Multicenter survey of *in vitro* antifungal resistance in yeasts of medical importance isolated from Spanish patients

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Summary

Twelve Spanish laboratories collected 325 yeast clinical isolates during a 30 day's period, among them 224 *Candida albicans*, 30 *Candida glabrata*, and 27 *Candida parapsilosis. In vitro* antifungal susceptibility to amphotericin B, ketoconazole, fluconazole and itraconazole was determined by an agar diffusion test (Neo-Sensitabs®, Rosco, Denmark). All the isolates tested were susceptible *in vitro* to amphotericin B and nearly all (97.2%) to itraconazole. *In vitro* susceptibility to fluconazole and ketoconazole was high (90.2% and 91.4% of isolates, respectively) but showed variations depending on the species tested. Resistance to fluconazole and ketoconazole was low in *C. albicans* (4% and 3%, respectively), but 30% of *Candida guilliermondii* and 36% of *C. glabrata* isolates were resistant to fluconazole. Ketoconazole resistance was observed in 40% of *C. glabrata*, and 17% of *Candida tropicalis*. Resistance to antifungal drugs is very low in Spain and it is related to non-*C. albicans* isolates.

Key words

Antifungal susceptibility, Agar diffusion, *Candida,* Yeasts, Spain, Multicenter, Cross-resistance

Estudio multicéntrico de la resistencia *in vitro* a los antifúngicos en levaduras de importancia médica aisladas de pacientes españoles

Se evaluaron 325 levaduras aisladas en 12 laboratorios españoles durante un periodo de 30 días, incluyendo 224 *Candida albicans*, 30 *Candida glabrata* y 27 *Candida parapsilosis*. Se valoró la sensibilidad *in vitro* a anfotericina B, ketoconazol, fluconazol e itraconazol por un método de difusión en agar con tabletas Neo-Sensitabs® (Rosco, Dinamarca). Todos los aislamientos fueron sensibles a anfotericina B y, casi todos también, a itraconazol (97,2%). La sensibilidad a fluconazol y ketoconazol fue elevada (90,2% y 91,4% de los aislamientos, respectivamente) pero mostraba variaciones importantes dependiendo de las especies estudiadas. La resistencia a fluconazol y ketoconazol era baja en *C. albicans* (4% y 3%, respectivamente), pero el 30% de los aislamientos de *Candida gui-*

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1130-1406/99/5.00 Euros

lliermondii y el 36% de los de *C. glabrata* eran resistentes a fluconazol. Se observó resistencia *in vitro* a ketoconazol en el 40% de los aislamientos de *C. glabrata* y el 17% de los de *Candida tropicalis*. La resistencia a los antifúngicos de uso habitual es muy baja en España y está asociada a los aislamientos de especies diferentes a *C. albicans*.

Sensibilidad a los antifúngicos, Difusión en agar, *Candida,* Levaduras, España, Multicéntrico, Resistencia cruzada

The increasing importance of severe mycoses caused by yeasts in hospitalized and immunocompromised patients has raised the use of systemic antifungal agents and the development of new drugs or new formulations of old antifungals [1-3]. Amphotericin B is still the gold standard for treatment but other new and less toxic antifungals have been developed and many studies reflect their utility in the clinical setting [2]. *In vitro* resistance to amphotericin B is reduced and no clinical evidence of this phenomenon is observed in therapeutical use of this antifungal agent [4]. Resistance to fluconazole has been reported in HIV-infected patients with oropharyngeal candidiasis and long-term treatment with this drug and a selection of natural resistant species, as *Candida krusei*, has been observed in bone-marrow transplant patients treated prophylactically with fluconazole [1-3]. *In vitro* susceptibility testing of yeasts with these antifungals is important to establish the susceptibility patterns of isolates recovered from different regions and countries and to detect the prevalence of resistant isolates.

A collaborative resistance survey was carried out with the aim of investigate the susceptibility patterns of Spanish yeast clinical isolates to the most commonly used systemic antifungals: amphotericin B, ketoconazole, fluconazole and itraconazole.

MATERIAL AND METHODS

Study design. Twelve Spanish hospital and university-affiliated laboratories collected 325 yeast clinical isolates during a 30 day's period in 1994, among them 224 *Candida albicans*, 30 *Candida glabrata*, and 27 *Candida parapsilosis* (Tables 1 and 2). Participants were asked to include consecutive isolates, only one isolate per species per patient. In Table 2, the number of isolates of each species included in the study are summarized. Assays were performed following the same protocol of identification and susceptibility testing, in the laboratory where the clinical strains were isolated and results reported to the coordinating laboratory.

Table 2. Species distribution for the 325 clinical isolates evaluated.

