Antifungal susceptibilities of *Candida* spp. isolated from blood in Spain and Argentina, 1996–1999

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The aim of this study was to identify retrospectively trends in species distribution and susceptibility patterns of *Candida* species causing bloodstream infections in 99 medical centres (55 in Spain and 44 in Argentina) from 1996 to 1999. A total of 744 *Candida* isolates were sent to the mycology reference laboratories during the study period (514 to the Spanish laboratory and 230 to the Argentinian laboratory). *Candida* non-*albicans* strains caused more episodes of fungaemia than *Candida albicans* isolates in both Spain and Argentina. *C. albicans* was isolated in 30.2% (155/514) and 40.9% (94/230) of episodes in Spain and in Argentina, respectively. In addition, *Candida parapsilosis* was the second most commonly isolated pathogen (36.4%). *Candida tropicalis* caused 13.7% of infections and *Candida glabrata* 7.4%. The amphotericin B MIC was ≤1 mg/L for 97.5% of isolates, and 8.3% of strains had decreased susceptibility to flucytosine. Regarding susceptibility to azole agents, 9.9% (74/744) and 21.9% (163/744) exhibited decreased susceptibility to fluconazole and itraconazole, respectively. For *Candida* species, some marked differences were found between countries, and decreased susceptibility to azole agents was detected significantly more frequently ($P < 0.05$) among Argentinian isolates of *C. albicans*, *C. parapsilosis* and *C. tropicalis*. These findings reinforced the need for continued surveillance programmes to analyse the factors that may have an influence on candidaemia incidence. Susceptibility patterns were obtained by means of the proposed reference procedure for antifungal susceptibility testing of the European Committee on Antibiotic Susceptibility Testing (EUCAST). Excellent interlaboratory agreement was achieved for MICs for quality control strains noted in Spain and in Argentina (intraclass correlation coefficient of 0.97), indicating that the EUCAST procedure is a reliable methodology for susceptibility testing.

Introduction

The increase in fungal infections has prompted an increase in the use of antifungal agents, and in practice the widespread clinical use of these agents has resulted in measurable rates of acquired or innate fungal resistance in *Candida* species.[1–3] The emergence of fungal resistance has also been reported among yeast isolates causing bloodstream infections.[4–6] However, comparative studies from an international perspective that address trends in species distribution and antifungal susceptibility profiles among strains collected from blood cultures are limited. The SENTRY Antimicrobial Resistance Surveillance Program is an international programme designed to study bloodstream infections in medical centres in North America and Europe.[3,7,8] The findings of the SENTRY programme have underscored geographical differences in species distribution and antifungal resistance of *Candida* spp., proving that continued surveillance at an international level is necessary to detect new trends among invasive strains of yeasts.

The NCCLS has published a reference methodology for susceptibility testing of yeasts.[9] The broth dilution procedures

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proposed by the NCCLS show good interlaboratory reproducibility, which is essential to identify organisms unlikely to respond to certain antifungal treatments. This reference methodology is not problem free, and some studies have been conducted to try to overcome the limitations.[10] The Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) has proposed a standard for susceptibility testing of fermentative yeasts.[11] This standard takes into account the reference broth microdilution method of the NCCLS, but also includes modifications such as RPMI supplemented with 2% glucose as assay medium, inoculum size of 10^5 cfu/mL, flat-bottomed trays, spectrophotometric reading and 50% inhibition as the endpoint for azole agents and flucytosine. The aim of these modifications is to obtain a less subjective, 24 h incubation methodology for antifungal susceptibility testing. Recent reports have indicated that these modifications have yielded a reproducible technique that shows good agreement with the NCCLS reference procedure.[12]

This work analyses species distribution and susceptibility patterns of *Candida* isolates causing fungaemia in Spain and Argentina. The study includes strains received by the mycology reference laboratories of Spain and Argentina from various medical centres. Antifungal susceptibility testing was performed following the EUCAST methodology. The details of these analyses and their implications are the subject of this work.

