ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1995, p. 1512–1516 Vol. 39, No. 7
0066-4804/95/$04.00+0

Patterns of In Vitro Activity of Itraconazole and Imidazole Antifungal Agents against *Candida albicans* with Decreased Susceptibility to Fluconazole from Spain

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Received 14 October 1994/Returned for modification 15 December 1994/Accepted 1 May 1995

Two groups of recent clinical isolates of *Candida albicans* consisting of 101 isolates for which fluconazole MICs were ≤0.5 μg/ml ($n = 50$) and ≥4.0 μg/ml ($n = 51$), respectively, were compared for their susceptibilities to fluconazole, clotrimazole, miconazole, ketoconazole, and itraconazole. Susceptibility tests were performed by a photometer-read broth microdilution method with an improved RPMI 1640 medium supplemented with 18 g of glucose per liter (RPMI–2% glucose; J. L. Rodríguez-Tudela and J. V. Martínez-Suárez, Antimicrob. Agents Chemother. 38:45–48, 1994). Preparation of drugs, basal medium, and inocula was done by the recommendations of the National Committee for Clinical Laboratory Standards. The MIC endpoint was calculated objectively from the turbidimetric data read at 24 h as the lowest drug concentration at which growth was just equal to or less than 20% of that in the positive control well (MIC 80%). In vitro susceptibility testing separated azole-susceptible strains from the strains with decreased susceptibilities to azoles if wide ranges of concentrations (20 doubling dilutions) were used for ketoconazole, miconazole, and clotrimazole. By comparison with isolates for which fluconazole MICs were ≤0.5 μg/ml, those isolates for which fluconazole MICs were ≥4.0 μg/ml were in general less susceptible to other azole drugs, but different patterns of decreased susceptibility were found, including uniform increases in the MICs of all azole derivatives, higher MICs of several azoles but not others, and elevated MICs of fluconazole only. On the other hand, decreased susceptibility to any other azole drug was never found among strains for which MICs of fluconazole were lower.

The azole-derivative antifungal agents include a large number of ergosterol synthesis inhibitors currently used to treat human fungal infections, including the imidazoles (clotrimazole [CZ], miconazole [MZ], ketoconazole [KZ], etc.) and the newer systemic triazoles (fluconazole [FZ] and itraconazole [IZ]) (11).

Secondary resistance to azole antifungal agents in *Candida albicans* first appeared in the 1980s in patients with chronic mucocutaneous candidiasis treated with KZ for very long periods of time (25, 33, 36). The isolates involved had markedly reduced susceptibilities to KZ and cross-resistance to other azole drugs. More recently, resistance to the triazole FZ has been described in *C. albicans* strains isolated from the oral cavities of AIDS patients in different parts of the world (2, 5–8, 15, 18, 22, 24, 25, 28, 29, 32, 34, 36). The absence in most cases of comparative susceptibility data for alternative azole antifungal agents in these isolates still leaves open the question of whether cross-resistance to azole drugs in *C. albicans* is a general phenomenon.

All of the azole antifungal agents share certain properties that makes in vitro susceptibility testing difficult, mainly partial inhibition of fungal growth that makes MIC endpoint determinations both very difficult and subjective (12, 29, 35). In the proposed standard of the National Committee for Clinical Laboratory Standards (NCCLS) (23), endpoints for azoles are determined visually at 48 h and the MIC is defined as the lowest drug concentration that reduces growth by 80% relative to the growth of the control (MIC 80%). We have previously shown that increased growth of *C. albicans* in RPMI 1640 medium supplemented with 18 g of glucose per liter (RPMI–2% glucose) compared with that in standard RPMI 1640 medium is an advantage which facilitates the reading of growth inhibition by azoles (30). As an alternative to the cumbersome macrodilution reference test (23), the microdilution method with RPMI–2% glucose and spectrophotometric MIC 80% calculation at 24 h is easier to perform and more objective, allowing similar results to be obtained by both methods (10, 27).

In the present study, we investigated the in vitro activities of five azole antifungal agents in current use against two groups of recent clinical isolates of *C. albicans* for which MICs of fluconazole were ≤0.5 and ≥4.0 μg/ml, respectively, by a broth microdilution adaptation of the proposed standard of NCCLS (23) with RPMI–2% glucose (30) and spectrophotometric MIC 80% endpoint calculation (9) at 24 h. The wide array of susceptibility patterns that we found reinforces the need for in vitro testing of azole drugs in cases of treatment failures in some clinical settings.

