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Oral candidosis: characterization of a sample of recurrent infections and study of resistance determinants

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SUMMARY

Recurrent oral candidosis is a common problem in immunocompromised patients, and it is frequently triggered by resistance induced by antifungal treatment. Knowledge of the mechanisms by which the yeast persists in the host could allow the management of this type of infection. This study used electrophoretic karyotyping and restriction fragment length polymorphism based on the use of 27A probe to study 12 pairs of *Candida albicans* isolates from patients with recurrent candidosis to distinguish new infections from relapses caused by the same strain responsible for the first episode. Subsequently, RT-PCR was used to evaluate expression of *CDR1*, *CDR2* and *MDR1* genes, which are involved in *C. albicans* azole resistance, in the three pairs that consisted of variants of the same strain. Restriction polymorphism resulted in better discrimination than with karyotyping in defining differences between strains. In one case, RT-PCR allowed us to identify deregulation of efflux pump genes as the possible underlying mechanism in recurrent candidosis. The techniques employed resulted effective for the characterization of recurrent oral candidosis. Broader analysis could help to control better these infections and choose adequate therapy.

KEY WORDS: Recurrent oral candidosis, Eectrophoretic karyotyping, Restriction fragment length polymorphism, Resistance determinants

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INTRODUCTION

In recent decades, fungal mucocutaneous infections have shown a global increase. This trend has been linked to the introduction of new technologies and therapies, which allow the survival of immunocompromised patients, and to the use of invasive procedures and broad-spectrum antibiotics (Fridkin and Jarvis, 1996; Richardson, 2005; Hobson, 2003; Banerjee *et al.*, 1991; Pfaller *et al.*, 1998). The majority of mycoses are caused by *Candida* spp., which are also mainly responsible for nosocomial fungal infections (CDC, 2001; Eggimann *et al.*, 2003; Ruhnke, 2006). *Candida albicans* is the most frequently isolated species.

The recent increase in candidoses caused by non-albicans species, particularly *Candida glabrata* and *Candida parapsilosis*, is related mainly to the widespread use of antifungal agents in prophylaxis and therapy (Fridkin and Jarvis, 1996; Ruhnke, 2006; Richardson, 2005).

Oral candidosis represents one of the more common mucocutaneous *Candida* infections, especially in newborns, older people and HIV-positive patients. These infections are most frequently caused by *C. albicans*, but other species as *C. glabrata*, *Candida tropicalis*, *C. parapsilosis*, *Candida krusei* and *Candida dubliniensis* have

