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Susceptibilities of Norwegian *Candida albicans* Strains to Fluconazole: Emergence of Resistance

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All *Candida albicans* isolates in Norwegian microbiological laboratories in 1991 judged clinically important (except vaginal isolates) were collected. The isolates were tested for susceptibility to fluconazole with an agar dilution test and a commercially available agar diffusion test. A total of 212 strains (95%) were susceptible to fluconazole, and MICs for most of the strains (92%) were ≤1.56 μg/ml. The agar diffusion test using a 15-μg tablet and a 48-h incubation period separated resistant from susceptible strains with a wide margin. The only exception was a strain for which the MIC was 6.25 μg/ml. The difference in zone size between the resistant and the susceptible populations of strains was 11 mm. Accordingly, it appears that the agar diffusion test is an appropriate method for detecting fluconazole resistance. The 12 fluconazole-resistant isolates originated from eight AIDS patients with oral or esophageal *Candida* infections. Seven of the patients had been given fluconazole for 1 month or more, often as self medication. Four had infections that were clinically resistant to fluconazole; one additional patient responded only when the dose was increased. All isolates recovered from these patients were analyzed by multilocus enzyme electrophoresis. The 12 *C. albicans* isolates belonged to five electrophoretic types, but three of four patients attending one hospital had isolates belonging to one electrophoretic type. One possible explanation for this finding could be that a nosocomial spread of resistant strains has occurred.

The increased importance of yeasts, and especially *Candida albicans*, as a cause of serious infections in hospitalized patients has been documented in several studies (12). This has resulted in an increased use of systemic antifungal agents. The drug of choice for the treatment of most serious fungal infections is still amphotericin B, but during the last few years several new less toxic antimycotic agents have been developed. In vitro testing of yeasts with these new antifungal agents is important in order to establish the susceptibility of wild-type strains recovered from different geographical regions and to detect the possible emergence of resistant strains.

One of these new antimycotic agents, fluconazole, was registered for use in Norway early in 1991. The aims of this study were twofold: firstly, to investigate the susceptibility pattern of Norwegian *C. albicans* strains to fluconazole, and secondly, to evaluate a commercial agar diffusion test method for testing of the susceptibilities of *C. albicans* strains to fluconazole.

MATERIALS AND METHODS

***C. albicans* strains.** All yeast isolates in Norwegian microbiological laboratories in 1991 from blood culture and from other specimens considered clinically important, with the exception of vaginal isolates, were collected. The strains were sent to the mycological laboratory at the National Institute of Public Health, Oslo, for identification and susceptibility testing. A total of 224 *C. albicans* strains from 180 patients were collected. Of the 224 isolates, 78 were recovered from blood, 7 were from central venous catheters, 37 were from abdominal pus specimens, 17 were from other pus specimens, 53 were from the upper respiratory tract and esophagus, 10 were from urine, and 22 were from other sources. Most of the strains were identified by the germ tube test. Germ tube-negative strains and all fluconazole-resistant strains were identified by assimilation and fermentation tests (15).

Enzyme genotypes of *C. albicans* strains. Both susceptible and resistant strains isolated from patients with fluconazole-resistant *C. albicans* strains were analyzed by multilocus enzyme electrophoresis as described by Caugant and Sandven (2). Electrophoretic type (ET) designations corresponded with those used previously.

Susceptibility testing. (i) Agar dilution. The method recommended by Pfizer Central Research was used (Pfizer Central

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Research, Sandwich, Kent, United Kingdom). Serial twofold dilutions of fluconazole were prepared in distilled water (0.1 to 250 μg/ml). Portions (1.5 ml) of each dilution were mixed with 13.5 ml of high-resolution antifungal assay medium to give final drug concentrations of 0.01 to 25 μg/ml in the agar plates. The *C. albicans* isolates were grown overnight at 37°C in Sabouraud dextrose broth. Each culture was diluted in sterile 0.85% saline to give a yeast cell suspension of 10^5 CFU/ml. The density of the inoculum was controlled with a spectrophotometer. The plates were inoculated with a Denley multipoint inoculator delivering 1 μl of each culture inoculum. A total of 24 isolates were inoculated on each dish. A control plate without fluconazole was inoculated at the beginning of each run. Two control strains were included in each run to ensure comparable results. The plates were incubated at 28°C for 48 h. The MIC was recorded as the lowest concentration of fluconazole that suppressed visible growth. A faint haze of colonies was ignored. The results were read independently by two persons. Strains for which the MIC was ≥12.5 μg/ml were defined as resistant.