(Part of this work was presented at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, La., 17 to 20 October 1993 [20].)

MATERIALS AND METHODS

Assay medium. RPMI–2% glucose, an improved medium that facilitates the reading of growth inhibition by azole drugs (30), was used in all tests. The basal medium was RPMI 1640 powder with L-glutamine (Sigma Aldrich Química, S.A., Madrid, Spain) (23). Preweighed aliquots of 10.4 g of medium powder were mixed with 900 ml of deionized water buffered with morpholinepropanesulfonic acid (MOPS; Sigma Aldrich Química). The buffer solution was prepared with 34.53 g of MOPS per 900 ml of water (final molarity of 0.165 when the solution was completed to 1,000 ml). The solutions were mixed by using magnetic spin bars at room temperature and were titrated by using 10 M sodium hydroxide to pH 7.0. After filter sterilization by using 0.22-μm-pore-size filters, 100 ml of an

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TABLE 1. MIC 80% of five azole antifungal agents for *C. albicans* control strains

C. albicans strain	MIC (μg/ml)[a]				
	FZ	CZ	MZ	KZ	IZ
ATCC 90028	0.5	≤0.0002	≤0.0002	≤0.0002	0.06
ATCC 90029	0.25	≤0.0002	≤0.0002	≤0.0002	0.12
ATCC 64548	0.25	≤0.0002	≤0.0002	≤0.0002	0.12
ATCC 64550	32.0	32.0	1.0	0.25	2.0

[a] The MIC 80% was read spectrophotometrically at 24 h by using RPMI–2% glucose, as described in Materials and Methods.

autoclaved solution of glucose at 180 g/liter was carefully added. After stirring, solutions of RPMI–2% glucose were divided into aliquots and were stored at 4°C. One aliquot was used for sterility checks, retesting the pH (6.9 to 7.1 was considered acceptable), and as a growth control with a reference strain of *C. albicans* according to the results of a previous study (30).

Antifungal agents. FZ (Pfizer S.A., Madrid, Spain), KZ and MZ (both from Janssen Farmaceútica, S.A., Madrid, Spain), and CZ (Química Farmaceútica Bayer, S.A., Barcelona, Spain) were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma Aldrich Química) at a starting concentration of 12,800 μg/ml. IZ (Janssen Farmaceútica) was also dissolved in 100% DMSO, but at 800 μg/ml. All of these stock solutions were frozen at −70°C until use.

Susceptibility testing procedure. MICs were determined by a microdilution test described previously (27, 30), with minor modifications. Two different microtiter plates were prepared for each antifungal agent except IZ. For CZ, MZ, and KZ, the final concentrations, ranging from 128.0 to 0.0002 μg/ml in the two plates used for each agent, were obtained by 20 twofold serial dilutions. FZ was tested in two different ranges of concentrations, from 128.0 to 0.25 μg/ml and from 8.0 to 0.015 μg/ml, respectively, that were each separately obtained by 10 twofold serial dilutions; all strains were tested with the plate containing the low concentration and those for which the FZ MIC was ≥8.0 μg/ml were confirmed with the plate containing the high concentration. IZ was tested at concentrations ranging from 8.0 to 0.015 μg/ml. Starting inocula of 10^6 CFU/ml were prepared by the spectrophotometric method recommended by NCCLS (23). The adjusted yeast suspensions were diluted 1:10 with RPMI–2% glucose medium, and microtiter plates were inoculated with this dilution by using an automatic pipettor programmed to dispense 10 μl into each well to obtain final inocula of approximately 10^4 CFU/ml (9). The inoculated plates were incubated at 35°C for 24 h in a humid atmosphere. After agitation of the plates with a microtiter plate shaker for 5 min (1), spectrophotometric readings were performed with a Mios Merck automatic plate reader (Merck Igoda, S.A., Madrid, Spain) set at 405 nm. The MIC endpoint was calculated as the lowest drug concentration giving rise to an inhibition of growth equal or greater than 80% of that of the drug-free control (MIC 80%), similar to the visual endpoint criterion recommended by NCCLS (23). We have also used the 50% inhibitory concentration ($IC_{1/2}$) (14) as the MIC endpoint criterion for azole drugs (20, 27, 30, 32), but we have shown that $IC_{1/2}$ and MIC 80% are equivalent when RPMI–2% glucose is used as the antifungal testing medium (31).

