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Nationwide Survey of In Vitro Activities of Itraconazole and Voriconazole against Clinical *Aspergillus fumigatus* Isolates Cultured between 1945 and 1998

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The isolates in a collection of 170 *Aspergillus fumigatus* isolates recovered from 114 patients and 21 different medical centers in The Netherlands over a period of 53 years were tested for the presence of resistance to itraconazole and voriconazole according to the guidelines of NCCLS document M38-P and by the E-test. Three isolates were highly resistant to itraconazole, and voriconazole MICs were low for all isolates.

Over the past few decades the incidence of invasive fungal infections has increased, especially those caused by *Aspergillus* species (11, 16). The treatment of choice for infected patients remains amphotericin B, although alternative drugs with activities against *Aspergillus* species are becoming available for clinical use, including the antifungal azoles itraconazole and voriconazole. Itraconazole is highly active against *Aspergillus* species but has been used almost exclusively as follow-up therapy in patients with invasive aspergillosis. However, with the availability of a new intravenous formulation of itraconazole and an oral solution, first-line therapy may become an option (1). Voriconazole is active against a wide range of filamentous molds including *Aspergillus* species. The drug can be administered both intravenously and orally and was found to be superior to amphotericin B as first-line therapy for the treatment of invasive aspergillosis (R. Herbrecht, D. W. Denning, T. F. Patterson, W. V. Kern, K. A. Marr, D. Caillot, E. Thiel, P. Ribaud, O. Lortholary, R. Greene, C. Durand, J. W. Oestmann, P. S. Stark, R. Sylvester, P. F. Troke, H. Schlamm, J. R. Wingard, R. H. Rubin, B. De Pauw, and J. E. Bennett, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 680, 2001).

Although testing of the susceptibilities of *Aspergillus fumigatus* isolates to amphotericin B in vitro is problematic, resistance to azoles can be detected (3, 5,6). The in vitro resistance of *A. fumigatus* to itraconazole was confirmed by use of animal experimental models (3, 5). However, the prevalence of resistance to itraconazole and voriconazole among *A. fumigatus* isolates is unknown. In the present study we investigated the prevalence of resistance among *A. fumigatus* isolates that had been cultured from patients admitted to hospitals throughout The Netherlands.

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Medical microbiology laboratories in The Netherlands were asked to send *A. fumigatus* isolates that had been cultured from clinical samples at their hospitals. In addition, the database of the collection of the Centraalbureau voor Schimmelcultures (CBS) was searched for *A. fumigatus* isolates that had been cultured from clinical specimens from Dutch patients. The isolates were subcultured onto Sabouraud glucose agar plates supplemented with 0.5% chloramphenicol; the plates were incubated at 29°C for 7 days, and the identifications were confirmed. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls. In addition, an itraconazole-susceptible *A. fumigatus* isolate (isolate AF71; MIC, 0.25 μg/ml) and an itraconazole-resistant *A. fumigatus* isolate (isolate AF91; MIC, >64 μg/ml) were included in each test (5).

Reference-grade lots of itraconazole (Janssen-Cilag, Tilburg, The Netherlands) and voriconazole (Pfizer Central Research, Sandwich, United Kingdom) were obtained from the manufacturers. Itraconazole Etest strips with concentrations ranging from 0.004 to 32 μg/ml were kindly provided by the manufacturer (AB Biodisk, Solna, Sweden).

Broth microdilution testing was performed essentially as described by the NCCLS in document M38-P (13). Twofold drug dilutions were prepared by making serial dilutions in RPMI 1640 medium (with L-glutamine and without bicarbonate; GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The final concentrations of both antifungal drugs ranged from 0.03 to 32 μg/ml.

A. fumigatus spores were harvested with a cotton stick for preparation of the inoculum. After heavy particles were allowed to settle, the density of the upper suspension was estimated by measuring the turbidities at 530 nm with a spectrophotometer (Spectronic 20D; Milton Roy, Rochester, N.Y.) (7). Spore suspensions were adjusted to an inoculum with a transmittance range of 80 to 82% (0.4×10^6 to 3.2×10^6

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CFU/ml). The adjusted suspension was diluted 1:50 with RPMI 1640 medium to obtain a twofold dilution of the test inoculum.