(ii) Comparison of agar and broth dilution methods. Twenty-three strains (all strains listed in Table 2 included) were tested both with the agar dilution method and with the reference broth dilution method proposed by the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Testing (10). The agar dilution method was the same as that described above except that the fluconazole dilution range used was the same as that recommended by the NCCLS (0.125 to 64 μg/ml).

The broth dilution method was performed according to the NCCLS Subcommittee recommendations (10). Liquid RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) was used. The medium was buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). The fluconazole dilutions were prepared to be 10 times the strength of the final drug concentration. These drug dilutions were dispensed in 0.1-ml volumes into sterile glass tubes. The inoculum was prepared by picking yeast cells from five 1-mm-diameter colonies from 24-h-old Sabouraud cultures and suspended in 5 ml of sterile 0.85% saline. The yeast cell suspension was vortexed and diluted in sterile 0.85% saline to give a yeast cell concentration of 1×10^6 to 5×10^6 CFU/ml. The density of the inoculum was controlled with a spectrophotometer. A working suspension was made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 medium, giving a dilution of 0.5×10^3 to 2.5×10^3 CFU/ml. The inoculum size of each isolate was controlled by removing 0.01 ml with a calibrated loop and inoculating this quantity onto a Sabouraud agar plate. The tubes with the fluconazole dilutions and two control tubes each containing 0.1 ml of drug-free medium were inoculated by adding 0.9 ml of the yeast inoculum to each tube. All tubes were incubated at 35°C and observed for the presence or absence of visible turbidity or growth at 48 h. One of the growth controls was diluted 1:5 to give an 80% inhibition standard. The MIC was defined as the lowest drug concentration which resulted in a culture with turbidity less than or equal to the 80% inhibition standard.

(iii) Agar diffusion. A commercial agar diffusion test from Rosco Laboratory (Rosco Diagnostica, Taastrup, Denmark) was used (1). The inoculum was standardized by using a spectrophotometer to give a concentration of approximately 5×10^5 CFU/ml. Plates containing buffered yeast nitrogen agar with glucose and asparagine were flooded with yeast cell suspension. Excess fluid was removed immediately with a pipette. The plates were dried for 15 min, and a fluconazole tablet (15 μg) was placed on the agar surface. The plates were incubated at 37°C, and the zone sizes were measured after 24 and 48 h of incubation. The zones were measured up to the point at which the colonies reached normal size. A faint growth closer to the tablet was disregarded. Large colonies within the inhibition zone were subcultured and retested.

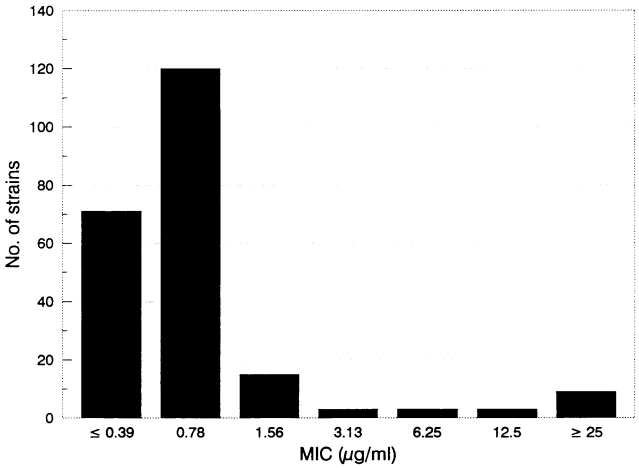

FIG. 1. MICs of fluconazole for 224 *C. albicans* isolates obtained by agar dilution testing.

Clinical information. Detailed information regarding underlying disease, treatment, and treatment results was obtained from the clinicians in charge of the patients with fluconazole-resistant isolates.