Definition of decreased susceptibility to FZ. In the case of FZ, we (32) have proposed 16.0 μg/ml as the breakpoint for resistance in strains causing oropharyngeal candidiasis in AIDS patients receiving standard doses of the drug. Other investigators have proposed similar breakpoints, that is, between 12.5 μg/ml (2, 36) and 32.0 μg/ml (22, 28). Nevertheless, we chose isolates for which FZ MICs were ≤0.5 μg/ml as representing susceptible isolates and isolates for which FZ MICs were ≥4.0 μg/ml as representing isolates with decreased susceptibility in order to define two different groups of strains not overlapping with regard to their susceptibilities to FZ and also on the basis of our own data for the strains of *C. albicans* susceptible to FZ that were always susceptible to the other azole antifungal agents (see below). Changes in the MICs of different azoles could be better seen in strains for which FZ MICs were between 4.0 and 16.0 μg/ml that were clearly different from the susceptible strains, although they were not defined as resistant.

Organisms. One hundred one clinical isolates of *C. albicans* were tested. These were isolated from 101 individual patients at different Spanish hospitals and were sent to our laboratory for identification or antifungal susceptibility testing. All but one of the *C. albicans* strains with decreased susceptibility to FZ were isolated from human immunodeficiency virus-positive patients. These 51 isolates for which FZ MICs were greater came from 14 different centers in eight provinces across Spain, and some of them have been described previously (8, 10, 20, 32). Culture, identification, and preservation of strains were carried out by standard methods (4). Suitable susceptible control organisms included *C. albicans* ATCC 90028 (23), *C. albicans* ATCC 90029 (23), and *C. albicans* ATCC 64548 (1). One resistant strain was also used as a control in all susceptibility tests: *C. albicans* ATCC 64550 (*C. albicans* AD), which was clinically resistant to KZ; this resistance has been confirmed in vitro as well as in vivo (13, 17, 33).

RESULTS

Comparative activities of azole drugs against reference strains. Each time that a set of MICs was determined, one resistant (*C. albicans* ATCC 64550) plus one of the susceptible (*C. albicans* ATCC 90028, *C. albicans* ATCC 90029, or *C. albicans* ATCC 64548) control strains were included. The MICs of the five azole drugs tested for the four control strains are given in Table 1. Variability among consecutive determinations was found to fall within a twofold range (±1 dilution) for FZ and IZ with the four strains and for CZ, MZ, and KZ with *C. albicans* ATCC 64550. It is noteworthy that the wide range of concentrations used allowed us to differentiate the greater in vitro activities of the imidazole derivatives (CZ, MZ, and KZ) compared with those of the newer systemic triazoles (FZ and IZ). In comparison with susceptible control strains, *C. albicans* ATCC 64550 (13, 17, 33) displayed decreased susceptibility to all of the azoles tested (Table 1), but at a very different range of concentrations.

Comparative activities of azole drugs against clinical isolates of *C. albicans* for which FZ MICs were lower. The in vitro activities of five azole derivatives against 50 clinical isolates of *C. albicans* for which FZ MICs were ≤0.5 μg/ml are given in Table 2. As for the susceptible control strains, the MICs of CZ, MZ, and KZ were much more lower than those of FZ and IZ. By this test method, IZ showed a slightly greater in vitro activity than FZ against *C. albicans* isolates for which FZ MICs were lower. We did not find isolates of *C. albicans* within this group that showed decreased susceptibility to any other azole (Table 2). Our previous results with 236 *C. albicans* strains

TABLE 2. Susceptibilities to five azole antifungal agents of *C. albicans* isolates for which FZ MICs were low and high

Isolate	Agent	MIC (μg/ml)[a]		
		Range	50%	90%
C. albicans for which FZ MICs were ≤0.5 μg/ml (n = 50)	FZ	0.12–0.5	0.25	0.25
	CZ	≤0.0002–≤0.0002	≤0.0002	≤0.0002
	MZ	≤0.0002–≤0.0002	≤0.0002	≤0.0002
	KZ	≤0.0002–≤0.0002	≤0.0002	≤0.0002
	IZ	0.03–0.25	0.12	0.12
C. albicans for which FZ MICs were ≥4.0 μg/ml (n = 51)	FZ	4.0–>128.0	16.0	128.0
	CZ	≤0.0002–128.0	8.0	64.0
	MZ	≤0.0002–>128.0	1.0	8.0
	KZ	≤0.0002–4.0	0.06	0.5
	IZ	0.06–>8.0	0.5	2.0

[a] The MIC 80% was determined by the microdilution test described in Materials and Methods.