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been isolated from saliva of infected subjects (Akpan and Morgan, 2002; Coogan *et al.*, 2006; Thompson *et al.*, 2010).
Risk factors for oral candidosis are the pathogenic characteristics of the yeast, such as the ability to adhere to the oral mucosa, to form hyphae and to bind fibronectin, and the condition of the host (use of prostheses, lack of functioning salivary glands, old age, use of antibiotics, or presence of other diseases) (Akpan and Morgan, 2002; Fanello *et al.*, 2006).
The treatment of oral candidosis is based on hygiene procedures and use of antifungal agents; prognosis is generally good, but wrong diagnosis, use of inadequate therapies and the persistence of risk factors predispose to recurrent infections (Akpan and Morgan, 2002). Recurrent episodes of candidosis in immunocompromised patients are determined by an increased avidity of the cells of the oral mucosa and by genotypical and phenotypical changes in the yeast cells, especially those included in a biofilm (Samaranayake *et al.*, 2001, Samaranayake *et al.*, 2003; Thompson *et al.*, 2010).
Two types of resistance are detectable in fungi: innate, such as that shown by *C. krusei* against fluconazole; or acquired after antifungal treatment, such as that shown by *C. albicans* strains responsible for oral infections in HIV-positive patients undergoing azole treatment (Yang and Lo, 2001). Resistance is determined by different mechanisms, and sometimes it is caused by more than one mechanism.
Resistance to azoles can be determined by overproduction, modification or substitution of cytochrome *P-450* enzyme (lanosterol 14-α-demethylase). Alternatively, azoles can be blocked out of the cell or expelled via multiple carriers or altered (Yang and Lo, 2001; Sanglard and Odds, 2002). The ability to expel azoles from the cytoplasm is based on the activity of two families of carrier proteins: ATP-binding cassette (ABC) and major facilitator (MF) proteins. *Candida* drug resistance (*CDR*) genes encode ABC proteins. The presence of CDR mRNA in the cytoplasm of fungal cells has been correlated with resistance to azoles (Kofla and Ruhnke, 2007; Park and Perlin, 2005). Among *MF* genes, *MDR1* (multidrug resistance 1) has been identified as being responsible for resistance to fluconazole in *C. albicans* (Yang and Lo, 2001).
Although evaluation of antifungal susceptibility through reliable methods is fundamental in the choice of therapy, it is still performed less than analysis of antimicrobial resistance in bacteria. However, the onset of antifungal resistance can be prevented only through correct management of patients and administration of adequate therapy. The rapid identification of strains, correct evaluation and interpretation of MIC values, together with consideration of all the factors that can influence the results of therapy are the main instruments to control complicated infections. When this is not carried out at first diagnosis, at least the strains that are isolated from infections that do not respond to therapy should be analyzed, to evaluate potential resistance mechanisms and address subsequent treatments (Rex and Pfaller, 2002).
In recent years, many molecular techniques have been applied to the analysis of the *Candida* genome. Some of these are based on the nucleic acid amplification, even directly in clinical samples; other methods need colony growth to compare specific strains.
These techniques can also be employed in the study of genetic modifications that cause antifungal resistance (López-Ribot *et al.*, 2000). Electrophoretic karyotyping (EK) and restriction endonuclease analysis of genomic DNA (REAG) are considered sufficiently discriminating and they are commonly used in epidemiological studies (Vasquez *et al.*, 1991; Chen *et al.*, 2005). Techniques based on the analysis of restriction polymorphism (restriction fragment length polymorphism; RFLP) have been used in conjunction with karyotyping in several epidemiological studies, and have given useful indications in differentiating strains (Scherer and Stevens, 1998).
Although the effectiveness of these methods has been demonstrated in several studies, they are not yet standardized, and many of them are still used with difficulty due to their costs and management. The present experiment used two different molecular techniques to study recurrent candidosis to evaluate which of them was more reliable to distinguish new from relapsed infections. Subsequently, the expression of genes that were involved in *C. albicans* azole resistance was evaluated to identify the mechanisms responsible for infection relapse.

MATERIALS AND METHODS

Samples collection

Oral rinses were collected among patients from the Department of Head and Neck Pathology of the Second University of Naples, Italy, admitted to the laboratory of the Hygiene Section, Department of Public, Clinical and Preventive Medicine, on the basis of a clinical suspicion of oral mycosis. All of them presented cancerous or pre-cancerous oral lesions, but at the time of sample collection, they were not taking antibiotics and were not under chemo- or radiotherapic treatments. Each patient was asked to collect a gargle (approximately 1 ml of saliva) with 9 ml sterile 0.9% NaCl solution for 60 s in a Petri dish (dilution 1:10).

This sample was placed in Sabouraud agar plus gentamicin and chloramphenicol and plates were incubated at 37°C for 24-48 h. Colonies were identified by multiplex-PCR without DNA extraction, as previously described (Liguori *et al.*, 2007; Liguori *et al.*, 2009; Liguori *et al.*, 2010). Strains isolated from those subjects who presented at least two episodes of oral candidosis were stored. For each patient with at least two episodes of infection caused by the same species, the strain isolated from the first infection before treatment, and that responsible for the second episode after antifungal therapy, were analyzed.

The analysis was based first on comparison of susceptibility patterns of *C. albicans* to more common antifungal drugs, and subsequently on the study of their karyotypes and restriction polymorphisms. Finally, RT-PCR was used to study efflux pump gene expression in *C. albicans* isolates.

