

Fluconazole Susceptibility of *Candida* Species in Singapore by Disc Diffusion Test

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Abstract

Introduction: Resistance of *Candida* species to fluconazole has been increasingly reported worldwide. To date, the prevalence of resistance to fluconazole in Singapore is unknown. The aim of this study was to use a newly described agar disc diffusion method to study levels of susceptibility of *Candida* species to fluconazole in several hospitals in Singapore. **Materials and Methods:** Three hundred and ninety *Candida* isolates from clinical specimens collected from different sites were tested, of which 191 isolates (49.0%) were *C. albicans*, 69 (17.7%) were *C. parapsilosis*, 59 (15.1%) were *C. glabrata*, 51 (13.1%) were *C. tropicalis* and 4 (1.0%) were *C. krusei*. Susceptibility testing was performed using 25 µg fluconazole discs and standard Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/mL of methylene blue. **Results:** Overall, 381 (97.7%) isolates were susceptible, 6 (1.5%) were susceptible dose-dependent, and 3 (0.8%) were resistant to fluconazole. Of the individual species, 99.5% of *C. albicans*, 93.2% of *C. glabrata*, 0% of *C. krusei*, and 100% of *C. parapsilosis*, *C. tropicalis* and other *Candida* species were susceptible. **Conclusion:** The resistance of *Candida* species to fluconazole, as measured using a new disc diffusion method, is low in Singapore, with the exception of *C. krusei*. Fluconazole remains a useful agent for the treatment of candidiasis in this country.

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Key words: Antifungal susceptibility testing, Disc diffusion method, Resistance

Introduction

Candida species is becoming increasingly important as a cause of serious infections particularly in immunocompromised patients. Surveillance in the USA between April 1995 and June 1996 revealed that *Candida* was the fourth leading cause of nosocomial bloodstream infections, accounting for 8% of such infections.¹ In Singapore, *Candida* ranks as the 10th most common isolate causing significant bloodstream infections in a study of over 1700 consecutive positive blood cultures in 1999 at the Singapore General Hospital, a tertiary referral hospital (Sng LH and Oon LLE, unpublished data). Human immunodeficiency virus (HIV) infection, organ and bone marrow transplants and the use of prosthetic devices have all contributed to this increasing incidence. This has resulted in the growing use of systemic antifungal agents, especially fluconazole.

Fluconazole is a triazole compound which acts by inhibiting ergosterol synthesis. The ease of administration, the availability of both oral and intravenous forms and the relative lack of toxicity have seen fluconazole emerging as an attractive alternative to amphotericin B in the treatment of *Candida* and *Cryptococcus* infections. However, reports of fluconazole-resistant *Candida* strains are now appearing.²

C. krusei is intrinsically resistant to fluconazole while *C. glabrata* is less susceptible² and these species are now increasing in incidence.³

Antifungal susceptibility testing is currently not routinely performed in the majority of clinical laboratories worldwide. The reference broth dilution method has only recently been standardised by the National Committee for Clinical Laboratory Standards (NCCLS)⁴ and is too labour-intensive and costly for many clinical laboratories. There is therefore a need for an easy, rapid and reliable method for screening of resistance to fluconazole. A disc diffusion method analogous to that used for testing antibacterial agents could be easily incorporated into a clinical laboratory. A few such methods have been developed and these have shown good correlation with the standard reference NCCLS broth dilution method.⁵⁻⁸

To date, the prevalence of resistance to fluconazole in Singapore is unknown. Such information is useful to guide local clinicians in the treatment of serious *Candida* infections. The aim of this study was to determine the level of susceptibility of *Candida* species to fluconazole in several hospitals in Singapore with the use of a newly described agar disc diffusion method. We also wanted to study the level of fluconazole susceptibility in blood cultures

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in particular, and also look at the epidemiological factors associated with fluconazole resistance if the numbers of resistant isolates permitted statistical analysis.