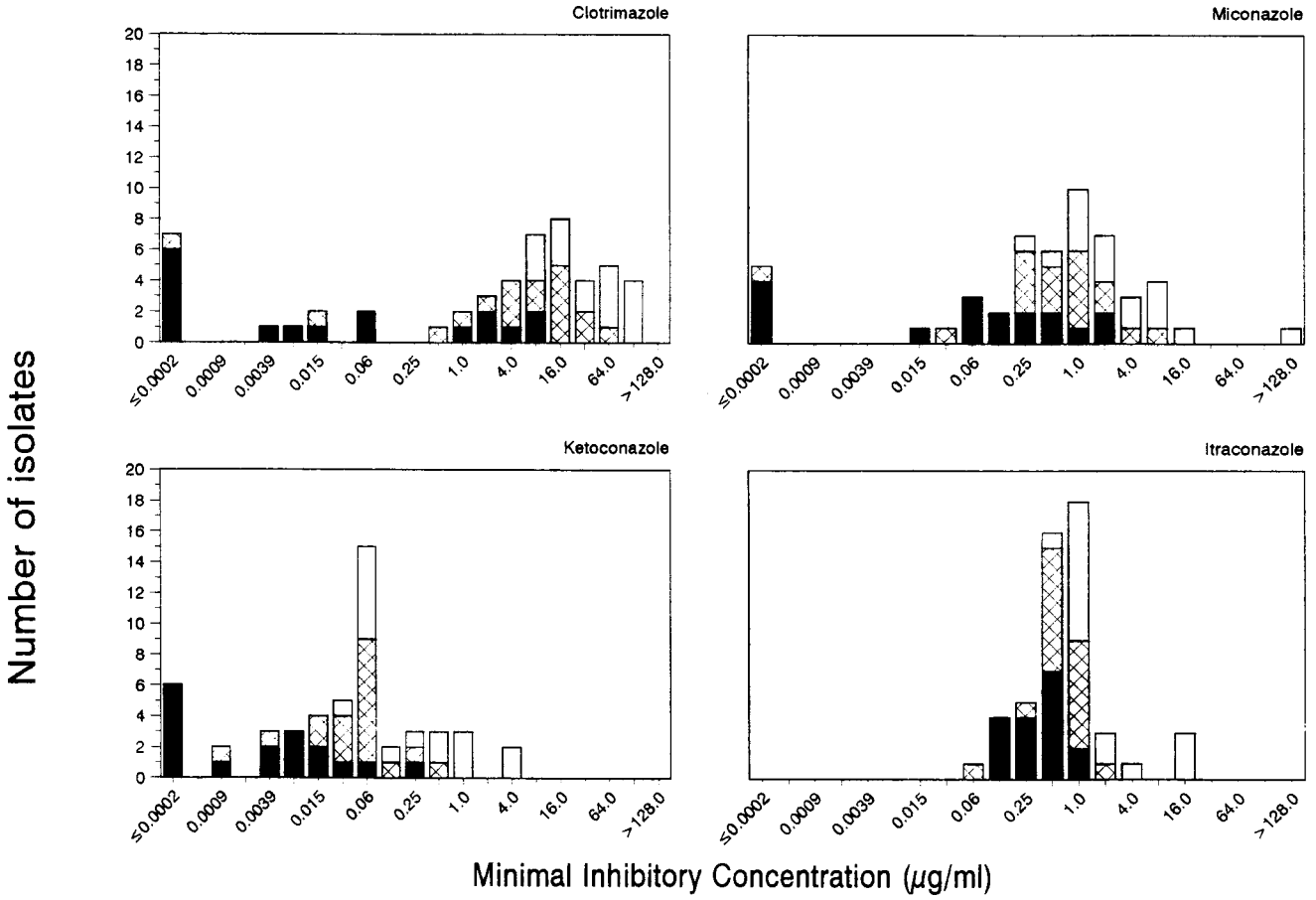

FIG. 1. Distributions of susceptibilities to four azole antifungal agents of 51 *C. albicans* isolates for which FZ MICs were ≥4.0 μg/ml. MICs of FZ were arbitrarily grouped into three classes to show a general parallel decrease in susceptibility and minor exceptions. The IZ MIC of 16 μg/ml represents an MIC of >8.0 μg/ml. ■, FZ MIC range, 4.0 to 8.0 μg/ml; ▨, FZ MIC range, 16.0 to 32.0 μg/ml; □, FZ MIC range, 64.0 to >128.0 μg/ml.