Antifungal susceptibility testing

Antifungal resistance analysis was carried out through a colorimetric microdilution test (YO3; Trek Diagnostic Systems, Westlake, OH, USA) as recommended by the manufacturer. It comprised a dilution series of six antifungal compounds and MICs were determined on the basis of yeast growth in the wells, as shown by Alamar Blue. Isolates were defined as susceptible, dose-dependently susceptible, intermediate or resistant on the basis of limit values defined by Clinical and Laboratory Standard Institute (CLSI) reported in Table 1 (Rex *et al.*, 1997).

TABLE 1 - *CLSI breakpoints (µg/ml) for susceptibility to antifungal compounds.*

	S	*SDD*	*I*	*R*
Fluconazole	<8	16-32	/	>64
Flucytosine	<4	/	8 - 16	>32
Itraconazole	<0.12	0.25-0.5	/	>1

PFGE

Pulsed-field gel electrophoresis (PFGE) was employed to detect any genomic differences in pairs of strains through direct comparison between karyotypes.

For the electrophoresis, plugs were prepared and blocked onto a 0.8% agarose gel in the electrophoretic chamber CHEF DR II (Bio-Rad Laboratories, Segrate, MI, Italy) as recommended by the manufacturer.

A *Saccharomyces cerevisiae* marker (Bio- Rad Laboratories, Segrate, MI, Italy), with a range between 225 and 2200 kb, was used as a molecular weight marker. Conditions which gave the best results were a switch time of 120 s/24 h and 240 s/36 h, voltage 3.5 V/cm, and rotation angle 106°. Visual comparison was carried out between chromosomal band patterns. Isolates that did not show differences in number or size were considered equal (Shin *et al.*, 2004-b, Shin *et al.*, 2005; Rho *et al.*, 2004).

RFLPs

Restriction analysis was carried out as described by Scherer and Stevens (Scherer and Stevens, 1998).

The method was based on the hybridization of the 27A probe to *C. albicans* DNA, which was extracted with the EZ1 DNA Tissue Kit (Qiagen S.p.A., Milano, Italy) and digested with 10 U/µL *Eco*RI (Roche Applied Science, Monza, Italy). Light emission at 466 nm was evaluated by VersaDoc MP 4000 (Bio- Rad Laboratories, Segrate, MI, Italy) and images were enhanced with QuantityOne 4.6.5 software (Bio-Rad Laboratories, Segrate, MI - Italy), by considering as equal isolates which presented 100% similarity, different those strains which showed ≤90% similarity and subtypes those strains with similarity patterns between 90 and 99% (Di Francesco *et al.*, 1999).

Analysis of resistance determinants

For pairs of isolates with similar karyotypes and restriction patterns, we used a protocol of RT-PCR based on the quantification of *CDR1*, *CDR2* and *MDR1* gene expression in relation to that of *TEF3* (translation elongation factor 3) housekeeping gene.

Sequences of primers and probes employed are shown in Table 2. RNA extraction was carried out using the RNeasy Mini Kit (Qiagen S.p.A., Milano, Italy) from sub-cultures in Yeast extract Peptone Dextrose (YPD) medium (Difco, Becton Dickinson Italia, Buccinasco - MI - Italy).

Each amplification reaction was performed three times for each isolate, in a 25-µL solution that contained 14 µL Qiagen OneStep Real Time-PCR mixture, 1 µL each primer (10 µM), 1 µL of probe (2 µM), 2 µL RNA and 6 µL RNase-free water. Reverse transcription of 45 min at 52°C was followed by inactivation at 95°C for 5 min, 40 amplification cycles of 15 s at 95°C and 1 min at 54°C, and a final extension at 54°C for 10 min. Fluorescence emission was measured using iCycler iQ software (Bio-Rad Laboratories, Segrate, MI, Italy).

Results were expressed as the mean of three measures obtained for each sample.

For each gene, a twofold increase in expression compared to that of TEF3 was considered significant.

Exposure of yeast isolates to fluconazole

For fluconazole exposure, we followed the protocol described by Sanguinetti *et al.* (Sanguinetti *et a1.*, 2005). Suspensions of *C. glabrata* cells (OD_{600}, 0.1) freshly prepared in YEPD medium were grown at 30°C to reach an OD_{600} of 0.3. Then fluconazole was added at a final concentration of 50 µg/ml and the cultures were incubated at 30°C for a further 4 h. After the cells were exposed to fluconazole, they were centrifuged at 10,000 x *g* for 10 min and washed twice with sterile water, and the pellet was used for quantitative RT-PCR analysis (see above).

