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Molecular Epidemiology of *Candida* Isolates from AIDS Patients Showing Different Fluconazole Resistance Profiles

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Thirty *Candida* isolates obtained from the oropharynxes of three AIDS patients were genotypically characterized. In vitro fluconazole MIC determination revealed increasing fluconazole resistances during treatment, thereby confirming the in vivo situation. Pulsed-field gel electrophoresis karyotyping, randomly amplified polymorphic DNA analysis, and hybridizations with *Candida albicans* repetitive element 2 were used to determine possible genotypic changes. The isolates from two patients showed genetic homogeneity, suggesting the selection for resistant variants. One patient experienced a strain switch to *Candida krusei*. Horizontal spread of identical strains between the patients could be excluded. However, the molecular methods used might not be sufficient to detect the underlying genetic basis of resistance to fluconazole.

The opportunistic fungal pathogen *Candida albicans* is usually a harmless commensal organism found in the oral cavities of many healthy humans (4, 6). However, in immunocompromised patients, *C. albicans* can cause life-threatening infections (10, 12, 16, 17).

Fluconazole is a highly effective and well-tolerated antifungal drug (3) which is extensively used for the treatment of candidal infections. In recent years, cases of fluconazole resistance in *C. albicans* isolates after prolonged use have been reported (2, 12, 13, 26). Several studies have demonstrated a correlation between in vitro loss of susceptibility (12, 18) and clinical appearance of resistance.

The object of our research was to follow the in vitro findings of resistance on a molecular level with assays determining the genetic profiles of *Candida* isolates. For this purpose, three methods with different inherent sensitivities were applied, as most studies examining genotypic variations in *C. albicans* isolates so far have relied on a limited repertoire of methods (12, 16). This approach seemed reasonable to us in order to possibly detect resistance-related DNA deletion or insertion events, as reported for a variety of procaryotic organisms (14). Following preliminary assessment of the isolate karyotypes by pulsed-field gel electrophoresis, we compared the hybridization patterns of these strains by use of the *Candida albicans* repetitive element 2 (CARE-2); additional experiments included a randomly amplified polymorphic DNA (RAPD) analysis.

Isolates of *Candida* spp. obtained from three patients (two males and one female) in advanced stages of human immunodeficiency virus (HIV) infection were selected for further studies from a cohort of 65 subjects with symptomatic HIV infection and recurrent oropharyngeal candidiasis as described earlier (18). *Candida* specimens from the beginning as well as the end of the treatment period (ranging from 15 to 21 months) were included. The initial isolates of all three patients were fluconazole susceptible, according to their MIC (measured in micrograms per milliliter) profile.

TABLE 1. Characteristics of isolates from HIV-infected patients with recurrent oropharyngeal candidiasis

Patient and isolate no.	Date of isolation (mo/day/yr)	Species	Fluconazole MIC (μg/ml)
Patient Gr			
1	10/20/89	*C. albicans*	0.78
2	01/24/90	*C. albicans*	0.78
3	02/23/90	*C. albicans*	0.39
4	05/04/90	*C. albicans*	0.39
7	11/07/90	*C. albicans*	0.78
10	11/20/90	*C. albicans*	1.56
11	11/27/90	*C. albicans*	0.39
13	04/15/91	*C. albicans*	6.25
14	05/23/91	*C. albicans*	25
15	07/13/91	*C. albicans*	≥50
Patient F			
1	11/02/89	*C. albicans*	0.78
4	06/27/90	*C. albicans*	3.12
6	07/10/90	*C. albicans*	6.25
7	07/11/90	*C. albicans*	6.25
10	09/07/90	*C. krusei*	12.5
11	09/07/90	*C. albicans*	12.5
14	10/12/90	*C. albicans*	25
16	10/26/90	*C. albicans*	12.5
17	01/14/91	*C. albicans*	≥50
18	02/11/91	*C. albicans*	6.25
Patient H			
1	08/28/89	*C. albicans*	0.39
5g	07/02/90	*C. albicans*	6.25
6	07/03/90	*C. albicans*	1.56
7	07/04/90	*C. glabrata*	0.78
12	09/10/90	*C. albicans*	≥50
14	10/11/90	*C. krusei*	≥50
16	10/16/90	*C. krusei*	≥50
17r	11/18/90	*C. krusei*	ND[a]
25	02/20/91	*C. krusei*	≥50
26	03/20/91	*C. krusei*	≥50

[a] ND, not determined.