tested for their susceptibilities to FZ and KZ (32) showed identical results, i.e., lack of decreased susceptibility to KZ among the strains for which FZ MICs were ≤0.5 μg/ml.

Comparative activities of azole drugs against clinical isolates of *C. albicans* for which FZ MICs were higher. The isolates that were selected because the FZ MICs were ≥4.0 μg/ml showed different levels and patterns of decreased susceptibility to the other azole derivatives. They were in general less susceptible to CZ, MZ, KZ, and IZ (Table 2), but there were also *C. albicans* strains for which FZ MICs were higher but for which the lower MICs of the other azole drugs were retained (Table 2; Fig. 1). Compared with the group for which FZ MICs were low (Table 2), the most active compound against *C. albicans* strains with decreased susceptibility to FZ was IZ, with MICs that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of isolates tested closer to those for the group for which MICs of FZ were lower, although there were also some strains for which IZ MICs were >8.0 μg/ml (Table 2; Fig. 1). On the other hand, KZ, MZ, and CZ, showed a more pronounced increase in their MIC_{50}s and MIC_{90}s (which could be even greater because we do not know the lower limit of the MICs for the group of strains for which FZ MICs were lower).

Patterns of decreased susceptibility to azoles in clinical *C. albicans* isolates. The distribution of FZ MICs for the group of isolates more resistant to FZ generally paralleled those of the other azoles (Fig. 1), and uniform decreased susceptibility could be considered the rule. Minor exceptions such as the strains for which CZ, MZ, and KZ MICs were outside of the general distribution of CZ, MZ, and KZ MICs (Fig. 1) need further investigation; i.e., even lower concentrations of imidazoles need to be tested to see if the MICs for these strains are exactly the same as the MICs for the strains for which FZ MICs were lower.

On the other hand, studies of the in vitro-in vivo correlation for the activity of the azole drugs are just beginning (29, 32), and the defined breakpoints for FZ and KZ are tentative (32). Trying to describe the different patterns of susceptibility found in the group of 51 *C. albicans* isolates for which FZ MICs were higher, we compared only those isolates for which a MIC of at least one azole that was the same as that for the group for which FZ MICs were lower was retained (Table 2). The result, provided in Table 3, is that the different patterns were analyzed only in 11 strains for which different combinations of low and increased MICs of the five azoles were shown, but there was a wide array of different combinations of MICs for the remaining 40 strains, from small to large increases in the MICs of all five azole antifungal agents (Table 2; Fig. 1). Overall, the proportion (and number) of strains for which the MICs of each azole was increased for the 51 *C. albicans* isolates more resistant to FZ was 90% (n = 46) for MZ, 88% (n = 45) for KZ, 86% (n = 44) for CZ, and 80% (n = 41) for IZ.

TABLE 3. *C. albicans* isolates with decreased susceptibilities to FZ and for which MICs of other azoles were increased in a nonparallel manner

Strain	MIC (μg/ml)					Pattern of increased MICs	No. of isolates
	FZ	CZ	MZ	KZ	IZ		
73	8.0	≤0.0002	≤0.0002	≤0.0002	0.12	FZ	3
65	8.0	0.007	≤0.0002	≤0.0002	0.25	FZ-CZ	1
58	8.0	≤0.0002	0.015	≤0.0002	0.12	FZ-MZ	1
85	8.0	0.015	0.06	≤0.0002	0.25	FZ-CZ-MZ	1
66	32.0	0.015	≤0.0002	0.003	0.25	FZ-CZ-KZ	1
77	8.0	≤0.0002	0.25	0.007	0.25	FZ-MZ-KZ	2
75	8.0	0.003	0.12	0.003	0.12	FZ-CZ-MZ-KZ	1
88	8.0	≤0.0002	1.0	0.015	0.5	FZ-MZ-KZ-IZ	1