RESULTS

Samples collection

Out of the 113 patients analyzed, 23 recurrent episodes of infection were recorded (20.3%), and each of them was sustained by a single *Candida* species. In seven (30.4%) cases, the second isolate was different from that responsible for the first episode, whereas in the remaining 16 (69.5%), the species was the same. In the latter group, 12 pairs of isolates consisted of *C. albicans*, two of C. *glabrata*, and one each of *C. tropicalis* and *C. parapsilosis*. Pairs of *C. albicans* strains (named A-L) were analyzed to distinguish re-infections caused by resistant strains from new

TABLE 2 - *Primers and probes employed in real-time PCR.*

Gene (Accession No.)	*Primer/probe*	*Sequence*[a]	*Gene location (5'-3')*
CDR1 (X77589)	CDR1a	AACCGTTTACGTTGAACACGATAT	2504-2527
	CDR1b	ACCAACTTCACCATCTTCAATGAC	2565-2588
	CDR1pr	6FAM- ACTCACGCCGACACCACCGTTGTT-TAMRA	2535-2558
CDR2 (U63812)	CDR2a	TGGCTAGTGTTTATATGGCAACCT	1725-1748
	CDR2b	AAGCTTCAGCAATTGACACTCTTT	1821-1844
	CDR2pr	6FAM- TCACCACCGGAAACACCACGCACA-TAMRA	1792-1815
MDR1 (Y14703)	MDR1a	TCTCGGGTGGATTCTTTGCTAAT	2659-2681
	MDR1b	AATGGACCAAAACTAGGACCACA	2775-2797
	MDR1pr	6FAM- ACGGCACCCAAACTCCAAGCGGC-TAMRA	2751-2773
TEF3 (Z12822)	TEF3a	AACCGTTTACGTTGAACACGATAT	2480-2504
	TEF3b	ACCAACTTCACCATCTTCAATGAC	2565-2588
	TEF3pr	Texas Red- ACTCACGCCGACACCACCGTTGTT-BHQ2	2535-2558

[a]Abbreviations: 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N',N'-tetramethylrhodamine; Texas Red, trademark product from Molecular Probes; BHQ2, Black Hole Quencer 2.

infections caused by other strains after antifungal treatment. Gene expression was studied in pairs of the same strains to evaluate possible mechanisms involved in resistance. Information on corresponding patients and the time elapsed between the first and second episodes of infection for each of them are reported in Table 3.

Antifungal susceptibility

MIC values of *C. albicans* strains before and after antifungal therapy, together with the type of treatment for each patient, are shown in Table 4. Overall, 20 (83.3%) strains were susceptible and four (16.6%) were dose-dependently susceptible towards fluconazole, 21 (87.5%) strains were sus-

TABLE 3 - *Information about patients who were source of strains isolation.*

Patient	*Sex*	*Age*	*Smoke*	*Alcool*	*Prostheses*	*Other diseases*	*Time interval among infections*
A	F	65	N	N	N	tyroid disfunction	2 months
B	F	43	Y	N	Y	depression	5 months
C	M	52	N	Y	N	no other	2 months
D	M	64	Y	Y	Y	hypertension	5 months
E	F	62	N	N	Y	hypertension	5 months
F	F	30	Y	N	Y	no other	1 months
G	M	63	Y	N	Y	hypertension	1 months
H	F	58	N	N	N	hypertension	1 months
I	M	74	N	N	Y	hypertension	2 months
J	F	55	Y	N	N	hypertension	2 months
K	M	64	Y	Y	Y	no other	4 months
L	F	56	Y	N	Y	diabetes	2 months

TABLE 4 - *MIC values (g/mL) of antifungal compounds used (AB: amphotericin B, FZ: fluconazole, IZ: itraconazole, KZ: ketoconazole, FC: flucytosine, VOR: voriconazole) showed by C. albicans strains isolated before and after therapy (1: fluconazole for 10 days; 2: itraconazole for 7 days; 3: itraconazole for 14 days) and correspondence/discordance showed by each couple of strains.*