RESULTS

The results of the agar dilution test are shown in Fig. 1. A total of 212 strains (95%) were susceptible to fluconazole, and MICs for most of these strains (92%) were ≤1.56 μg/ml. The median MIC for the two control strains included in each run was 0.78 μg/ml, and the discrepancies between tests were within 2 dilutions. Twenty other isolates were tested on three different occasions. The range of MICs for all these isolates was within 1 dilution.

The comparison of broth and agar dilution MIC pairs for the 23 isolates tested by both methods demonstrated a good agreement. A total of 13 MIC pairs (7 susceptible and 6 resistant isolates) were in complete agreement. For the remaining 10 MIC pairs, a discrepancy of 1 dilution was observed for 7 of them, and a discrepancy of 2 dilutions was observed for 3 of them.

The results of the agar diffusion test after 24 and 48 h of incubation are shown in Fig. 2a and b, respectively. Strains with a zone size of ≥25 mm after incubation for 24 h showed no marked reduction in zone size when the incubation period was prolonged for another 24 h. Strains with a zone size of <25 mm after 24 h of incubation all showed a reduction in zone size after an additional 24 h of incubation. The 48-h test results (Fig. 2b) consequently showed a distribution of two well-defined populations: one dominant population with zone diameters of >26 mm and a smaller population with zones of ≤15 mm. The 48-h agar diffusion results were compared with the MICs (Table 1). The 13 strains with a zone size of ≤15 mm all proved resistant except for one strain, for which the MIC was 6.25 μg/ml, indicating an intermediate result (see the data for patient 2 in Table 2).

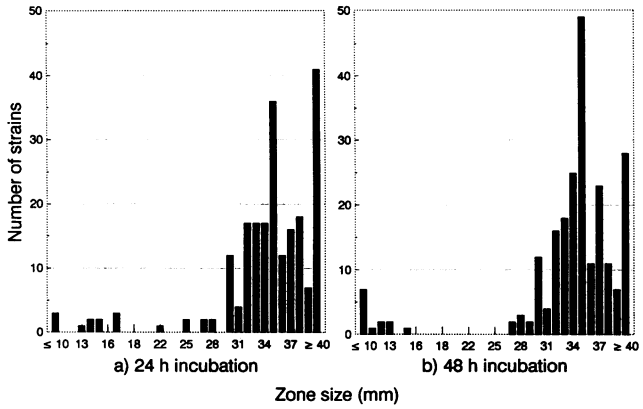

FIG. 2. Agar diffusion testing of 224 *C. albicans* isolates with fluconazole. Distributions of inhibition zone sizes after 24 h (a) and 48 h (b) of incubation.

Four of the five other strains for which MICs were 3.13 or 6.25 μg/ml had zone diameters in the range of 25 to 29 mm. All strains for which MICs were ≤1.56 μg/ml had zone sizes of ≥29 mm.

Growth of a few large colonies appearing within the inhibition zones occurred for two strains. Retesting of these demonstrated that the original culture consisted of two subpopulations, one resistant and one susceptible.

The 12 fluconazole-resistant isolates originated from eight patients treated in two different hospitals (four patients in each hospital). A total of 14 isolates from these patients were analyzed by using multilocus enzyme electrophoresis (Table 2). The eight patients had *C. albicans* isolates belonging to five ETs. Isolates from three of four patients attending one hospital were detected within a time span of 3 weeks and belonged to ET 4. Two patients concomitantly attending the second hospital had isolates belonging to ET 11. From patient 3, two isolates were recovered 4 months apart. Both belonged to the same ET; the first isolate was susceptible to fluconazole, while the second isolate was resistant. The isolates recovered from patient 2 also showed an increase in resistance during a period of 7 months.

