Candida glabrata complex from patients with healthcare-associated infections in Mansoura University Hospitals, Egypt: distribution, antifungal susceptibility and effect of fluconazole and polymyxin B combination

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Abstract

Introduction *Candida glabrata* complex is composed of three cryptic species, *Candida glabrata* sensu stricto, *C. bracarensis* and *C. nivariensis*. Its reduced susceptibility to fluconazole is responsible for treatment failure of its infections. Combination therapy is recommended to treat these resistant strains. This study assessed the distribution of *C. glabrata* complex collected from patients in Mansoura University Hospitals and their antifungal susceptibility, with evaluation of fluconazole and polymyxin B combination against them.

Methods *C. glabrata* complex was collected from patients with healthcare-associated infections. The isolates were identified biochemically then the species were detected using multiplex PCR. The susceptibility of isolates to antifungals and polymyxin B was tested by the microdilution assay. The effect of fluconazole and polymyxin B combination was assessed by checkerboard microdilution and time kill assays.

Results This study included 45 isolates of *Candida glabrata* complex. The common isolate was *Candida glabrata* sensu stricto (38 isolates). There were 4 isolates of *C. bracarensis* and three isolates of *C. nivariensis*. All isolates were susceptible to amphotericin B, and 17.8% were susceptible to itraconazole. Regarding fluconazole, 48.9% of isolates were susceptible dose-dependent and 51.1% were resistant. Synergistic effect of the combination was observed in 68.9% of isolates by the checkerboard method and in 66.7% of isolates using the time kill assay.

Conclusions *Candida glabrata* sensu stricto is the main member of the complex. Combination of fluconazole and polymyxin B can be considered a treatment option for fluconazole resistant *C. glabrata* complex infections. In vivo studies are needed to validate these results.

Keywords *Candida glabrata*, fluconazole, polymyxin B, time kill assay.

Introduction

Candida glabrata is one of the clinically significant *Candida* species causing 25% of superficial and systemic *Candida* infections in many groups of patients.¹ As a result of the introduction of new taxonomic species, *C. glabrata* is considered a cryptic complex consisting of *C. glabrata* sensu stricto, *C. nivariensis*, and *C. bracarensis* (known as *C. glabrata* complex).

Limited knowledge is available about the prevalence and antifungal resistance pattern of *C. glabrata* complex.²

Azoles are the most inexpensive and commonly used antifungals to treat serious *Candida* infections. Reports from the Centers for Disease Control and Prevention (CDC) have documented fluconazole (FLC) resistance in 7% of *Candida* species including *C. glabrata* complex.³

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The wide use of FLC in treatment and prophylaxis is the main factor responsible for the development of resistance. *Candida glabrata* complex has acquired resistance to other drugs used in the treatment of serious infections such as amphotericin B (AMB) and echinocandins.⁴

With the limited number of antifungal medications available, there is a crucial need for new, less common, lines of therapy. Some clinical agents other than antifungal drugs have fungicidal activity, one of these agents is polymyxin B. Polymyxin B is an antibiotic that is used for the treatment of multidrug resistant Gram negative bacteria and that possesses fungicidal activity in high concentration. Combination of polymyxin B at low concentrations with other antifungal agents shows killing activity against many fungal agents.⁵

The aim of this work was to determine the distribution of *C. glabrata* cryptic species among the clinical isolates collected from patients in Mansoura University Hospitals (MUHs) and to assess their antifungal susceptibility pattern, in addition to evaluating the effect of FLC and polymyxin B combination against FLC resistant *C. glabrata* complex.

Methods

Candida species were collected from clinical samples of patients at MUHs from January 2014 to January 2017. Mansoura University Hospitals include General, Emergency and Critical Situations and Convalescence Hospitals and are located at 60-El Gomhoria street, Mansoura, Egypt. Clinical samples were collected from patients with healthcare-associated infections as identified by CDC.⁶ In case of collection of consecutive samples from the patients, non-repetitive *Candida* species were included in the study. This work was approved by Mansoura Ethical Committee (R/17.05.45) and informed consent was obtained from each patient.

Germ tube test was done to differentiate *Candida albicans* from non-*albicans* *Candida* species.⁷ *Candida glabrata* complex was further identified biochemically using API 20 C (BioMérieux, Marcy-l'Étoile, France) according to the manufacturer's guide.