The twofold drug concentrations were dispensed in sterile, 96-well flat-bottom microtitration plates on the day of the test. Each well was inoculated with 100 μl of a suspension of the twofold dilution of the conidial test inoculum. The trays were incubated at 35°C and examined after 24 and 48 h of incubation. The growth was assessed by visual observation with the aid of a concave mirror. The growth was scaled according to the guideline in NCCLS document M38-P (13).

The in vitro activity of itraconazole against 125 *A. fumigatus* isolates was determined by the Etest according to the instructions of the manufacturer. The plates were incubated in plastic bags at 35°C, and the MICs were read after 24 and 48 h.

For the broth microdilution method, the MIC endpoint was determined as the lowest drug concentration that resulted in an approximately 75% or greater reduction in growth (MIC-1) (8). Furthermore, the MIC for each isolate was compared with that for the strain resistant to itraconazole. For the Etest, the MIC was defined as the location where the inhibition ellipse intersected with the MIC scale on the strip.

A total of 930 MICs for the 170 clinical *A. fumigatus* isolates were obtained and analyzed. Two readings (at 24 and 48 h) of the MIC of each drug for each isolate were obtained by both methods. Because the Etest strips contain a continuous gradient of itraconazole instead of the established twofold drug dilutions, the MIC endpoint obtained by the Etest was elevated to the next twofold dilution concentration which matched the drug dilution in the schema used for the broth microdilution method to facilitate comparisons. Agreement occurred when the MIC results by the Etest and broth microdilution method were in exact agreement or were within ±1 twofold dilution. Comparisons were made by the Mann-Whitney test. Differences were considered significant if P was <0.05.

The collection of 170 *A. fumigatus* isolates included 54 isolates that were obtained from CBS. These isolates had been cultured from 54 Dutch patients between 1945 and 1990, but most of the isolates were cultured between 1961 and 1970. The origins of 19 of the CBS isolates were not known; these isolates had been cultured from patients at 10 different hospitals in The Netherlands. The remaining 116 *A. fumigatus* isolates were obtained from 11 hospitals in The Netherlands, including 5 tertiary care centers. These isolates were cultured from 95 patients after 1990. Overall, the collection represented *A. fumigtus* isolates cultured over a period of 53 years (1945 to 1998) from specimens from 114 patients at 21 different hospitals in The Netherlands.

The in vitro activities of voriconazole and itraconazole are summarized in Table 1. An excellent correlation between the readings obtained at 24 and those obtained at 48 h was observed. Overall, voriconazole appeared to be more active than itraconazole against these isolates (Table 1). High voriconazole MICs were not observed for any of the isolates. Itraconazole showed no in vitro activity against three *A. fumigatus* isolates (isolates AZG05, AZG06, and AZG07). These itraconazole-resistant isolates were recovered from respiratory secretions from a lung transplant recipient who developed respiratory tract colonization with *A. fumigatus* posttransplantation. Treatment with itraconazole was given for several months, and during this period cultures of sputum and bronchoalveolar lavage fluid remained positive. Voriconazole was active in vitro against these itraconazole-resistant isolates. The MICs for isolates ($n = 51$) cultured before the introduction of itraconazole for clinical use in 1990 were not significantly different from those for isolates ($n = 119$) cultured after 1990 ($P = 0.65$).

TABLE 1. Comparison of itraconazole and voriconazole MICs for 170 *A. fumigatus* isolates

Antifungal agent	Test method[a]	MIC (μg/ml)[b]			
		Geometric mean	Range	50%	90%
Itraconazole	Broth micro (24 h)	1	0.5–64	1	1
	Broth micro (48 h)	1	0.5–64	1	1
	Etest (24 h)	0.6	0.03–64	0.5	0.75
	Etest (48 h)	0.7	0.13–64	0.5	0.75
Voriconazole	Broth micro (24 h)	0.17	0.06–0.5	0.13	0.25
	Broth micro (48 h)	0.25	0.06–1	0.25	0.5

[a] Broth micro, broth microdilution method. The results of both tests were read after 24 or 48 h.
[b] 50% and 90%, MICs at which 50 and 90% of isolates, respectively, are inhibited.

Excellent correlations between the results of the Etest and those of the NCCLS broth microdilution method were found at both 24 and 48 h, although two isolates showed insufficient growth at 24 h to allow reading of the MIC. The levels of agreement between the Etest and the broth microdilution method for itraconazole were 91.0 and 93.5% at 24 and 48 h, respectively. The itraconazole MICs for the three *A. fumigatus* isolates resistant to itraconazole were also high (MICs were >64 μg/ml) by the Etest.