The clinical information on these eight patients is shown in Table 2. All isolates were from AIDS patients with oral or esophageal *Candida* infections. Seven of the patients had been given fluconazole for 1 month or more, often as self medication (Table 2). Four patients had infections that were clinically resistant to fluconazole. Two of these were subsequently treated with intravenous amphotericin B, and two were treated with ketoconazole. One additional patient (patient 8) with an oral *Candida* infection did not respond to fluconazole at a dosage of 50 mg two to three times a week, and the dosage had to be increased to 400 mg daily before a satisfactory clinical response was achieved. One severely ill patient (patient 1) showed some improvement in response to a combination of 50 mg of fluconazole daily and amphotericin B lozenges, and one patient (patient 7) with oral candidiasis responded satisfactorily to a daily dose of 100 mg of fluconazole. The last patient (patient 4) was not treated with fluconazole at the time of isolation of the resistant strain. She was, however, treated with fluconazole 1/2 year later because of a severe oral *Candida* infection and did not respond to a dosage of 150 mg daily. Unfortunately, no yeast isolate from the last episode was preserved.

TABLE 1. Comparison of agar dilution MICs of fluconazole with agar diffusion inhibition zones for 224 *C. albicans* strains

MIC (μg/ml)	No. of strains with zone size (mm)[a] of:		
	≥30	29–25	≤15
≥25			9
12.5			3
6.25	1	1	1
3.13		3	
1.56	14	1	
≤0.78	191		

[a] Zone sizes were measured after 48 h of incubation. No strains had zone sizes in the range 15 to 24 mm.

DISCUSSION

Susceptibility testing of yeast is difficult. The results are influenced by various test conditions such as the culture medium used, inoculum size, incubation temperature, and duration of incubation (3, 4, 7, 8, 14), and it is therefore necessary that the methods used be standardized as far as possible to obtain reproducible results. For the last few years, progress has been made in the United States by the NCCLS Subcommittee on Antifungal Susceptibility Tests in the development of standardized reference methods for yeast susceptibility testing, and a proposed standard has recently been published (10). When the present study was planned, there was, however, no generally recommended method for testing of susceptibility to fluconazole, and we therefore decided to use the agar dilution method developed by Pfizer, the manufacturer of fluconazole. Using this method, we obtained an acceptable reproducibility. The discrepancies between tests for the two control strains used in each run were within 2 dilutions, and discrepancies were within 1 dilution for the 20 other strains tested on three separate occasions.

The agar dilution test used by us differs in several aspects from the broth dilution method proposed by the NCCLS, and it is therefore not obvious that MIC results obtained with the two methods are comparable. The agreement between the two methods was, however, found to be good. The MIC pairs for all 23 isolates tested by both methods were within 2 dilutions. All isolates, except one, found to be resistant by the agar dilution method were also found to be resistant by the NCCLS method (Table 2). The MIC for one strain was found to be 16 μg/ml by the agar dilution method and 8 μg/ml by the NCCLS method.

As fluconazole usage is increasing, it is important to monitor susceptibility of yeast strains (11). It is difficult to do this on a large scale by the presently recommended NCCLS method or the agar dilution method used by us, since both methods are quite work intensive. It is unlikely that smaller laboratories will have the capacity to perform MIC determinations with these methods. We therefore thought it important to evaluate an agar diffusion test suitable for use in smaller laboratories and for susceptibility testing of a larger number of strains. A disc diffusion test has previously been evaluated in a study by Pfaller et al. (13). A 25-μg fluconazole disc was used, and the plates were incubated at 28°C for 24 h. The intralaboratory reproducibility was reported to be good, with 91% of the results within 4 mm of others in the same set, whereas the interlaboratory agreement was poor, as only 59% of the disc test results agreed within 4 mm (13). In the present study, we have evaluated the ability of the agar diffusion test to separate strains into susceptibility

TABLE 2. Clinical information regarding eight AIDS patients with fluconazole-resistant *C. albicans* and characteristics of all isolates recovered from these patients