Molecular identification of *C. glabrata* cryptic species

Isolates identified as *C. glabrata* complex were subjected to multiplex PCR for species identification after DNA extraction using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Amplification of ITS1 was performed using the following primers: forward primers for *C. glabrata sensu stricto* [GLA-f (5'-CGGTTGGTGGGTGTTCTGC-3')], *C.*

bracarensis [BRA-f (5'-GGGACGGTAAGTCTCCCG-3')] and *C. nivariensis* [NIV-f (5'-AGGGAGGAGTTTGTATCTTTCAAC-3')], and the reverse primer UNI-5.8S (5'-ACCAGAGGGCGCAATGTG-3'), which amplifies the 5.8S rDNA region. The reaction technique was performed according to the previously described protocol.⁸

Antifungal susceptibility testing

Antifungal susceptibilities of the isolated strains to FLC, itraconazole (ITC) and AMB were tested by the broth microdilution method according to revised CLSI guidelines for antifungal susceptibility.⁹ The final AMB and ITC concentrations were 0.0313-16 µg/mL and 0.125-128 µg/mL for FLC. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 strains were included in each test for quality control.

No reported interpretive guidelines for testing polymyxin B against *C. glabrata* complex are available. The minimal inhibitory concentration (MIC) of polymyxin B was measured as the lowest concentration of the drug resulting in complete inhibition of *C. glabrata* complex visual growth.

Synergy testing

The effect of FLC and polymyxin B combination on susceptible-dose dependent (SDD) and resistant *Candida glabrata* complex strains was assessed by the checkerboard titration method and time kill assay as previously described.^{10,11}

Checkerboard titration method:

The in vitro interaction between fluconazole and polymyxin B was done by a two-dimensional checkerboard microdilution technique.¹¹ The MIC of each drug (fluconazole and polymyxin B) was detected using broth microdilution method as described before. For FLC the MIC value was used and for polymyxin B 1/2 MIC value was used. In the checkerboard titration assay, four to eight times to at least 1/8–1/16 times the expected MIC were included for each drug.

The fractional inhibitory concentration index (FICI) was estimated for each antimicrobial agent in each combination using the following formula:

FICA=MIC of drug A in combination/MIC of drug A alone

FICB=MIC of drug B in combination/MIC of drug B alone

FICI=FICA+FICB

FICI was interpreted as follows: ≤ 0.5 synergy, >0.5 to ≤ 1 additive effect, >1 to <4 indifferent, and ≥ 4 antagonism.

Time-kill assay:

Time-kill studies were done according to the method described before.¹² Before the time kill assay, each *C. glabrata* complex strain was subcultured twice on Sabouraud dextrose agar (SDA) plates. For each strain, the experiment was performed twice. For determination of time-kill assay, the MIC of FLC and 0.5 MIC of polymyxin B alone and in combination were used. Three to five colonies from a 24-48 h growth plate were suspended in 9 mL of sterile water. The starting fungal inoculum was adjusted to 0.5 McFarland standard turbidity. One milliliter of the adjusted suspension was mixed with nine milliliters of either RPMI 1640 medium alone (control) or RPMI 1640 with appropriate concentration of single or drug combination. The resulting inoculum was 1×10^5 to 5×10^5 colony forming units (CFU)/mL.

Serial two-fold dilutions of FLC and polymyxin alone and in combination were prepared in RPMI 1640 medium with morpholinepropanesulfonic acid (MOPS). An aliquot of the prepared organism inoculum was added to each tube. Quality control tubes were

included. One tube with the organism without the drug was included. Another sterility control tube, which contained growth medium with no drug or organism, was included each day. Tubes were incubated at 35°C then vortexed prior to the detection of colony counts.

At specific time points (0, 24 and 48 h), 100 μ L from each solution were serially diluted in sterile water then 30 μ L were cultured on SDA plates in duplicate for determination of CFU counts. Plates were incubated at 35°C for 48 h. All time-kill experiments were performed in duplicate. Determination of synergy was done using the mean CFU/mL from the duplicate plates. Prior to the start of the time-kill curve studies, assessment of antifungal carryover was performed according to the method described before.¹² No carryover was detected for fluconazole at the MIC.