Materials and Methods

Isolates

One hundred and ninety-one isolates of *C. albicans* and 199 isolates of non-*albicans* species of *Candida* were collected from December 1997 to December 1998. These isolates were taken from clinical specimens sent to the mycology laboratory at the Singapore General Hospital. Duplicate isolates (same patient-same species within the same hospitalisation period) were excluded from the study. Two hundred and thirty-one (59.2%) isolates were from the Singapore General Hospital and the rest were sent from other government and restructured hospitals (148; 37.9%) and private laboratories (11; 2.8%). The isolates came from patients in a broad range of medical specialties, with 120 (30.8%) from internal medicine specialties, 75 (19.2%) from surgical specialties, 43 (11.0%) from intensive care units, 39 (10.0%) from obstetrics and gynaecology, 37 (9.5%) from haematology/oncology, 23 (5.9%) from paediatrics and the rest of the 53 (13.6%) from patients in other or unknown wards. One hundred and sixteen (29.7%) isolates were recovered from respiratory sites, 115 (29.5%) from blood and other sterile sites, 44 (11.3%) from genital sites, 30 (7.7%) from urine and the rest of the 85 isolates (21.8%) were from other sources.

Identification Methods

All isolates were identified to species level based on a conventional scheme that included determination of germ tube production, microscopic morphology on cornmeal agar and the use of a commercial kit, the API20C AUX (bioMérieux, France).

Susceptibility Testing

The agar disc diffusion test described by Meis et al⁷ was used in this study. This is a modification of the NCCLS M2-A6 disc diffusion method for bacteria susceptibility testing.⁹ The media used was standard Mueller-Hinton agar (BBL, Becton Dickinson, USA) supplemented with 2% glucose and 0.5 µg/mL of methylene blue. Testing was performed with a 25-µg fluconazole disc (Becton Dickinson, Sparks, MD, USA). The plates were then incubated at 35°C in ambient air for 18 to 24 hours. Slow growing isolates were read after 48 hours incubation only if there was insufficient growth at 24 hours.

Quality control (QC) strains were *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019. Acceptable ranges for zone diameters were 32 to 43 mm and 26 to 37 mm, respectively.⁷ QC testing was carried out with the performance of each batch of tests.

All test and QC plate zone endpoints were electronically read by a BIOMIC Plate Reader System (Giles Scientific Inc., Santa Barbara, CA, USA), which also interpreted and recorded the results.^{7,10} Zone diameter endpoints were read at 80% growth inhibition. To reduce operator variability in the reading of zone diameters, 2 technologists who were specially trained in using the BIOMIC system were involved in the susceptibility testing of the isolates in this study.

Zone sizes were interpreted as susceptible, susceptible-dose dependent or resistant. Interpretive breakpoints were based on zones that correlated, by means of regression analysis, with NCCLS recommended category breakpoints for minimum inhibitory concentrations (MICs) by the reference broth dilution method.⁷ The category susceptible-dose dependent implies the need to use sufficient doses of fluconazole to achieve clinical response for these isolates.¹¹ Minimum inhibitory concentrations of 50% and 90% (MIC₅₀ and MIC₉₀, respectively) of isolates of the individual *Candida* species were extrapolated from zone diameters by the BIOMIC computer system.

Epidemiological Data

The medical records of all patients who had *Candida* isolates with reduced susceptibility to fluconazole were studied for principal diagnosis and duration of antifungal therapy in the previous year.

Statistical Method

The chi-square test was used to compare the susceptibility pattern of *Candida* species from the Singapore General Hospital and other hospitals.

Results

Susceptibility of *Candida* species to Fluconazole

The distribution of *Candida* species was as follows: *C. albicans* (191; 49.0%), *C. parapsilosis* (69; 17.7%), *C. glabrata* (59; 15.1%), *C. tropicalis* (51; 13.1%), *C. rugosa* (6; 1.5%), *C. krusei* (4; 1.0%), *C. lusitanae* (3; 0.8%), *C. guilliermondii* (2; 0.5%), and one isolate each of *C. kefyr*, *C. maris*, *C. pelliculosa* and *C. zeylanoides*. One isolate could not be speciated. Table I shows the distribution of *Candida* species in the Singapore General Hospital and those from other restructured hospitals and private laboratories.