Species	No. of isolates
Candida albicans	224
Candida glabrata	30
Candida parapsilosis	27
Candida tropicalis	12
Candida guilliermondii	10
Cryptococcus neoformans	4
Other fungal species	18

Identification of yeasts. The isolates were identified by conventional mycological methods, such as germ tube test, chlamydoconidia production and microscopic morphology on corn meal agar (Oxoid, UK) with Tween 80 by the Dalmau method [5], as well as biochemical characterization with the ATB ID 32C system (BioMérieux, France).

Agar diffusion test. The growth inhibition zone and the category (resistant, intermediate and susceptible) were determined for amphotericin B (AMB), ketoconazole (KTZ), fluconazole (FLZ) and itraconazole (ITZ) using a commercial agar diffusion test with Neo-Sensitabs® tablets (Rosco, Denmark) [6-15]. Neo-Sensitabs® sensitivity testing is a standardized agar diffusion method which includes antifungal agents in tablets of 9 mm of diameter with AMB (10 µg), KTZ (15 µg), FLZ (15 µg) and ITZ (10 µg). Inocula contained $5x10^5$ cells/ml and was prepared from an overnight subculture on Sabouraud Glucose Agar (Difco, USA), obtaining suspensions corresponding to McFarland 0.5 standard and then diluted (1:2) with sterile saline solutions. For *Candida krusei* strains, inocula was equivalent to McFarland 0.5 standard, diluted 1:10 in saline solutions. Two milliliters of each inoculum suspensions were poured onto the agar surface and later removing the liquid in excess. Plates were dried at 35∞C for 15 min and tablets were placed onto the agar surface. Plates were incubated at 35∞C for all the species but *Cryptococcus* spp. isolates were incubated at 30∞C.

Table 1. Sources of the studied isolates.

Town	No. of isolates	Clinical specimen							
		ODT	VAG	BLO	URI	PER	S&N	RTR	CSF
Alcoy (Alicante)	30	1	14	0	8	1	2	4	0
Barcelona	27	0	13	0	6	0	7	1	0
Bilbao	56	45	11	0	0	0	0	0	0
Córdoba	30	2	0	9	5	2	9	3	0
Huércal-Overa (Almería)	13	1	7	0	0	0	1	4	0
Huesca	16	3	8	0	0	0	2	3	0
La Laguna (Tenerife)	24	3	3	2	4	0	2	10	0
Santander	24	2	7	1	1	0	2	11	0
Santiago (La Coruña)	18	12	0	0	0	0	6	0	0
Sevilla	30	23	0	1	3	1	0	2	0
Vigo (Pontevedra)	31	11	4	1	0	1	0	12	2
Zaragoza	26	2	2	21	1	0	0	0	0

ODT= Mouth and digestive tract; VAG= Vagina; BLO= Blood; URI= Urine; PER= Peritoneum; S&N= Skin and nails; RTR= Respiratory tract; and CSF= cerebrospinal fluid.

Table 3. Susceptibility of yeast isolates to amphotericin B, ketoconazole, fluconazole and itraconazole.

Species (No. of isolates)	Percent of isolates susceptible to			
	Amphotericin B (10)	Ketoconazole (22)	Fluconazole (22)	Itraconazole (11)
Candida albicans (224)	100	96.89	95.98	97.77
Candida glabrata (30)	100	60	63.33	96.67
Candida parapsilosis (27)	100	92.59	96.30	100
Candida tropicalis (12)	100	83.33	91.67	100
Candida guilliermondii (10)	100	100	70	100
Cryptococcus neoformans (4)	100	75	75	100
Candida inconspicua (2)	100	50	100	100
Candida famata (2)	100	100	100	100
Candida krusei (2)	100	0	0	100

Figures in parentheses indicate the chosen breakpoint in millimeters.

Table 4. Cross-resistance to other antifungals amongst *Candida* resistant to fluconazole.

Yeasts resistant to (No. of isolates)	No. of these isolates also resistant to fluconazole		
	Ketoconazole	Itraconazole	both azoles
Candida albicans (9)	4	5	3
Candida glabrata (11)	7	1	1
Candida parapsilosis (1)	0	0	0
Candida tropicalis (1)	0	0	0
Candida guilliermondii (3)	0	0	0
Candida krusei (2)	2	0	0