Synergy demonstrated a $\geq 2 \log_{10}$ reduction in the colony count after 48 h with the drug combination compared to the most active single agent alone, indifference showed a $<2 \log_{10}$ increase or decrease in colony count at 48 h with the combination compared to that with the most active single agent alone, and antagonism revealed a $\geq 2 \log_{10}$ increase in colony count after 48 h with the combination compared to that with the most active single agent alone.¹³

Results

This study included 45 *Candida glabrata* complex isolates recovered from patients with healthcare-associated fungal infections at MUHs during January 2014 to January 2017. The isolates were collected from 23 female (51.1%) and 22 male patients (48.9%) with mean age 45.6 years ($\pm$ SD 15.7, range 12-68 years).

The most common isolated species was *C. glabrata sensu stricto* (38) representing 84.4%. There were 4 strains of *C. bracarensis* and 3 strains of *C. nivariensis* (8.9% and 6.7% respectively). All *C. glabrata* complex species were mostly isolated from urine samples (Table 1).

Susceptibility testing

The sensitivity of *C. glabrata* complex to antifungal agents and polymyxin B were

Table 1. Distribution of *C. glabrata* cryptic complex species in different clinical samples

Clinical sample	No. (%) of <i>C. glabrata</i> cryptic complex species			Total
	<i>C. glabrata</i> sensu stricto	<i>C. nivariensis</i>	<i>C. bracarensis</i>	
Urine	20	3	2	25
Respiratory tract	4	0	0	4
Blood	5	0	2	7
Mucosal surfaces	9	0	0	9
Total	38 (84.4)	3 (6.7)	4 (8.9)	45 (100)

Table 2. In vitro susceptibilities profile of different *C. glabrata* cryptic species and their antifungal MIC range

Species (no. of isolates)	Antifungal agent	MIC ($\mu\text{g/mL}$) range	Mean $\pm$ SD	MIC 90	MIC 50	No (%) of isolates		
						S	SDD	R
<i>C. glabrata</i> sensu stricto (38)	AMB	0.062-1	0.44 $\pm$ 0.35	1	0.25	38 (100)	0	0
	FLC	4-128	5.63 $\pm$ 45.4	128	32	0	18 (47.4)	20 (52.6)
	ITC	0.031-2	0.73 $\pm$ 0.63	2	0.5	6 (15.8)	18 (47.4)	14 (36.8)
<i>C. bracarensis</i> (4)	AMB	0.125-0.5	0.31 $\pm$ 0.22	0.5	0.31	4 (100)	0	0
	FLC	32-64	4 $\pm$ 16	64	32	0	1 (25)	3 (75)
	ITC	0.25-1	0.5 $\pm$ 0.35	1	0.38	2 (50)	2 (50)	0
<i>C. nivariensis</i> (3)	AMB	0.25-1	0.5 $\pm$ 0.43	1	0.25	3 (100)	0	0
	FLC	16	16 $\pm$ 0	16	16	0	3 (100)	0
	ITC	0.5	0.5 $\pm$ 0	0.5	0.5	0	3 (100)	0
Total (45)	AMB	0.06-1	0.42 $\pm$ 0.34	0.25	0.25	45 (100)	0	0
	FLC	4-128	5 $\pm$ 43	128	32	0	22 (48.9)	23 (51.1)
	ITC	0.03-2	0.38 $\pm$ 0.4	1	0.25	8 (17.8)	23 (51.1)	14 (31.1)

AMB – amphotericin B; FLC – fluconazole; ITC – itraconazole; MIC – minimal inhibitory concentration; SD – standard deviation; S – susceptible; SDD – susceptible-dose dependent; R – resistant.

examined using the standard microdilution assay. The MIC values ranged from 4 to 128 $\mu\text{g/mL}$ for FLC; 0.03 to 2 $\mu\text{g/mL}$ for ITC, 0.06 to 1 $\mu\text{g/mL}$ for AMB and 64 to 1024 $\mu\text{g/mL}$ for polymyxin B. All isolates were sensitive to AMB and 15.8% of *C. glabrata* sensu stricto strains were susceptible to ITC. Regarding FLC, 47.4% of *C. glabrata* sensu stricto isolates were SDD and 52.6% were resistant as presented in Table 2. Half of *C. bracarensis* isolates were susceptible to ITC and the other half were SDD, while 75% of them were resistant to FLC and 25% were

SDD. All *C. nivariensis* isolates were SDD to ITC and FLC. Revised CLSI guidelines for antifungal susceptibility (CLSI M27-S4 and M27-S3) were used in this study.