Materials and methods

Organisms

A total of 744 isolates were included. All *Candida* strains were recovered from blood cultures between 1996 and 1999 at 99 different medical centres (55 in Spain and 44 in Argentina). There were 514 Spanish and 230 Argentinian isolates. Each strain represented a unique isolate from a patient. The isolates were sent to the mycology reference laboratories for antifungal susceptibility testing. Table 1 shows the species distribution and country of origin. Before susceptibility testing, all isolates were identified by morphological and biochemical tests.[13] *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were incorporated as quality control strains in each set of experiments.[9]

Assay medium

The assay medium was RPMI 1640 without sodium bicarbonate and with L-glutamine (Sigma–Aldrich Química, Madrid, Spain; Gibco-BRL Life Technologies, Buenos Aires, Argentina) buffered to pH 7 with 0.165 M morpholinepropanesulphonic acid (MOPS; Sigma–Aldrich, Spain; Sigma Chemical Co., Buenos Aires, Argentina) and supplemented with glucose 18 g/L to reach a final concentration of 2% (RPMI–2% glucose). Culture medium was prepared as a double-strength solution and was sterilized by filtration.[12]

Table 1. Species distribution of *Candida* isolates causing bloodstream infections in Spain and Argentina

Candida species	Spain (%)	Argentina (%)	Total (%)
C. albicans	155 (30.2)	94 (40.9)	249 (33.5)
C. parapsilosis	201 (39.1)	70 (30.4)	271 (36.4)
C. tropicalis	55 (10.7)	47 (20.4)	102 (13.7)
C. glabrata	49 (9.5)	6 (2.6)	55 (7.4)
C. krusei	26 (5.1)	5 (2.2)	31 (4.2)
C. guilliermondii	17 (3.3)	0	17 (2.3)
C. lusitaniae	5 (1)	0	5 (0.7)
C. famata	1 (0.2)	4 (1.7)	5 (0.7)
C. pelliculosa	1 (0.2)	3 (1.3)	4 (0.5)
C. pintolopesii	3 (0.6)	0	3 (0.4)
C. kefyr	1 (0.2)	0	1 (0.1)
C. haemulonii	0	1 (0.4)	1 (0.1)
All	514	230	744

Antifungal agents

The antifungal agents used in this study were as follows: amphotericin B, flucytosine (Sigma-Aldrich Química, Spain; Sigma Chemical Co., Argentina), fluconazole (Pfizer, Madrid, Spain; Pfizer, Buenos Aires, Argentina) and itraconazole (Janssen Farmacéutica, Madrid, Spain; Janssen Farmaceútica, Buenos Aires, Argentina). Stock solutions were prepared in 100% dimethylsulphoxide (DMSO; Sigma-Aldrich Química, Spain; Sigma Chemical Co., Argentina) apart from flucytosine, which was dissolved in sterile distilled water. Stock solutions were prepared at concentrations 100 times the highest to be tested and were frozen at −70°C until used.

Preparation of inoculum

The yeast isolates were grown on Sabouraud dextrose agar (Oxoid, Madrid, Spain; Gibco-BRL Life Technologies, Argentina) for 24 h at 35°C. Suspensions were prepared by picking five distinct colonies of ≥1 mm diameter and suspending them in 5 mL of distilled sterile water. A spectrophotometric procedure for inoculum preparation was used.[9] The final inoculum suspension contained between 0.5×10^5 and 2.5×10^5 cfu/mL.

Susceptibility testing

Sterile plastic microtitration plates containing flat-bottomed wells were utilized (Labcenter, Madrid, Spain; Nunclon, Nunc, Buenos Aires, Argentina). The plates contained two-fold serial dilutions of the antifungal drugs with a volume of assay medium of 100 µL/well. Two drug-free medium wells

for sterility and growth controls were used. The trays were inoculated with 100 μL/well of the final inoculum, with the exception of sterility control wells. Final concentrations of drugs were 0.03–16 mg/L for amphotericin B, 0.12–64 mg/L for flucytosine and fluconazole, and 0.015–8 mg/L for itraconazole. The microtitration plates were incubated at 35°C for 48 h in a humid atmosphere.