Isolate	*Correlation*	*Therapy*	*AB*	*FZ*	*IZ*	*KZ*	*FC*	*VOR*
A1	NO	2	0.03	0.125	0.008	0.008	0.03	0.008
A2			0.03	8	0.5	0.25	0.03	0.125
B1	NO	3	0.008	32	0.008	0.008	0.03	0.008
B2			0.06	0.125	0.008	0.008	0.03	0.008
C1	NO	1	0.06	0.125	0.008	0.008	0.125	0.008
C2			0.03	0.125	0.008	0.008	0.125	0.008
D1	YES	2	0.06	0.125	0.008	0.008	0.03	0.008
D2			0.06	0.125	0.008	0.008	0.03	0.008
E1	YES	2	0.03	0.125	0.008	0.008	0.03	0.008
E2			0.06	0.125	0.008	0.008	0.03	0.008
F1	NO	1	0.06	0.125	0.008	0.008	0.03	0.008
F2			0.06	2	0.008	0.008	0.03	0.008
G1	NO	2	0.008	0.125	0.008	0.008	0.03	0.008
G2			0.03	0.125	0.008	0.008	0.03	0.008
H1	NO	1	0.06	0.125	0.008	0.008	0.03	0.008
H2			0.03	2	0.008	0.008	0.03	0.008
I1	YES	3	0.03	32	0.25	0.25	0.03	0.25
I2			0.06	32	0.25	0.25	0.03	0.25
J1	NO	2	0.008	0.125	0.008	0.008	0.03	0.008
J2			0.06	0.125	0.008	0.008	0.03	0.008
K1	NO	3	0.008	32	0.008	0.008	0.03	0.008
K2			0.06	0.125	0.008	0.008	0.03	0.008
L1	NO	2	0.03	0.125	0.008	0.008	0.03	0.008
L2			0.06	8	0.008	0.008	0.03	0.008

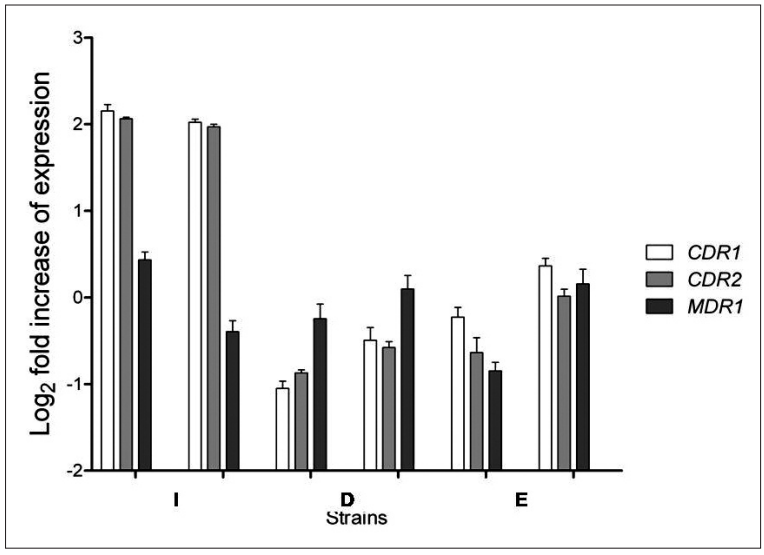

FIGURE 1 - *Levels of CDR1, CDR2 and MDR1 expression for the pairs I, D and E before and after therapy, compared with those of TEF3.*

ceptible and three (12.5%) were dose-dependently susceptible towards itraconazole, and 24 (100%) strains were susceptible towards 5-fluorocytosine. No isolates showed development of resistance after treatment, but in four cases (A, F, H and L) a decrease in susceptibility was recorded, whereas two cases (B and K) showed an increase between the first and second infections.