Synergy testing

The synergy of FLC and polymyxin B combination was tested by two methods; FICI and time-kill assay. Results of synergy testing are presented in Table 3. Synergy was observed in 68.9% of the isolates by FICI. By time-kill assay, synergy between both agents was observed in

Table 3. Synergy testing of fluconazole and polymyxin B by checkerboard titration method and time-kill assay

Strain no.	FLC MIC	FLC MIC in combination	Polymyxin MIC	Polymyxin MIC in combination	FICI	Result	Log ₁₀ change in time-kill assay	Result
1.	64	32	128	64	1	Additive	-1.5	Indifference
2.	32	2	128	64	0.6	Additive	-1.7	Indifference
3.	128	16	512	64	0.25	Synergy	-2.1	Synergy
4.	32	16	512	8	0.5	Synergy	-2	Synergy
5.	16	8	256	16	0.6	Additive	-2.6	Synergy
6.	16	2	128	2	0.1	Synergy	-2	Synergy
7.	32	2	64	16	0.3	Synergy	-2.3	Synergy
8.	16	16	256	128	1.5	Indifference	-2.5	Synergy
9.	64	4	128	128	1.1	Indifference	-1.4	Indifference
10.	32	16	256	16	0.6	Additive	-1.8	Indifference
11.	8	2	128	8	0.3	Synergy	-1.7	Indifference
12.	128	64	256	4	0.5	Synergy	-2.5	Synergy
13.	32	16	256	16	0.6	Additive	-0.8	Indifference
14.	16	8	256	32	0.6	Additive	-0.6	Indifference
15.	32	8	512	32	0.3	Synergy	-3	Synergy
16.	16	2	128	2	0.1	Synergy	-3	Synergy
17.	16	8	128	2	0.5	Synergy	-2.4	Synergy
18.	128	32	512	8	0.3	Synergy	-2.6	Synergy
19.	64	8	256	32	0.3	Synergy	-2.2	Synergy
20.	4	1	128	4	0.3	Synergy	-2.4	Synergy
21.	64	32	1024	8	0.5	Synergy	-2.2	Synergy
22.	32	8	128	32	0.5	Synergy	-2.4	Synergy
23.	64	2	128	32	0.3	Synergy	-2.8	Synergy
24.	128	32	1024	16	0.3	Synergy	-3	Synergy
25.	32	16	512	32	0.6	Additive	-1.6	Indifference
26.	16	4	512	64	0.4	Synergy	-3	Synergy
27.	16	8	512	128	0.8	Additive	-0.6	Indifference
28.	32	4	1024	8	0.1	Synergy	-3.2	Synergy
29.	128	2	64	1	0.03	Synergy	-4	Synergy
30.	64	2	256	4	0.05	Synergy	-3.5	Synergy
31.	64	32	1024	8	0.5	Synergy	-3	Synergy
32.	32	8	128	32	0.5	Synergy	-2.8	Synergy
33.	8	2	128	32	0.5	Synergy	-2.4	Synergy
34.	128	32	1024	16	0.3	Synergy	-2.4	Synergy
35.	32	16	512	32	0.6	Additive	-0.8	Indifference
36.	16	4	512	64	0.4	Synergy	-3	Synergy
37.	128	16	512	64	0.3	Synergy	-1.8	Indifference
38.	8	4	128	8	0.6	Additive	-0.7	Indifference
39.	128	64	256	4	0.5	Synergy	-2.4	Synergy
40.	32	16	512	8	0.5	Synergy	-1.6	Indifference
41.	16	8	256	32	0.6	Additive	-2.5	Synergy
42.	32	8	512	32	0.3	Synergy	-1.6	Indifference
43.	128	16	512	64	0.3	Synergy	-2.1	Synergy
44.	8	4	128	8	0.6	Additive	-1.5	Indifference
45.	32	16	512	8	0.5	Synergy	-2.5	Synergy

FICI – fractional inhibitory concentration index; **FLC** – fluconazole; **MIC** – minimal inhibitory concentration. See text for FICI formula and interpretation.

66.7% of the isolates. The FLC MIC 50 and MIC 90 of *C. glabrata* complex were 32 µg/mL and 128 µg/mL respectively, higher than the MIC 50 and MIC 90 of fluconazole and polymyxin B combination: 8 µg/mL and 32 µg/mL respectively.