Endpoint determination

The MICs were determined at 24 and 48 h. Both laboratories determined the endpoint using a spectrophotometer method. In Spain the spectrophotometer was a MRXII (Dynatech, Cultek, Madrid, Spain) and the wavelength used was 530 nm. In Argentina the spectrophotometer was a Multiskan RC (Labsystem, Helsinki, Finland) and the wavelength employed was 450 nm.

For amphotericin B, the endpoint was the lowest drug concentration exhibiting reduction in growth of 95% or more compared with that of the control growth. For flucytosine and azoles the endpoint was the lowest drug concentration exhibiting reduction in growth of 50% or more compared with that of the control growth.

Interpretive criteria for susceptibility to flucytosine, fluconazole and itraconazole followed the recommendations of the NCCLS.[9] For analysis of the results of susceptibility testing, strains were classified as being susceptible or having decreased susceptibility. Decreased susceptibility to antifungal agents included the dose-dependently susceptible, intermediate and resistant categories of the NCCLS. Separate analysis of the dose-dependently susceptible or intermediate and resistant strains showed no differences in species distribution and epidemiological patterns between these categories.

Statistical analysis

Differences in proportions were determined by Fisher's exact test or by χ^2 analysis. A P value of <0.05 was considered significant. The results were not compared when one of the values was 0%.

The reproducibility of the results obtained from the two laboratories (Spain and Argentina) was evaluated by calculating an intraclass correlation coefficient (ICC), which compared the results of 23 consecutive determinations of the MICs for the two quality control strains included. A two-way random effect model was utilized to calculate the ICC, which was expressed over a maximum value of 1 with a confidence interval of 95% (95% CI). This coefficient is a measure of the reproducibility of and the correlation between MICs from the Spanish and Argentinian laboratories. In addition, the ICC is an assessment of the reliability of EUCAST methodology. MICs were transformed on $\log_2$ data. All statistical analysis was carried out with the Statistical Package for the Social Sciences (SPSS, version 10.0; SPSS S.L., Madrid, Spain).

Results

Species distribution

Table 1 shows the distribution of *Candida* species in each country. Both reference laboratories received a majority of non-*albicans* strains during the study period. The percentage of *Candida albicans* strains causing bloodstream infections was 30.2% (155/514) among Spanish isolates and 40.9% (94/230) among Argentinean isolates ($P = 0.003$, χ^2 test). In addition, *C. parapsilosis* was collected more commonly than *C. albicans* (39.1% versus 30.2%) in the Spanish laboratory.

C. parapsilosis, *Candida glabrata* and *C. krusei* were more frequently encountered in blood cultures in Spain than in Argentina (39.1% versus 30.4%, $P = 0.023$; 9.5% versus 2.6%, $P < 0.001$; and 5.1% versus 2.2%, $P > 0.05$, respectively). On the other hand, *Candida tropicalis* was isolated more frequently in Argentina (20.4%) than in Spain (10.7%, $P < 0.001$). Data for other species are displayed in Table 1.

Antifungal susceptibility testing results for blood isolates from Argentina and Spain

In Table 2, the MIC_{50}s, MIC_{90}s and MIC ranges of all antifungal agents are shown. The MIC_{50} and the range are displayed for those species with <20 isolates. For those with fewer than 10 isolates, only the range is included. Those species with fewer than five isolates are not included in Table 3.

Table 2 shows that amphotericin B exhibited good *in vitro* activity against the majority of isolates (MIC < 1 mg/L). For some isolates however, the MIC of amphotericin was 2 mg/L. Among Spanish isolates, 4.5% (nine of 201) of *C. parapsilosis*, 1.8% (one of 55) of *C. tropicalis*, 2% (one of 49) of *C. glabrata* and 11.5% (three of 26) of *C. krusei* strains had an MIC of amphotericin B of 2 mg/L. Among Argentinean isolates, only 4.3% (two of 47) of *C. tropicalis* showed an MIC of 2 mg/L. Among unusual species, amphotericin B MICs ranged from 0.12 to 1 mg/L, except for the *Candida haemulonii* strain. The MIC of amphotericin B obtained for this isolate was 8 mg/L.