Patient[a]	Date[b]	ET[c] of isolate	MIC of fluconazole (μg/ml) for isolate by:		Clinical condition(s)	Drug(s) and dosage(s)	Result
			Agar[d]	Broth[e]			
1	7/90				Esophagitis	Ketoconazole	Unknown
	12/90				Esophagitis	Fluconazole, 50 mg daily, + amphotericin B lozenges	Dysphagia somewhat improved
	1/91	12	16	32	Esophagitis	Fluconazole, 50 mg daily, + amphotericin B lozenges	Dysphagia somewhat improved
2	12/89				Oral candidiasis	Ketoconazole, 200 mg daily	Cured
	10/90				Oral candidiasis	Fluconazole, 50 mg daily	Improved
	3/91	5	8	8	Esophagitis	Fluconazole, 100–150 mg daily	Some improvement
	5/91	5	16	8	Oropharyngeal candidiasis, esophagitis	Fluconazole, 100–150 mg daily	Probably no effect
	10/91				Oropharyngeal candidiasis, esophagitis	Fluconazole, 100–150 mg daily	Clinical resistance
	10/91	5	64	64	Oropharyngeal candidiasis, esophagitis	Amphotericin B, i.v.[f] for 10 days	Clinical response, short lasting
	10/91	2	64	64	Oropharyngeal candidiasis, esophagitis	Amphotericin B, i.v. for 10 days	Clinical response, short lasting
	11/91	5	64	64	Oropharyngeal candidiasis, esophagitis	Fluconazole, 100–200 mg daily	No certain clinical effect
3	9/89				Oral candidiasis	Ketoconazole, 200 mg daily	Clinical response
	12/90				Oral candidiasis	Fluconazole, 50 mg daily for 3 months	Clinical response
	3/91	11	2	2	Severe oral candidiasis	Fluconazole, 200 mg daily	Clinical response
	8/91[g]	11	128	128	Oropharyngeal candidiasis, esophagitis	Fluconazole, 100 mg daily	Clinical resistance
	8/91				Oropharyngeal candidiasis, esophagitis	Amphotericin B i.v., 15 mg daily	Clinical response
4	3/89				Oral candidiasis	Ketoconazole, 200 mg daily	Satisfactory
	4/91	11	32	32	Oral candidiasis	Ketoconazole, 200 mg daily	Satisfactory
	10/91				Severe oral candidiasis	Fluconazole, 100–150 mg daily	Clinical resistance
5	10/90				Oral candidiasis	Miconazole gel	Satisfactory
	5/91				Oral candidiasis	Fluconazole, 50 mg 3 times a week	Satisfactory
	11/91	4	32	32	Oral candidiasis	Fluconazole, 50 mg daily	Clinical resistance
	11/91				Oral candidiasis	Ketoconazole, 200 mg; later 400 mg daily	Unsatisfactory
	12/91				Oral candidiasis	Amphotericin B lozenges	Satisfactory response for 5 months
6	12/89				Oral candidiasis	Miconazole gel	Satisfactory
	2/91				Oral candidiasis	Fluconazole, 50 mg daily for 1- to 2-week periods	Satisfactory
	11/91	4	16	64	Oral candidiasis	Fluconazole, 50 mg daily	Clinical resistance
	12/91				Oral candidiasis	Ketoconazole, 400 mg daily	Clinical response; later development of esophagitis
7	10/90				Oral candidiasis	Miconazol gel	Unknown
	12/90				Oral candidiasis	Ketoconazol, 400 mg for 10 days	Unknown
	4/91				Oral candidiasis	Fluconazole, 100 mg daily for 1 month	Satisfactory
	10/91				Oral candidiasis	Fluconazole, 100 mg daily	Satisfactory
	12/91	2	64	32	Oral candidiasis	Fluconazole, 50 mg 3 times a week	Satisfactory
8	12/88				Oral candidiasis	Miconazole gel	Unknown
	12/89				Oral candidiasis	Ketoconazole, 200 mg 3 times a week	Satisfactory
	1/90				Oral candidiasis	Fluconazole, 50 mg daily for 2 weeks; later periodical treatment with 50–100 mg 2 or 3 times a week	Satisfactory
	11/91				Oral candidiasis	Fluconazole, 100 mg daily	Unsatisfactory
	12/91	4	32	32	Oral candidiasis	Fluconazole, 200 mg daily; later, 400 mg daily	Clinical resistance on 200 mg of fluconazole; satisfactory result on 400 mg of fluconazole