Overall, 381 (97.7%) isolates were susceptible, while 6 (1.5%) were susceptible-dose dependent and 3 (0.8%) were resistant. In the Singapore General Hospital, 228 (98.7%) were susceptible, 2 (0.9%) were susceptible-dose dependent and 1 (0.4%) was resistant. In the combined data from other hospitals and private laboratories, 153 (96.3%) isolates were susceptible, 4 (2.5%) were susceptible-dose dependent and 2 (1.3%) were resistant. Using the chi-

TABLE I: DISTRIBUTION OF *CANDIDA* SPECIES IN THE SINGAPORE GENERAL HOSPITAL AND OTHER HOSPITALS

Species	Singapore General Hospital n (%)	Other hospitals & private laboratories n (%)	Total n (%)
<i>C. albicans</i>	112 (48.5)	79 (49.7)	191 (49.0)
<i>C. parapsilosis</i>	39 (16.9)	30 (18.9)	69 (17.7)
<i>C. glabrata</i>	38 (16.5)	21 (13.2)	59 (15.1)
<i>C. tropicalis</i>	36 (15.6)	15 (9.4)	51 (13.1)
<i>C. krusei</i>	2 (0.9)	2 (1.3)	4 (1.0)
Others	4 (1.7)	12 (7.5)	16 (4.1)
Total	231	159	390

TABLE II: FLUCONAZOLE SUSCEPTIBILITIES OF *CANDIDA* SPECIES

Species	Total no. of isolates	Type		
		Susceptible n (%)	Susceptible-dose dependent n (%)	Resistant n (%)
<i>C. albicans</i>	191	190 (99.5)	1 (0.5)	0 (0)
<i>C. parapsilosis</i>	69	69 (100)	0 (0)	0 (0)
<i>C. glabrata</i>	59	55 (93.2)	2 (3.4)	2 (3.4)
<i>C. tropicalis</i>	51	51 (100)	0 (0)	0 (0)
<i>C. krusei</i>	4	0 (0)	3 (75)	1 (25)
Others	16	16 (100)	0 (0)	0 (0)
Total	390	381 (97.7)	6 (1.5)	3 (0.8)

square test, there was no statistical significance in the difference in the susceptibilities of *Candida* species between the Singapore General Hospital and the other hospitals ($P = 0.168$). A decision was therefore made to analyse the data from all the hospitals together.

The susceptibilities of individual species to fluconazole are shown in Table II. Almost all isolates of *C. albicans* (99.5%) were fully susceptible to fluconazole, while non-*albicans* species of *Candida* were less susceptible (96.0%). Of the non-*albicans* species of *Candida*, all species were fully susceptible except for *C. glabrata* (93.2%) and *C. krusei* (0%).

Table III shows the MIC₅₀ and MIC₉₀ values of the most common *Candida* species. *C. albicans* had the lowest and *C. krusei* had the highest MIC₅₀ and MIC₉₀ values.

Blood Culture Isolates

There were 92 blood culture isolates. The distribution of species was as follows: *C. tropicalis* (26; 28.3%), *C. albicans* (21; 22.8%), *C. parapsilosis* (18; 19.6%), *C. glabrata* (13; 14.1%), *C. krusei* (3; 3.3%) and other *Candida* species (11; 12.0%). Twenty-four (26.1%) were from patients in internal medical wards, 20 (21.7%) from patients in surgical wards, 16 (17.4%) from patients in

TABLE III: MIC₅₀ AND MIC₉₀ VALUES OF *CANDIDA* SPECIES TO FLUCONAZOLE

Species	No. of isolates	MIC ₅₀ (µg/mL)	MIC ₉₀ (µg/mL)	Range (µg/mL)
<i>C. albicans</i>	191	0.25	0.57	<0.25 - 45
<i>C. parapsilosis</i>	69	0.25	2.46	<0.25 - 7.0
<i>C. glabrata</i>	59	1.07	3.73	<0.25 - 128
<i>C. tropicalis</i>	51	0.47	1.32	<0.25 - 2.5
<i>C. krusei</i>	4	24.3	104	24.3 - 104

MIC₅₀: minimum inhibitory concentration of 50% of the strains;
MIC₉₀: minimum inhibitory concentration of 90% of the strains
(MIC values extrapolated from zone diameters by BIOMIC reader system)

intensive care units, 17 (18.5%) from patients in haematology and oncology wards and the rest (15; 16.3%) from other or unknown specialties. In total, 95.7% of the blood culture isolates were susceptible, 3.3% were susceptible-dose dependent and 1.1% was resistant to fluconazole. All *C. albicans* isolates and 94.4% of the non-*albicans* species of *Candida* were fully susceptible to fluconazole. Two isolates of *C. krusei* and one isolate of *C. glabrata* tested susceptible-dose dependent, while one *C. krusei* isolate was resistant.