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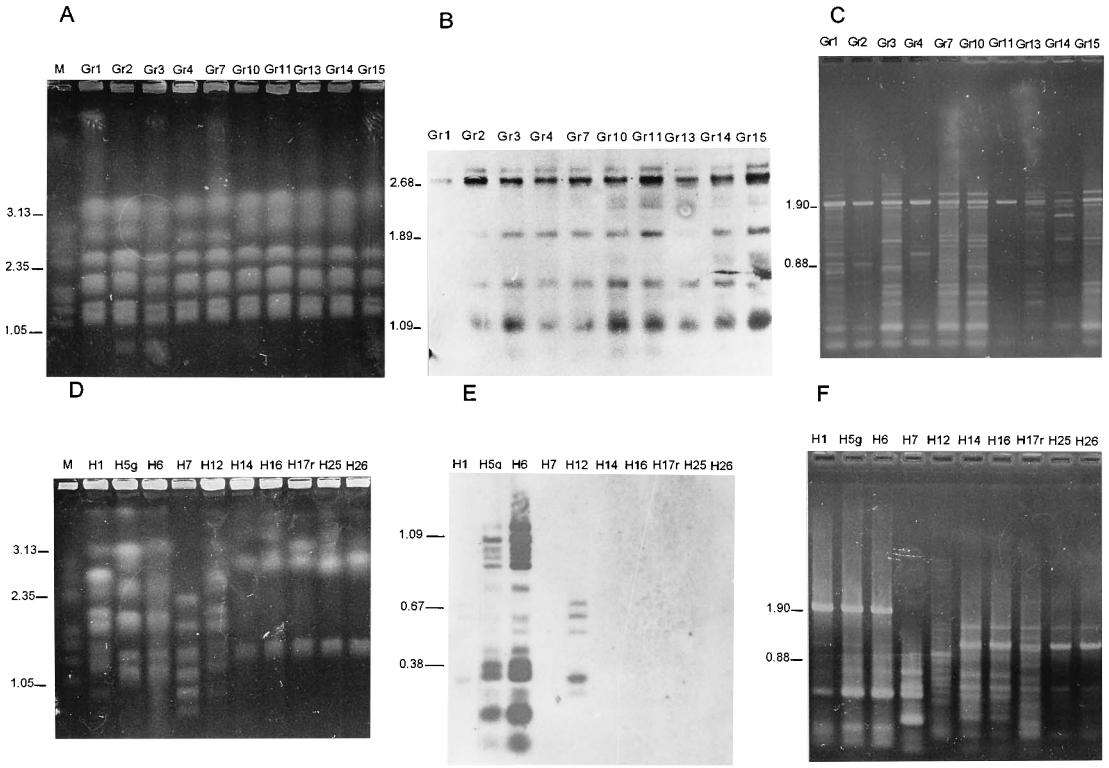

FIG. 1. Genotypic characterization of 10 individual isolates from two HIV-infected patients with recurrent oropharyngeal candidiasis treated with fluconazole. (A through C) Patient Gr. (D through F) Patient H. Sizes in megabase pairs (A and D) and kilobase pairs (B, C, E, and F) are denoted at the left of the respective panels. (A and D) Karyotype obtained by pulsed-field gel electrophoresis (size markers [M] are whole *Hansenula wingei* chromosomes). (B and E) Hybridization of chromosomal *Candida* DNA with the ECL-labelled CARE-2 probe. (C and F) RAPD analysis with the M13 primer.

Samples from HIV-infected patients were obtained by rinsing the mouth with tap water, followed by oral washings with 10 ml of a sterile 0.9% NaCl solution. After streaking on Sabouraud dextrose agar plates (4% dextrose), the numbers of CFU were determined. All *Candida* isolates were identified on the basis of rice agar morphology, germ tube production, and biochemical profiles by the API 20C AUX diagnostic system (bioMérieux, Freiburg, Germany). Tests for susceptibility of all specimens were performed at the same time by using a broth microdilution technique as described previously (18). *Candida* isolates displaying MICs of ≥25 μg/ml were defined as fluconazole resistant according to recent suggestions (18). *Candida* intact chromosomes were isolated (7) and separated by contour-clamped homogeneous electric field electrophoresis (CHEF-DR III system; Bio-Rad, Munich, Germany) by using 0.7% FastLane agarose (FMC Bioproducts, Rockland, Maine) under the following conditions: 150 to 400 s for 10 h, 2 V/cm, 106° electrode angle followed by 400 to 900 s for 38 h, 2.5 V/cm, 106° electrode angle; electrophoresis was carried out at 11°C. For hybridizations and RAPD analysis, yeast chromosomal DNA was prepared as described by Millon et al. (12). DNA was isolated from a 30-ml overnight culture grown in Sabouraud liquid medium and stored in sterile water.

Two synthetic primers (5′-CTCTAAAACTGTGCTTGGTG-3′ and 5′-AATTTGCACTCATCGAGAGC-3′) corresponding to the flanking ends of CARE-2 (8) were used to amplify the desired 954-bp product by PCR (21). Following amplification, the products were separated by electrophoresis through a 1% agarose gel; the appropriate DNA band was isolated and eluted with GeneClean (Bio 101 Inc., La Jolla, Calif.).