Shadomy modified medium was used (Yeast Nitrogen Base, asparagine and glucose) with phosphate buffer to pH 7 and containing cloramphenicol to avoid bacterial contamination. Media was heated at 100∞C during 15 min, and then cooled to 60∞C before pouring the medium in Petri dishes (12 cm x 12 cm). A pre-diffusion drying was made at 37∞C during 15 min. After an incubation period of 20-24 h, the inhibition diameter zones were read in mm with a caliper (Mitutoyo, Japan). For AMB the presence of a clear and visible zone was measured, with no colonies inside it. For the azole derivatives inhibition zone was measured up to the limit of colonies of normal size. This test allowed the categorization of the isolates into susceptible, intermediate or resistant, according to the zone diameter interpretative standards. For AMB and ITZ, susceptible strains were those with diameter zones more or equal to 15 mm; intermediate, between 10 mm and 14 mm; and resistant those with no zone at all. For FLZ and KTZ, susceptible strains were those with diameter zones of more or equal to 20 mm; intermediate, between 12 mm and 19 mm; and resistant, with less than 12 mm according to manufacturers instructions [6,7]. The collection and manufacturers reference strains used in the study included C. albicans American Type Culture Collection (ATCC) 90028, *C. albicans* ATCC 90029, *C. albicans* Y01.09 (Pfizer, UK) (susceptible to FTZ), *C. albicans* Y01.19 (Pfizer)(resistant to FLZ), *C. albicans* ATCC 64548 (susceptible to all the antifungal tested), *C. albicans* ATCC 64550 (resistant to ITZ), *C. glabrata* ATCC 90030 (resistant to FTZ) and *C. glabrata* 2238NL (Rosco) (resistant to AMB), and *C. parapsilosis* ATCC 22019 (susceptible to all the antifungal tested) and *C. krusei* ATCC 6258 (resistant to FLZ) as NCCLS Quality Control strains [16].

RESULTS AND DISCUSSION

In vitro susceptibility values of reference strains were inside the limits obtained in previous studies of standardization of antifungal susceptibility tests [10-12,14,16].

The results of this study have shown that *in vitro* resistance to antifungal drugs is very low in Spain. Influence of geographical factors has been pointed out about resistance levels to AMB and 5-fluorocytosine. All the isolates tested were susceptible *in vitro* to AMB and nearly all (97.2%) to ITZ (Table 3). This high AMB susceptibility rate observed in this study contrasts with the results reported by Torres-Rodríguez *et al.* [17] who described a rate of 2% and 65.5% of resistant and intermediate isolates, respectively, by using the same method. The present results are closer to the therapeutic reality, as clinical resistance to AMB is exceptionally observed with yeasts. Other authors [15,18,19] have observed this absence of AMB resistance by using the same agar diffusion method or broth microdilution methods but some intermediate isolates has been described.

In vitro susceptibility to FLZ and KTZ was high (90.2% and 91.4% of isolates, respectively) but showed variations depending on the species tested. Resistance to FLZ and KTZ was low in *C. albicans* (4% and 3%, respectively), but 30% of *Candida guilliermondii* and 36% of *C. glabrata* isolates were resistant to FLZ. Fluconazole *in vitro* resistance (7.7%) is related to isolates from *C. glabrata, C. krusei* or *C. guilliermondii* (comprising <15% of all the isolates tested). These species of *Candida* are characterized by their reduced susceptibility to triazole antifungals, as has been previously described [reviewed in 2,4]. The rate of FLZ resistance is very similar to those previously published in local studies [15,18,20]. Discrepancies between *in vitro* susceptibility to ITZ rate in this multicenter study and that observed by Carrillo *et al.* [15] (97,23% versus 89.1%) could be associated to differences in species distribution of isolates tested in both studies. Ketoconazole resistance was observed in 40% of *C. glabrata*, and 17% of *Candida tropicalis*. The *in vitro* cross-resistance to other azole antifungals amongst yeast resistant to FLZ is shown in the Table 4. Only six resistant strains to ITZ were observed in the 25 resistant isolates to FLZ studied and 13 of them were resistant to KTZ. The differences between susceptibility to ITZ and FLZ could be attributed to the better ability of ITZ to cross the cell membrane in comparison with FLZ [4].

CONCLUSIONS

Resistance to antifungal drugs is very low in Spain. FLZ *in vitro* resistance is related to isolates from *C. glabrata*, *C. krusei* or *C. guilliermondii* (comprising <15% of all the isolates tested). *in vitro* resistance to KTZ is associated to *C. glabrata*, *C tropicalis* and *C. parapsilosis* (comprising <25% of all the isolates tested).

The material used for susceptibility testing in this multicenter study was funded by Rosco, Taastrup, Denmark and Izasa SA, Barcelona, Spain. We thank Dr. Antonio Ansó (Izasa SA) for providing materials, and technical assistance.
Guillermo Quindós was partially financed by grants 97/2052 (Ministerio de Sanidad y Consumo / Fondo de Investigación Sanitaria - Plan Nacional de Investigación Científica y Desarrollo Tecnológico, modalidad C para 1997) and UPV 093.327-EC233/97 (Universidad del País Vasco).

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