Susceptible-Dose Dependent and Resistant Isolates

There were 9 isolates which were susceptible-dose dependent and resistant to fluconazole. These isolates and the epidemiological data of the patients infected with these isolates are shown in Table IV. There were 4 isolates each of *C. krusei* and *C. glabrata* and 1 isolate of *C. albicans*. Four (44.4%) isolates were from blood cultures. All the patients had underlying conditions that predisposed them to *Candida* infections. Three of the patients had haematological malignancies, 2 had diabetes mellitus, 1 had the acquired immunodeficiency syndrome (AIDS) and 1 patient had total parenteral nutrition via a central line. The last patient was on high-dose steroid treatment and had 1 isolate each of *C. krusei* and *C. glabrata*.

All patients with *C. krusei* infection had not received any prior fluconazole therapy. Three out of 4 patients with *C. glabrata* infection had prior fluconazole therapy, ranging from 2 to 26 days at doses of 100 to 200 mg once a day. The last patient with *C. glabrata* infection was on voriconazole for 21 days at 200 mg once a day. The *C. albicans* isolate which was susceptible-dose dependent was isolated from a patient with AIDS who was on prolonged 100 mg fluconazole once a day for the treatment of oral candidiasis.

Discussion

Fluconazole Susceptibility of *Candida* Species

This is the first study looking at the fluconazole susceptibility of *Candida* species in Singapore. A high

TABLE IV: EPIDEMIOLOGICAL DATA OF PATIENTS WITH SUSCEPTIBLE-DOSE DEPENDENT AND RESISTANT ISOLATES

Patient	Isolate	Susceptibility to fluconazole	Site	Diagnosis	Fluconazole use (days)	Voriconazole use (days)	Amphotericin B use (days)
1	<i>C. krusei</i>	S-DD	Blood	ALL & BMT	0	0	22
2	<i>C. krusei</i>	S-DD	Skin	Sjogren's syndrome; on steroids	0	0	0
3	<i>C. krusei</i>	S-DD	Blood	Malabsorption; on TPN	0	0	0
4	<i>C. krusei</i>	R	Blood	CGL & BMT	0	0	19
2	<i>C. glabrata</i>	S-DD	Lymph node	Sjogren's syndrome; on steroids	26	0	0
5	<i>C. glabrata</i>	S-DD	Blood	AML & BMT	0	21	0
6	<i>C. glabrata</i>	R	Genital	Diabetes mellitus	2	0	0
7	<i>C. glabrata</i>	R	Urine	Diabetes mellitus	4	0	0
8	<i>C. albicans</i>	S-DD	Oral cavity	AIDS	>300	0	0

AIDS: acquired immunodeficiency syndrome; ALL: acute lymphoid leukaemia; AML: acute myeloid leukaemia; BMT: bone marrow transplant; CGL: chronic granulocytic leukaemia; R: resistant; S-DD: susceptible-dose dependent; TPN: total parenteral nutrition

Note: Patient 2 had two different *Candida* isolates from different sites at different times

percentage of our isolates (97.7%) was fully susceptible to fluconazole. However, this figure may not be an accurate reflection of the overall susceptibility of *Candida* species in Singapore. *C. albicans* accounted for 49.0% of all isolates in this study whereas in 1998-1999 in the mycology laboratory of the Singapore General Hospital, this species accounted for 55.2% of all *Candida* isolates. The overall susceptibility may well be higher as *C. albicans* is the most susceptible *Candida* species in this study.