Regarding results for susceptibility to flucytosine, overall 8.3% (62/744) of isolates showed decreased susceptibility to flucytosine. No significant differences were encountered between the percentages of Spanish and Argentinian isolates intermediate or resistant to flucytosine. However, several significant differences were found when results were analysed by yeast species. Briefly, the percentage of *C. albicans* with decreased susceptibility to flucytosine among Argentinean isolates was 5.3% (five of 94), and among Spanish isolates was 0.6% (one of 155) ($P = 0.019$). The percentage of *C. tropicalis* isolates exhibiting a flucytosine MIC ≥ 8 mg/L among Argentinian isolates was 10.6% (five of 47), and among Spanish isolates was 1.8% (one of 55) ($P = 0.05$). Finally, 40.8% (20/49) of Spanish *C. glabrata* isolates showed decreased susceptibility to this agent, whereas among Argentinian

Table 2. *In vitro* susceptibilities to amphotericin B, flucytosine, fluconazole and itraconazole of strains from blood cultures of *Candida* species isolated in Argentina and Spain from 1996 to 1999

		MIC (mg/L)							
		Spain				Argentina			
Candida species		*n*	MIC_{50}	MIC_{90}	range	*n*	MIC_{50}	MIC_{90}	range
C. albicans	amphotericin B	155	0.50	1	0.03–1	94	0.5	0.5	0.06–1
	flucytosine		0.25	0.5	0.12–128		0.12	2	0.12–128
	fluconazole		0.25	0.5	0.12–128		0.25	8	0.12–128
	itraconazole		0.03	0.12	0.015–0.12		0.015	0.5	0.015–16
C. parapsilosis	amphotericin B	201	0.5	1	0.03–2	70	0.5	1	0.12–1
	flucytosine		0.25	1	0.03–64		0.12	1	0.12–128
	fluconazole		1	2	0.12–8		2	4	0.12–16
	itraconazole		0.03	0.12	0.015–1		0.06	0.5	0.015–8
C. tropicalis	amphotericin B	55	0.5	1	0.03–2	47	0.5	1	0.12–2
	flucytosine		0.25	1	0.12–8		0.25	16	0.12–128
	fluconazole		0.5	1	0.06–4		0.5	4	0.12–128
	itraconazole		0.06	0.25	0.015–1		0.03	1	0.015–16
C. glabrata	amphotericin B	49	0.5	1	0.03–2	6			0.25–0.5
	flucytosine		0.25	8	0.06–16				0.12–0.12
	fluconazole		8	64	1–128				0.25–128
	itraconazole		0.5	2	0.12–16				0.015–4
C. krusei	amphotericin B	26	1	2	0.03–2	5			0.5-0.5
	flucytosine		8	16	1–16				0.12–8
	fluconazole		32	128	8–128				1–128
	itraconazole		0.25	1	0.015–1				0.03–16
C. guilliermondii	amphotericin B	17	0.5		0.06–1				
	flucytosine		0.5		0.03–16				
	fluconazole		8		0.25–32				
	itraconazole		0.5		0.015–1				
C. lusitaniae	amphotericin B	5			0.25–1				
	flucytosine				0.12–0.5				
	fluconazole				0.12–2				
	itraconazole				0.03–0.25				
All	amphotericin B	514	0.5	1	0.03–2	230	0.5	1	0.06–8
	flucytosine		0.25	4	0.12–128		0.12	2	0.12–128
	fluconazole		0.5	16	0.06–128		0.5	8	0.12–128
	itraconazole		0.06	0.5	0.015–16		0.03	0.5	0.015–16