DISCUSSION

The lack of standardized reference methods for susceptibility testing of yeasts before 1992 (23) has precluded the comparison of the present data concerning FZ-resistant *C. albicans* with data from previous studies of the in vitro activities of imidazole antifungal agents. When susceptible strains were tested, discrepant results were the rule (29), although there are reports of spectrophotometric determination of 50% growth inhibition with very low MICs for most imidazoles (19), as we also found (Tables 1 and 2). Nevertheless, for the best-documented strains clinically resistant to KZ, most results indicated that the strains were cross-resistant in vitro to other azoles (17, 25, 33). In vivo cross-resistance to azoles has also been described for some of these KZ-resistant *C. albicans* strains in experimental models of animal infection (26, 33). With regard to the in vitro activity of IZ against *C. albicans* isolates with decreased susceptibility to FZ, our results are very similar to those of Barchiesi et al. (3), who showed increased MICs of IZ for most but not all strains, although in the case of FZ-susceptible strains, the IZ MICs of Barchiesi et al. (3) were lower than ours.

Against the general idea of cross-resistance to azoles in *C. albicans*, there are now reports of responses to KZ and IZ among AIDS patients whose isolates have developed FZ resistance (7, 15, 36). These responses could be due to infrequent *C. albicans* isolates displaying resistance to FZ only (Table 3). An explanation for this independent FZ resistance might be related to two different or both of the phenomena described below.

First, the development of azole resistance may be a progressive phenomenon that is related, for example, to the accumulation of stepwise mutations, with the MICs of FZ being the first to increase after the selective pressure made by any azole. We have never seen *C. albicans* isolates for which MICs of other azole drugs are increased in the absence of increased MICs of FZ (Table 2) (32), even for those isolates from AIDS patients treated with KZ only (32), and to our knowledge this has yet to be reported. AIDS patients bearing strains with decreased susceptibilities to FZ only (Table 3) for which the MIC is less than 16.0 μg/ml may respond to elevated doses of FZ, KZ, or IZ (7, 15, 24, 32, 36), but in our experience most patients will have relapses of infections with isolates highly resistant to FZ and cross-resistant to other azoles (32). Changes in the properties of the cell membranes as a result of greater amounts of nonesterified sterol giving them a phospholipid/sterol ratio that is lower than that of azole-susceptible strains, as reported for *C. albicans* ATCC 64550 (16, 33), could easily explain this cross-resistance (Table 1).

The second phenomenon may be that FZ-specific resistance really exists and that it originates from a specific decrease in the intracellular FZ content of some resistant *C. albicans* isolates (36). Other proposed mechanisms of resistance for *C. albicans* isolates resistant to azoles are decreased activities of the enzymes of the ergosterol biosynthesis pathway and a reduced affinity of cytochrome P-450-dependent 14α-demethylase for azoles (16). If these differences in the mechanisms of resistance to azoles in *C. albicans* can indeed originate resistance to selective azoles, it could explain why strains without cross-resistance really exist and would make in vitro testing even more important.

Nevertheless, our results suggest that decreased susceptibility to one different azole antifungal agent in *C. albicans* is usually shown in a way parallel to that for other drugs but with a different degree of full expression of decreased susceptibility for each antifungal agent, probably depending on the intrinsic properties of each drug together with the specific yeast strain involved and the particular clinical setting. Furthermore, differences in in vitro susceptibility do not necessarily predict differences in in vivo response (21), as can be seen with isolates of *C. albicans* for which KZ or FZ MICs are increased and that are clinically sensitive to the drugs (32).

From a practical point of view, we can conclude that the detection of decreased susceptibility to azole drugs requires the use of lower concentrations of imidazole derivatives than those proposed previously (23), that increased MICs of the other azoles cannot always be presumed for those isolates of *C. albicans* with decreased susceptibilities to FZ, and that in our experience, the strains of *C. albicans* susceptible in vitro to FZ are susceptible in vitro to CZ, MZ, KZ, and IZ as well.

ACKNOWLEDGMENTS

This work was supported in part by grants 93/0002/01 from the Fondo de Investigaciones Sanitarias and BI094-0181 from the Comisión Interministerial de Ciencia y Tecnología, both of Spain.

We thank Pfizer S.A., Janssen Farmaceútica S.A., and Química Farmaceútica Bayer S.A., for supplying the antifungal powders.

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