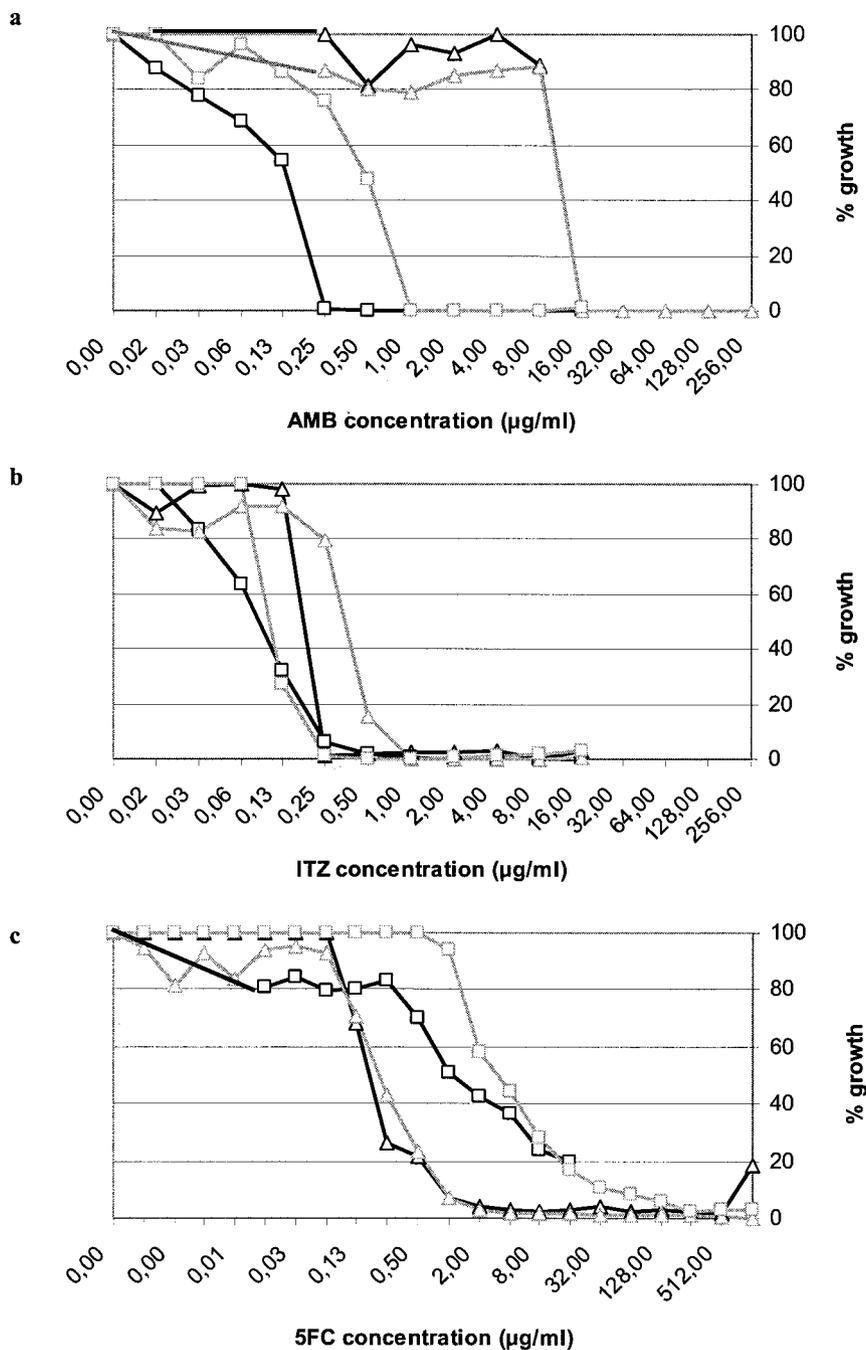
These results were in agreement with results found in a limited number of other studies in which the effect of pH on antifungal activity was also investigated. Both Minagawa et al. (2) and Odds et al. (4) showed that the in vitro activity of the imidazole ketoconazole against yeast isolates decreased when the medium pH decreased. Odds et al. also showed that for yeast isolates the activity of AMB decreased and that of 5FC increased when the medium pH decreased. The increase in activity for 5FC was also found by Viviani et al. for *Cryptococcus neoformans* (8). The mechanism that causes pH-dependent change of activity of these antifungal agents is not clear and warrants further study.