PFGE

On the basis of the number and migration distances of DNA bands, PFGE showed two to five bands of different sizes in chromosomal patterns of strains from the same couple in eight cases (couples A-C, F, H, and J-L). Visual analysis of electrophoretic chromosomal patterns revealed a correspondence between strains in the other four *C. albicans* pairs (D, E, G, I).

RFLPs

RFLPs generated by EcoRI produced patterns with similarity coefficients comprised between 76.5 and 99.5%. First and second isolates from individual patients were assigned to the same DNA type in three of twelve cases (couples D, E, I), while sequential isolates from couples A-C, F-H and J-L showed a similarity <90% and were assigned to different DNA types.

Analysis of resistance determinants

The three pairs of *C. albicans* isolates (D, E and I) that comprised strains with the same chromosomal and restriction patterns and a similar susceptibility profile were submitted to quantitative RT-PCR, in relation to the expression of *TEF3* (Figure 1). D and E showed a slight, non-significant increase in the expression of all the genes considered. On the contrary, in I, both isolates showed high levels of expression, with a decrease in *MDR1* expression between the first and second strains. As expected on the basis of MIC values, overexpression of *CDR1* and *CDR2* was recorded in the first and second isolates, which allowed us to identify the potential mechanism responsible for re-infection.

Expression of CDR1, CDR2, and MDR1 in isolates grown in the presence of fluconazole

To evaluate whether the expression profiles observed for the three pairs of isolates analysed (D, E, and I) could change after exposure to fluconazole, we performed experiments in which the expression of the *CDR1*, *CDR2*, and *MDR1* genes by the isolates grown in the presence of fluconazole at a final concentration of 50 µg/ml was analyzed. The expression levels were compared to those obtained with the same isolates grown in the absence of fluconazole (Table 5). Interestingly, the profiles of expression of all target genes by the resistant isolates (pair I) remained unchanged, whereas marked increases in the levels of gene expression, particularly of *CDR1* and *CDR2*, were observed for the susceptible pairs (D and E).

TABLE 5 - *Expression profiles of resistance-related genes for the 3 pairs of related isolates grown in the presence of fluconazole*[a].

Isolate	*Fluconazole susceptibility category*[b]	*Genes expression (fold increase*[c]*)*		
		CDR1	*CDR2*	*MDR1*
D1	S	5.32	4.32	3.29
D2	S	5.21	4.44	2.92
E1	S	4.11	3.25	1.83
E2	S	4.20	3.07	2.21
I1	R	1.04	0.98	2.42
I2	R	1.12	1.03	2.60

[a]Fluconazole was used at a final concentration of 50 g/ml, as described previously (1). [b]S-DD, susceptible dose dependent (MIC, 16 to 32 g/ml); R, resistant (MIC, 64 g/ml). [c]Quantification was performed by real-time RT-PCR (see text for details). The values are averages of four independent experiments and represent increases in the level of gene expression relative to that for the same isolates grown in the absence of fluconazole (set equal to 1.00).

DISCUSSION

The correct and rapid diagnosis of candidosis together with the identification of species represent two fundamental aspects in the control of this type of infection, both for the patient and the community. Moreover, the molecular characterization of yeasts might be necessary in certain circumstances, as in recurrent oral infections (Thompson *et al.*, 2010). Nowadays, several methods are available for the epidemiological analysis and control of candidosis. In many fungal species, unequal rearrangements of chromosomes, or their breakage and re-joining can lead to a length polymorphism that can be analyzed by EK. If the resulting polymorphism is not discriminatory, DNA can be digested with rare-cutting restriction enzymes before electrophoresis, which allow analysis of a major number of fragments (REAG). Genomic DNA can also be submitted to digestion by frequent-cutting restriction enzymes to analyze RFLPs, which can be caused by variability in the cutting sites or insertions/deletions among them. This analysis is rapid, simple and cost-saving, but the patterns obtained can be difficult to interpret and compare (Anderson *et al.*, 1993; Soll, 2000).

Sequencing represents the most reliable comparison method, but it is not yet applicable routinely (Chen *et al.*, 2002). Currently, multilocus sequence typing, which is based on the sequencing of internal segments of housekeeping genes, seems to be the most promising technique because of its reproducibility (Tavanti *et al.*, 2005). It is still not sufficiently widespread, but progress in automation and decreased costs suggest that it will become more widely used in the future.