C. glabrata isolates, no intermediate or resistant organisms were encountered.

Concerning the results for susceptibility to azole agents, 9.9% (74/744) and 21.9% (163/744) of isolates exhibited decreased susceptibility to fluconazole and itraconazole, respectively. Overall, no marked differences were found between countries. When analysing by *Candida* species, however, some significant evidence of differences between laboratories was observed. Among Argentinian *C. albicans* isolates, 8.5% (eight of 94) showed decreased susceptibility to fluconazole and among Spanish isolates the MIC of fluconazole was ≥16 mg/L for 1.9% (three of 155) of strains ($P = 0.014$). Higher MICs of fluconazole were obtained for 6.3% (three of 47) of Argentinian isolates of *C. tropicalis*, whereas *C. tropicalis* with decreased susceptibility to fluconazole was not encountered among the Spanish isolates. Regarding itraconazole results, Argentinian *C. albicans* and *C. parapsilosis* isolates more frequently exhibited decreased susceptibility to itraconazole than Spanish isolates (14.8% versus 6.4%, $P = 0.02$; and 20% versus 8.9%, $P = 0.01$, respectively). In contrast to preceding results, the percentage of intermediate or resistant isolates of *C. glabrata* was significantly higher among Spanish than Argentinian isolates (93.8% versus 66.6%, $P = 0.012$). Table 3 summarizes the susceptibility results for azole agents categorized by *Candida* species.

An analysis including fluconazole- and itraconazole-resistant strains only showed no differences in species distribu-

Table 3. Percentages of strains exhibiting decreased susceptibility to azole agents grouped by *Candida* species

Candida species[a]	Laboratory	*n*	Decreased suseptibility to fluconazole (%)[b]	Decreased suseptibility to itraconazole (%)[c]
C. albicans	Spain	155	1.9	6.4
	Argentina	94	8.5	14.8
C. parapsilosis	Spain	201	0	8.9
	Argentina	70	1.4	20
C. tropicalis	Spain	55	0	18.1
	Argentina	47	6.3	19.1
C. glabrata	Spain	49	42.8	93.8
	Argentina	6	50	66.6
C. krusei	Spain	26	88.4	57.6
	Argentina	5	80	60
C. guilliermondii	Spain	17	23.5	88.2
	Argentina	0		
C. lusitaniae	Spain	5	0	20
	Argentina	0		
All	Spain	514	10.3	22.5
	Argentina	230	9.1	20.4

[a]Unusual species (less than five isolates) are not included.
[b]Percentage of strains with MIC of fluconazole ≥16 mg/L.
[c]Percentage of strains with MIC of itraconazole ≥0.25 mg/L.

tion compared with analysis also including dose-dependently susceptible and resistant organisms. Among Spanish isolates, 3.1% (16/514) and 6% (31/514) exhibited resistance to fluconazole and itraconazole, respectively. Among Argentinian isolates, the percentage of strains resistant to fluconazole was 4.3% (10/230) and to itraconazole was 7.8% (18/230).

Reproducibility of results between Argentina and Spain

The two quality control strains recommended by NCCLS document M27-A[9] were used throughout this study. All MICs obtained in Argentina were inside the NCCLS reference range, except for three amphotericin B MICs obtained for *C. krusei* ATCC 6258. In Spain, some MICs of amphotericin B, flucytosine, fluconazole and itraconazole were one two-fold dilution below the reference range. The reproducibility and the correlation among MICs values of Spanish isolates and Argentinian isolates were high, with an ICC of 0.97 (95% CI 0.96–0.98). The ICC is a reverse measurement of the variability of the MICs for quality control strains, and is expressed over a maximum value of 1.