Buffered medium at a pH of 7.0 has been accepted as the standard for in vitro susceptibility testing of microorganisms. However, it is not yet clear that this is the optimal pH with regard to clinical outcome. In a patient the pH at the site of a fungal infection may very well be lower than the natural body pH of 7.4 due to necrosis (7), the production of organic acids by the fungus (6), or lysosome activity of granulocytes and macrophages (1). For this reason, it may very well be that the in vitro antifungal activity found at pH 5.0 corresponds better with clinical outcome than the in vitro activity found at the standard pH of 7.0. Considering our

TABLE 1. MIC$_{50}$s of AMB, ITZ, and 5FC against 21 *Aspergillus* isolates determined in two different media at two different pH values

Species (n)	MIC$_{50}$[a] (range)											
	AMB				ITZ				5FC			
	RPMI 1640		YNB		RPMI 1640		YNB		RPMI 1640		YNB	
	pH 5	pH 7	pH 5	pH 7	pH 5	pH 7	pH 5	pH 7	pH 5	pH 7	pH 5	pH 7
A. fumigatus												
ITZ susceptible (5)	16 (16–32)	0.25ND (0.25)	32 (16–64)	1* (0.25–4)	0.5 (0.25–0.5)	0.25 (0.25–0.5)	1 (0.5–2)	0.5* (0.125–1)	0.063 (0.031–0.25)	16* (2–128)	0.063 (0.016–0.25)	16* (1–64)
ITZ resistant (5)	16 (8–64)	0.25* (0.125–1)	32 (16–32)	0.75* (0.5–4)	>16 (>16)	>16 (>16)	>16 (>16)	>16 (>16)	0.188 (0.063–0.5)	24* (2->256)	1 (0.125–1)	48* (8–256)
A. flavus (5)	16 (16–64)	0.5* (0.25–0.5)	32 (16–64)	2* (1–2)	0.5 (0.25–0.5)	0.25* (0.25–0.5)	1 (1)	0.5ND (0.25–0.5)	0.25 (0.125–0.5)	1.5* (0.25–2)	0.25 (0.125–0.5)	3* (2–4)
A. terreus (6)	16 (8–64)	0.75* (0.5–2)	32 (32–128)	3* (2–4)	0.5 (0.25–0.5)	0.125* (0.062–0.25)	1 (0.5–1)	0.25ND (0.25)	0.063 (0.063–0.125)	2* (0.5->256)	0.25 (0.063–1)	4* (1–64)
All isolates (21)	**16 (8–64)**	**0.5 (0.125–2)**	**32 (16–128)**	**2 (0.25–4)**	**0.5 (0.25->16)**	**0.25 (0.062->16)**	**1 (0.5->16)**	**0.5 (0.125->16)**	**0.125 (0.031–0.5)**	**4 (0.25->256)**	**0.25 (0.016–1)**	**8 (1–256)**

[a] In micrograms per milliliter. MIC$_{50}$, MIC at which 50% of the isolates tested were inhibited. MICs of AMB and ITZ were read at the lowest concentration that showed no growth in comparison with that of the growth control (MIC-0). MICs of 5FC were read at the lowest concentration that showed no more than 50% growth in comparison with that of the growth control (MIC-2). Asterisks indicate a statistically significant difference between MICs found at pH 5.0 and 7.0 ($P < 0.01$). ND, P value could not be determined, because a standard deviation of zero was found.

a

b

c

FIG. 1. Effects of pH on the in vitro activities of AMB (a), ITZ (b), and 5FC (c) against an *A. flavus* isolate in either RPMI 1640 (black lines) or YNB (grey lines). Squares, pH 7.0; triangles, pH 5.0.

results, this means that for the treatment of invasive fungal infections it would be better to use 5FC or ITZ instead of AMB, because both of the former drugs show better in vitro activities at pH 5.0 than AMB. Further in vivo studies are necessary in order to determine the pH at the site of infection and to correlate in vitro results with in vivo response.

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