Microarrays offer sensitivity, specificity and high discriminatory power. They can be employed to detect and type yeasts from clinical samples, by defining the presence and quantity of gene variants, as well as identifying genes involved in pathogenesis (Spiess *et al.*, 2007; Wilson *et al.*, 2009).

In the present study, yeasts from patients with recurrent candidosis were studied. The strain isolated during the first infection before any treatment, and the strain responsible for the second episode, which occurred after antifungal therapy, were analyzed to verify if the recurrent infection was caused by different yeasts or by a variant of the first strain, to analyze their antifungal susceptibility spectrum and to highlight possible resistance mechanisms triggered by antifungal drug administration.

All 12 patients from whom pairs of *C. albicans* strains were isolated were treated with azoles. Susceptibility analysis did not show resistance onset in yeasts that caused the second episode of infection, but it disclosed a decrease in sensitivity in four cases. In another two cases, MIC decreased between the first and second infections. Moreover, only a few strains showed low susceptibility towards fluconazole and itraconazole, as reported in previous similar studies (Shin *et al.*, 2001, Shin *et al.*, 2005; Kuriyama *et al.*, 2005). However, *C. albicans* is not intrinsically resistant to azoles but it can became resistant through induction. In addition, patients included in the study were not submitted to immunosuppressive or antifungal therapies at the sampling time, although they had preexisting diseases. Thus, it can be supposed that resistance mechanisms and variant selection were not triggered because of a lack of therapy induction.

Isolates were typed through karyotype and restriction polymorphism analysis. Karyotyping represents a valid method to study the correla-

tion among isolates or to identify any variation in the number or length of chromosomes in relation to environmental conditions. Several studies have compared karyotyping with other typing methods, such as the inter-repeat PCR, REAG or Southern blotting, to determine if karyotype changes were due to chromosomal rearrangements in the same isolates, or to colonization by new strains. Some investigations demonstrated a correspondence between chromosomal differences showed by karyotyping and isolate diversity established by other techniques. However, in some cases recombinations which yielded visible variations in chromosomal patterns did not involve regions which represent target for other methods and then do not give similar results (Shin *et al.*, 2001; Shin *et al.*, 2004a; Huang *et al.*, 2004; Barchiesi *et al.*, 1999). In fact, small differences recorded by other techniques may also be presented by strains with equal karyotype (Barton *et al.*, 1995). Hybridization of digested DNA with the 27A probe is a highly sensitive typing method and is employed in many epidemiological studies (Barton *et al.*, 1995; Magee *et al.*, 1992; Boccia *et al.*, 2002). The sequence of the probe is contained by about 10 regions distributed across *C. albicans* chromosomes, and this produces polymorphisms that can be easily detected and can give useful indications on strain correlation (Scherer and Stevens, 1998).

Here, PFGE was used to determine the correspondence or discordance between each pair of isolates, or to identify possible variations due to rearrangements in the same chromosomal pattern (Shin *et al.*, 2001; Rho *et al.*, 2004). Comparisons showed differences between bands in eight cases; all the pairs of isolates with a similar karyotype demonstrated MICs without variation after therapy. A fingerprinting analysis was carried out in parallel on the isolates by utilizing the 27A probe. Computerized comparison among restriction patterns showed variations in nine pairs of isolates. Thus, the two typing techniques did not have completely corresponding results. For one pair of isolates, which showed similar antifungal susceptibility between strains, restriction polymorphism was more discriminant than karyotyping, as reported in similar previous studies (Huang *et al.*, 2004).

In 1991, Vasquez *et al.* suggested that karyotyping is simpler, more sensitive and reproducible than RFLP for *C. albicans* typing (Vasquez *et al.*, 1991). However, karyotype analysis is more time-consuming, and the number of isolates that can be processed at the same time is lower than that in a normal electrophoresis gel. Moreover, the use of a probe with a mid-repeated sequence like 27A gives higher sensitivity to RFLP, as demonstrated in the present study. However, because of their complexity, the interpretation of RFLP patterns needs computer-assisted analysis. These limitations and relative costs reduce its applicability (Williams and Lewis, 2000).