[a] Patients 1 through 4 were attending one hospital, and patients 5 through 8 were attending another hospital.
[b] Month/year.
[c] ETs are as given in reference 2.
[d] Agar dilution method.
[e] Broth dilution method (NCCLS).
[f] i.v., intravenously.
[g] Two isolates for which the ET and MICs were identical were recovered in this case.

groups. When the agar diffusion test was read after incubation for 48 h, two well-defined populations emerged. The difference in zone size between these populations was 11 mm. For strains with a zone size of ≤15 mm, MICs were ≥12.5 μg/ml. The only exception was an isolate for which the MIC was 6.25 μg/ml and which had a zone diameter of 12 mm. Fluconazole-resistant isolates were subsequently recovered from this patient (patient 2 in Table 2). There were no resistant strains within the group of strains with zone sizes of >26 mm. Even though interlaboratory variation may be substantial, it should be possible to separate susceptible from resistant strains with this method in all laboratories. Accordingly, it appears that the agar diffusion test used in this study is an appropriate method for detecting fluconazole resistance. Until the method has been evaluated in other studies, we will, however, advise that all strains with a zone size of <25 mm be tested by a reference method, preferably the broth dilution method as recommended by NCCLS.

In this study we have defined resistant strains as strains for which the MIC is ≥12.5 μg/ml. Twelve strains which were resistant in vitro were recovered from eight patients. Seven of these patients had been treated with fluconazole for various periods of time (1 month to more than 1 year) because of oral or esophageal *Candida* infections. The last patient had been treated with ketoconazole only. For two patients, it is likely that fluconazole resistance was induced by prolonged treatment since susceptible isolates belonging to the same ET had been recovered prior to the recovery of resistant isolates. The other patients may have acquired resistant isolates either as a result of prolonged fluconazole treatment or by nosocomial spread of isolates. If primary fluconazole-resistant isolates were common, one would have expected resistant isolates to be isolated also from patient groups other than AIDS patients.

MICs for the in vitro resistant strains were at least 10 times higher than those for the main population of *C. albicans* strains, and it is important to clarify whether infections caused by such strains are clinically resistant to fluconazole treatment. All seven fluconazole-treated patients initially responded to treatment. Five patients later developed various degrees of clinical resistance. Two were treated with intravenous amphotericin B, and two were treated with ketoconazole. One patient responded satisfactorily when the fluconazole dosage was increased to 400 mg daily. With AIDS patients, it therefore appears that treatment failure is likely to occur if infections caused by fluconazole-resistant isolates are treated with this antimycotic agent. This observation corroborates the observation of others (5, 6, 9, 16, 17). In previous studies, fluconazole-resistant strains of *C. albicans* were isolated from 13 AIDS patients not responding to fluconazole treatment. All these patients had received prolonged therapy or long-term prophylaxis with fluconazole. Consequently, available data support the notion that the use of prolonged fluconazole therapy or prophylaxis in AIDS patients should be avoided whenever possible. Whether this also applies to other patient groups remains to be seen.

All isolates recovered from patients with resistant *C. albicans* were characterized by using multilocus enzyme electrophoresis (2). Isolates from three patients belonged to ET 4, and isolates from two patients belonged to ET 11. It has been shown that these two ETs accounted for 13 and 4%, respectively, of 31 *C. albicans* isolates from different Norwegian laboratories (2) and 6 and 3% of 68 blood culture isolates from Norway in 1991 (unpublished results). The proportion of these two ETs among fluconazole-resistant strains was therefore higher than expected. Possible explanations for this could be that these strains cause infections in AIDS patients more frequently than do other strains or that certain *C. albicans* strains are more prone to develop resistance. Another possibility is that a nosocomial spread of resistant strains has occurred. The latter hypothesis is to some degree supported by the fact that the two patients with ET 11 strains were patients at the same hospital and the three patients with ET 4 strains were at another hospital. The resistant *C. albicans* strains from these three patients were also isolated within a short period of approximately 3 weeks.

In conclusion, the results of this study have shown that the development of fluconazole-resistant *C. albicans* strains may occur in AIDS patients receiving long-term fluconazole treatment and that a commercially available agar diffusion test appears to be a useful and easily performed method for detecting resistant *C. albicans* strains.

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