*Hin*fI (Pharmacia)-digested yeast DNA (10 μg) was electrophoresed through a 1.2% agarose gel for 4 h at 60 V. The gel was stained, photographed, and vacuum blotted onto a nylon membrane (Pall, Portsmuth, England) according to standard procedures (22). Following UV linking of the DNA to the membrane, the filter was hybridized overnight with approximately 200 ng of ECL-labelled CARE-2 DNA (25). Detection of the chemiluminescent signal was performed according to the manufacturer's (Amersham, Braunschweig, Germany) directions. All CARE-2 hybridizations were performed in duplicate to ensure reproducibility.

About 100 ng of yeast DNA served as the template for RAPD analysis. PCRs were performed with a synthetic primer (5′-GAGGGTGGCGGTTCT-3′) (23). DNA preparations and independent RAPD reactions were performed in duplicates.

The MICs for the *C. albicans* isolates of patient Gr increased strongly in the course of time, resulting in values of ≥50 μg/ml for fluconazole (Table 1). This is probably because of selection of the most resistant variant following long-term treatment with antifungal agents. The uniform karyotypic pattern obtained by pulsed-field gel electrophoresis (Fig. 1A) corroborates this observation. The homogeneity of the banding patterns following CARE-2 hybridizations (Fig. 1B) indicates that the patient was infected by a single strain that acquired resistance during treatment. Following RAPD analysis (Fig. 1C), some low-specificity bands occurred, but a major band at 1.9 kb and two main bands of low molecular weight were invariably present in all tested isolates. No correlation between high MICs and specific banding patterns could be demonstrated.

For patient F isolates, MICs (Table 1) also increased dramatically during the observation period. The exception is isolate F 10, in which apparently *Candida krusei* has transiently cocolonized the patient. In contrast, isolate F 17 exhibited an

extremely high MIC of ≥50 μg/ml, whereas isolate F18 was relatively fluconazole susceptible, indicating either a nonstable resistance mechanism or the isolation of a cocolonizing, susceptible *C. albicans* strain at this time of the infection. However, the genotypic assays (data not shown) confirmed that the genetic backgrounds of all *C. albicans* isolates were essentially identical. This again indicates the existence of one variant successfully mounting a fluconazole resistance.

The MICs for patient H isolates increased significantly after the species switch to the highly fluconazole-resistant *C. krusei* occurred (Table 1). In this patient, drug therapy obviously favored the oropharyngeal colonization by *C. krusei* strains, thereby eliminating *C. albicans*. The changes in the karyotypic banding patterns (Fig. 1D) highlight the transition from *C. albicans* to *C. krusei* via *C. glabrata*. The group of *C. krusei* isolates appeared genotypically stable. Interestingly, patient H seems to have been colonized by two *C. albicans* strains that differ markedly in their overall genotypic pattern (compare H5 with H12 in Fig. 1E); both are eventually replaced by *C. krusei*. The remarkably high fluconazole resistance of H12 (lane 5), in combination with an atypical RAPD profile (Fig. 1F), calls its biochemical identification as a *C. albicans* strain in question. All three patients were colonized by divergent *C. albicans* variants, as shown by their unique genomic profiles.

In conclusion, two profoundly different colonization strategies (26) are exemplified in this study, and both have to be considered. First, one strain can supersede another strain in the oral cavity in adaptation to new challenges in its environment. This might eventually lead to colonization by a *Candida* species that is per se resistant (2, 3, 26) to fluconazole, as in patient H, where *C. albicans* isolates were replaced by *C. krusei* isolates because of their natural fluconazole resistance (13). Second, some strains are capable of developing drug resistance under continuously maintained selective pressure (5, 12, 18). In this study, increases in resistance in patient Gr isolates were not accompanied by detectable genomic changes, yet fluconazole resistance gradually increased.

The inhibitory effect of azole compounds on 14α-demethylase (2, 27), which disturbs the cell membrane composition, is well characterized (3, 26). In contrast, the underlying mechanisms by which de novo resistance to fluconazole is mediated are still unclear. Selection of drug-decomposing enzymes is one possibility for the yeast to gain resistance. Several genomic alterations have been noted in *C. albicans*, some of which also affect the phenotypic morphology (1, 9, 19, 20, 24). A genomic difference as subtle as the existence of a 380-bp intron can determine susceptibility to flucytosine, a widely used antifungal drug (11). Additional molecular techniques may unravel the genomic bases for acquired resistances, but since small mutation events are beyond the sensitivities of the methods used in this study, a broader spectrum of techniques is necessary for the evaluation of drug resistance. The selective stress put on clinical isolates by repeated and prolonged, sometimes even combined, usage of certain drugs (13, 15) should be observed carefully with respect to the priming of new drug resistances.

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