Finally, expression analysis of genes responsible for the expulsion of azoles from the fungal cell was carried out on three pairs of isolates that comprised variants of the same strain: *CDR1* and *CDR2*, which are involved in resistance to all azoles, and *MDR1*, which encodes a fluconazole-specific efflux pump, to identify the possible mechanism responsible for resistance to therapy and recurrent infection. Real-time PCR is the technique of choice for quantitative study of resistance because of its higher sensitivity and rapidity compared with northern blotting (Kofla and Ruhnke, 2007; Park and Perlin, 2005). In the first case, both isolates showed high levels of gene expression, with a decrease between the first and second strains for *MDR1*. Overexpression of *CDR1* and *CDR2* observed in both the isolates allowed us to identify expulsion of itraconazole as the possible cause of relapse. It should be noted that the reduction in *MDR1* expression in the strain isolated after therapy could correspond to the lack of fluconazole induction. In the other two cases studied, we observed a slight increase in expression of all the genes considered between the two episodes of infection. Although this corresponded to the susceptibility test results, this increase was not significant. Interestingly, we observed that the upregulation of efflux pump-encoding genes was unchanged in the resistant isolates, suggesting that some genetic alteration that perhaps affects a regulatory gene occurred, thereby resulting in unaltered resistance profiles. By contrast, when cultures of the 4 susceptible isolates were treated with fluconazole, we found that all three transporter genes were upregulated (Tab. 5). For these patients, persistence of the yeast could be attributable to other resistance mechanisms, such as mutation in the genes involved in ergosterol synthesis, or other causes linked to col-

onization ability of the isolate (Rogers and Barker, 2003).

Moreover, when interpreting these results, differences between what happens in vitro and in vivo have to be considered. If therapy is taken correctly, it is possible that, with certain antifungal drug concentrations, overexpression mechanisms have been triggered that allowed the microorganisms to survive, but such a situation did not take place in the laboratory. In fact, the ability of *C. albicans* to become resistant in the presence of fluconazole and to re-acquire azole susceptibility in the absence of induction has been demonstrated, with a different level of expression for the different genes involved (Marr *et al.*, 1998, Marr *et al.*, 2001; Lepak *et al.*, 2006). Further studies are necessary to clarify which processes take part in relapse.

Even if the number of strains analyzed was small, our study represents a contribution to the characterization of recurrent oral candidosis. With regard to diagnosis, multiplex PCR can represent a useful instrument for prompt identification of the species of *Candida* responsible for infection, as demonstrated previously (Liguori *et al.*, 2007, Liguori *et al.*, 2009, Liguori *et al.*, 2010). An important goal is to establish a real-time PCR protocol for the rapid diagnosis of candidosis which will detect any species of *Candida* in clinical samples, and define their proportional role in the pathogenesis of infection. Moreover, molecular methods could be employed to detect azole resistance, as shown for *C. glabrata* through quantitative RT-PCR (Gygax *et al.*, 2008). With regard to strain typing, the techniques employed showed sufficient reliability, although there are disadvantages due to the time needed to carry out the procedures and the complex interpretation of the results.

Most pairs of isolates consisted of strains from different species, and our sample size was not large enough to carry out statistical analyses or to broaden the study to measure resistance determinants. Real-time PCR identified the deregulation of efflux pump genes as the possible mechanism involved in recurrent candidosis in one case. In similar studies using northern blotting on *C. albicans* and *C. glabrata* serial isolates from immunocompromised subjects treated with fluconazole, the same mechanism was identified to explain the increase in MIC after therapy (Marr *et al.*, 1998, Marr *et al.*, 2001; Bennett *et al.*, 2004). Analysis of gene expression seems to be necessary to identify the processes responsible for relapse, and real-time PCR, which is rapid and easy to perform, was shown to be suitable for this process. Its use should be extended to other genes, to define the mechanisms that determine the persistence of yeasts in the host and their control, especially in immunocompromised patients and in hospital settings.

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