Discussion

The distribution of *C. glabrata* complex infections has been changed recently due to the description of new cryptic species. The cryptic species of *C. glabrata* complex include *C. glabrata*

sensu stricto, *C. bracarensis* and *C. nivariensis*.² One of the aims of the present work was to study the distribution of *C. glabrata* cryptic species among clinical isolates of *C. glabrata* complex from MUHs.

Candida glabrata sensu stricto is responsible for 15-20% of *Candida* infections globally and represents the second cause of *Candida* bloodstream infections in the United States. In our work, *C. glabrata* sensu stricto was the principal cryptic species of the *C. glabrata* complex representing 84.4% of the total isolates, while the other two species of the complex accounted for 15.6%. This is in agreement with other studies from different countries where *C. glabrata* sensu stricto represented 100% and 93.4% of *C. glabrata* complex recovered from patients in Spain and Argentina, respectively, while *C. bracarensis* and *C. nivariensis* were recovered from 6.6% of clinical samples in the later study.^{14,15}

In the present work, *C. glabrata* complex showed reduced sensitivity to fluconazole and no susceptible isolate was identified; 48.9% of the isolates were SDD and 51.1% were resistant. This high resistance to FLC is in accordance with another study from the United States where using the microbroth dilution method, FLC MIC was 0.5-256 µg/mL and SDD and resistant strains represented 80% and 20%, respectively with no susceptible strains to FLC.¹⁶ Additionally, our results match those of other studies from Taiwan¹⁷ and Chile¹⁸ where FLC MIC ranged from 0.25-256 µg/mL with no susceptible strains to FLC.

Hou et al.¹⁹ have reported that all *C. nivariensis* and *C. bracarensis* isolates in their study are SDD to FLC, in accordance with our study for *C. nivariensis*, however 25% of *C. bracarensis* isolates in our study are SDD and 75% are resistant which requires more researches with large numbers of isolates to confirm these data. This increase in *C. glabrata* complex non susceptibility to FLC may be partially due to inherent low susceptibility to azoles and the selection caused by wide use of FLC.¹

There is a rise in the prevalence of systemic fungal infections, especially in the

immunocompromised population. This rise is associated with an increase in the antifungal resistance to the known antifungal agents. This necessitates the urgent search for new therapies.²⁰ Polymyxin B is an old antibacterial agent that possesses fungicidal activity in high concentration.⁵

This study searched the possible fungicidal effect of low sub-inhibitory concentration of polymyxin B together with fluconazole against fluconazole insusceptible strains of *C. glabrata* complex. Fluconazole combination with low concentration of polymyxin B (1/2 MIC) was assessed by FICI method and time-kill assay. A synergistic effect was observed in 68.9% and 66.7% of FLC non susceptible *C. glabrata* complex isolates using the FICI method and the time-kill assay, respectively. This result matches that of Pankey et al.¹⁶ who detected a synergy with FLC and polymyxin B combination against 60% of *C. glabrata* complex isolates using the time-kill assay with no antagonism recorded. Likewise, another study has demonstrated the fungicidal activity of polymyxin B with antifungal agents such as FLC and ITC against many fungi including *Aspergillus*, *Candida* species and *Cryptococcus*.²¹

Many theories are proposed to explain the exact mechanism of synergistic effect of polymyxin B and FLC. Similar to the action of polymyxin B against Gram negative bacilli, its fungicidal activity is based on disruption of membrane integrity through attachment to anionic lipids on fungal membrane forming channels to impair the cytoplasmic membrane unity.^{22,23}

Fluconazole decreases the formation of ergosterol, which is the main lipid component of the cell membranes rendering the fungal cell more susceptible to polymyxin B. It has been documented that FLC increases intracellular concentration of calcium and hydrogen ions which interfere with ionic homeostasis of cell membrane augmenting the effect of polymyxin B.^{10,24}

Polymyxin B alone exerts a low fungicidal activity and needs higher doses. This may be due to the presence of steroid in the fungal cell

membrane which decreases the insertion of cationic peptides in the anionic lipids of the membrane.^{23,25}

Conclusions

Candida glabrata sensu stricto is the main species of the *C. glabrata* complex causing healthcare-associated infections in MUHs. *C. glabrata* complex displays a high resistance to fluconazole. A synergy results from using fluconazole with polymyxin B against *C. glabrata* complex. Consequently, this combination can be considered a treatment alternative for fluconazole resistant *Candida glabrata* complex infections after validation of these results using in vivo studies.

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Conflicts of interest: All authors – none to declare.

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