Discussion

Numerous studies have demonstrated that the incidence of candidaemia has dramatically increased in the past 15 years.[14–16] Both American and European multicentre investigations have indicated that *Candida* spp. are an important cause of nosocomial bloodstream infection, and that the proportion of infections due to non-*albicans* species is increasing. The Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) programme documented that *Candida* spp. were the fourth most common bloodstream pathogen, and *C. glabrata* emerged as an important cause of infection in the USA (20% of episodes).[17] Other multicentre studies, such as the US National Epidemiology of Mycoses Survey (NEMIS) programme and several European surveys, have reported similar findings.[18–24] The SENTRY Antimicrobial Resistance Surveillance Program is an international study designed to analyse bloodstream infections in medical centres of North America and Europe.[25] The programme detected 1184 episodes of candidaemia in 71 medical centres in the USA, Canada, Latin America and Europe between 1997 and 1999. In the USA, 45% of candidaemias were due to non-*albicans* species. *C. glabrata* was the most common non-*albicans* species in the USA (21%). The proportion of non-*albicans* bloodstream infections was highest in Latin America (55%). *C. albicans* accounted for 60% of infections in Canada and 58% in Europe. *C. parapsilosis* was the most common non-*albicans* species in Latin America (25%), Canada (16%) and Europe (17%). Resistance to fluconazole and itraconazole was observed with *C. glabrata* and *C. krusei*, and was observed more rarely among other species, although a trend of increased

susceptibility of *C. glabrata* to fluconazole was noted over the 3 year period.

Focusing on the results of our study it can be seen that, overall, non-*albicans* species were isolated more frequently than *C. albicans*. In the Spanish laboratory, 69.8% of episodes of candidaemia were caused by non-*albicans* species, and in the Argentinian laboratory, 59.1% of episodes. The rate of the incidence of *C. parapsilosis* fungaemia in both Spain and Argentina is interesting to note. Among Spanish isolates, *C. parapsilosis* was the most common pathogen (39.1%) and among Argentinean isolates, the second most frequently isolated *Candida* spp. (30.4%). This organism is often a cause of clusters of nosocomial cases related to poor catheter care or poor infection control practices.[3,4] Compared with the incidence in other studies, it is remarkable that similar rates of *C. parapsilosis* were described in neonatal units included in the NEMIS programme and in Latin American medical centres in the SENTRY programme. In addition, a single US institution study has reported the emergence of *C. parapsilosis* as the predominant *Candida* species causing fungaemia in a children's hospital (49% of episodes), and an Italian study and a Brazilian multicentre study have reported a rate of nosocomial candidaemias caused by *C. parapsilosis* comparable to the values obtained in the present study.[26–28] These data support the hypothesis that geographical variation may have influenced the high incidence of *C. parapsilosis* fungaemia in some institutions, but whether true or not, given the rates of *C. parapsilosis* infection in Spanish and Argentinian medical centres, good catheter care and infection control practices should be implemented in both countries.

The *in vitro* susceptibility results obtained for amphotericin B and flucytosine are consistent with other reports.[8,17,22,25] MICs of amphotericin B for the great majority of isolates (97.5%) ranged from 0.12 to 1 mg/L. In addition, 8.3% of strains had decreased susceptibility to flucytosine, a rate similar to those recorded by the SCOPE and SENTRY programmes and some European surveys.[17,25] Regarding susceptibility results for azole agents, as others have reported,[8,24] the percentages of fluconazole- and itraconazole-resistant organisms causing candidaemia were low, 3.5% and 6.6%, respectively. However, the rates of decreased susceptibility to azole agents (category including dose-dependently susceptible and resistant organisms) were significantly higher. Overall, the percentages of decreased susceptibility to fluconazole and itraconazole were 9.9% and 21.9%, respectively. On the other hand, our data indicated that although the rates of resistance to azole agents are low, there were regional differences in resistance to these agents when results were analysed by species. Decreased susceptibility to azole agents was detected more frequently among Argentinian isolates of *C. albicans*, *C. parapsilosis* and *C. tropicalis*. The explanation for this resistance trend is unknown. These findings reinforce the need for active surveillance programmes that analyse multiple factors such as patient population, hospital infection control practices, antifungal and antimicrobial therapies, cytotoxic treatments and underlying diseases.

Finally, the reproducibility of the susceptibility results using the EUCAST procedure was very high. A high correlation was observed between MICs for quality control strains obtained in Spain and Argentinian laboratories (ICC = 0.97), indicating that the EUCAST methodology is a reliable technique that gives good interlaboratory agreement. In addition, the MICs determined by the EUCAST method show a good agreement with the NCCLS reference MICs for quality control strains.

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