Since the detection of itraconazole resistance in *A. fumigatus*, significant progress has been made in the establishment of reproducible in vitro test systems for the detection of resistance to antifungal azoles in molds (6, 10, 12). However, although large surveillance programs to monitor trends in resistance in bacteria and yeasts have been established (15), the prevalence of itraconazole resistance among *A. fumigatus* isolates is unknown. Previous studies with *A. fumigatus* isolates from a single center showed that 4 of 156 (2.5%) *A. fumigatus* isolates were highly resistant to itraconazole (MICs, >16 μg/mg) (2). We found that the prevalence of resistance to itraconazole among *A. fumigatus* isolates was low and was similar to that found in single medical centers (2). No itraconazole resistance was detected among isolates cultured before 1990, which indicates that primary resistance of *A. fumigatus* to itraconazole is uncommon or nonexistent. Furthermore, even after itraconazole became available for treatment, the prevalence of resistance to itraconazole remained low. However, at least two factors could bias these findings. First, it is unknown whether phenotypic resistance is preserved during storage. All isolates were stored for various periods of time, with some isolates stored for up to more than 50 years. Although CBS takes great care to preserve its isolates (17), the effect of cryopreservation or freezing-drying on phenotypic resistance is unknown. Although the mechanism of azole resistance in *A. fumigatus* has not been fully elucidated, reduced intracellular concentrations of itraconazole have been found in resistant isolates, and these concentrations may be mediated by efflux pumps (4, 5, 14). If resistance were lost during storage, the

prevalence of resistance that we detected would underestimate the true prevalence of resistance. Second, selection of the appropriate isolates for in vitro susceptibility testing is essential. The level of exposure to itraconazole of the *A. fumigatus* isolates that we tested is probably very low. Itraconazole is not used for prophylaxis in most high-risk patient groups in The Netherlands, and amphotericin B is considered first-line therapy for the treatment of invasive aspergillosis. Furthermore, in contrast to *Candida*, *Aspergillus* species are infrequently cultured from patients with invasive disease. Isolates are cultured from respiratory specimens either before specific antifungal treatment is initiated or at autopsy, after primary therapy with amphotericin B has failed. Nevertheless, among the isolates that we analyzed, three proved to be highly resistant in vitro. The in vitro results corresponded with failure of the patient to respond clinically to treatment with itraconazole. However, itraconazole-resistant *A. fumigatus* isolates have been recovered both from patients who were being treated with itraconazole and from patients who had not previously been exposed to the drug (2, 5), indicating that both primary and secondary resistance might exist.

Overall, the MICs of voriconazole were lower than those of itraconazole when standard RPMI 1640 medium and the MIC-1 endpoint were used. Resistance of *A. fumigatus* to voriconazole was not observed. Voriconazole MICs were low even for isolates for which itraconazole MICs were high and were similar to those for isolates for which itraconazole MICs were low. This is in accordance with the results of a recent study that evaluated two itraconazole-resistant *A. fumigatus* isolates (10) that were originally described by Denning et al. (5). The voriconazole MICs for the itraconazole-resistant isolates were similar to those for the itraconazole-susceptible isolates (10).

There is some debate regarding the optimal conditions for the detection of azole resistance in *Aspergillus* species. Although azole resistance was originally detected by using an optically clear endpoint (MIC-0) (6), other multicenter reproducibility studies indicated good reproducibilities when the MIC-1 endpoint (8) and the 50% growth inhibition (MIC-2) endpoint (9) were used. However, increased rates of interlaboratory agreement (90 to 92%) were recently found in a multicenter evaluation that used standard RPMI 1640 medium, incubation for 48 h, and complete growth inhibition as the endpoint (10). These conditions are now being considered for inclusion in a future version of NCCLS document M38-A (the document describing the approved method) (10). Awaiting further developments in this respect, we included in each assay itraconazole-susceptible isolate *A. fumigatus* AF71 and itraconazole-resistant *A. fumigatus* isolate AF91 described by Denning et al. (5) to ascertain whether itraconazole resistance could be detected. By using the MIC-1 endpoint, the MIC for *A. fumigatus* isolate AF91 was consistently ≥64 μg/mg.

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