Only 3 *Candida* species, *C. krusei*, *C. glabrata* and *C. albicans*, showed decreased susceptibility to fluconazole. The susceptibilities of the individual *Candida* species to fluconazole in our study are very similar to the results in a surveillance programme (the SENTRY programme) conducted in the US, Canada and South America in 1997. In this latter study, fluconazole resistance was found in 0.6% of *C. albicans*, 8.7% of *C. glabrata* and 100% of *C. krusei* isolates while all other species were susceptible.¹² However, the number of isolates in this study was small. In comparison, a large international study conducted in 1997-1998 found that 98.5% of *C. albicans*, 94.0% of *C. parapsilosis*, 90.1% of *C. tropicalis*, 67.1% of *C. glabrata*, and 26.2% of *C. krusei* were susceptible to fluconazole.⁷

Three out of the 4 isolates of *C. krusei* were tested as susceptible-dose dependent. *C. krusei* isolates that were susceptible-dose dependent were also seen in the global worldwide study using this method⁷ and in other studies using the disc diffusion method.^{6,8} *C. krusei* appears to be clinically resistant to fluconazole^{2,13} and experts recommend that this species should be reported as resistant, over-riding the *in vitro* result, and that susceptibility testing for this species is not required.¹¹

For the blood culture isolates, a smaller percentage (95.7%) was fully susceptible to fluconazole. The main reason was the bigger percentage of *C. krusei* isolates (3.3% in blood cultures but only 1.0% in all specimens).

There were only 9 *Candida* isolates with decreased susceptibility to fluconazole in this study and the small number made it difficult to perform statistical analysis on the risk factors associated with fluconazole resistance. However, it is noted that all patients with *C. krusei* infection had no prior fluconazole therapy and 2 out of 4 of these patients were on amphotericin B prior to the isolation of *C. krusei*. A few studies have previously noted that patients not on fluconazole and those on amphotericin B can develop *C. krusei* infection.^{14,15} Our only *C. albicans* isolate with decreased susceptibility to fluconazole was from an AIDS patient who was on prolonged fluconazole therapy. It is not surprising as it is known that resistant *C. albicans* strains most commonly occur in patients with oropharyngeal candidiasis and AIDS.²

Disc Diffusion Method

The disc diffusion test used in this study proved to be technically simple and rapid to perform. Zone diameters could also be easily read and interpreted manually without the aid of the BIOMIC reader system. As zone diameters were read at 80% inhibition, reading was subject to operator variability, even with the BIOMIC reader system, though to a lesser extent with the latter. On the other hand, the reading of MIC endpoints in the NCCLS reference broth dilution method also present difficulty as they similarly have to be read at 80% inhibition.

The majority of isolates in our study required the standard 24 hours of incubation before zone diameters are read. This presents a distinct advantage over the NCCLS broth dilution method which requires 48 hours of incubation. However, certain *Candida* species, e.g. *C. glabrata*, had poorer growth after 24 hours incubation and required the full 48 hours of incubation.

The use of this disc diffusion test in a routine microbiology laboratory appears very promising, due with its simplicity

of performance and use of readily available media. However, the test is currently still not a standard test and further studies validating the correlation between this test and the NCCLS reference broth dilution method may be required.

The implications of this study pertain to both the microbiologist and the clinician. Most microbiology laboratories in Singapore currently do not perform fluconazole susceptibility testing. Due to expense and technical time needed, speciation of *Candida* isolates is also rarely performed routinely except in isolates from sterile sites. With an enlarging patient population with immunodeficiencies and the increasing use of fluconazole, there may be a need for the clinical microbiology laboratory to monitor the trend of non-*albicans* species of *Candida* as well as the susceptibility of the common *Candida* species, especially *C. albicans* and *C. glabrata*. Speciation of the *Candida* isolates should be undertaken for important specimens from sterile sites to identify *C. glabrata* and *C. krusei* isolates. *C. glabrata* and *Candida* isolates not responding to fluconazole may require susceptibility testing following consultation with the microbiologist.

For the local clinician, fluconazole remains a useful agent for the treatment of candidiasis. However, one also has to take into account host factors which can independently influence the response to antifungal therapy.¹⁶

Conclusion

On the whole, *Candida* species in Singapore, with the exception of *C. krusei*, are very susceptible to fluconazole using the new disc diffusion test. There is, however, a need for both the careful dosing of this agent as well as the monitoring of the trend of individual *Candida* species and their fluconazole susceptibilities to prevent the development of resistance.

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