

Increase of Non-Albicans *Candida* Species and Their Antifungal Susceptibility in Intensive Care Unit Patients (Mexico)

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Abstract

In Mexico little is known about candidemia by non-*albicans Candida* species and regarding their antifungal susceptibility. Besides without antifungal tests, fluconazole is one of the most used in empirical therapy. In the present study, we included patients from intensive care unit of one hospital in Mexico (2019–2020) with compatible yeast infection clinical signs, symptoms. Based on cultivable isolates, yeasts were identified by automated instrument and by molecular method (PCR), and their susceptibilities to six antifungals were characterized at different concentrations. From 105 patients, yeast cultures were recovered and identified mainly non-*albicans Candida* species (57.2%); and the most prevalent was *C. glabrata* (41.9%). Followed by *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* and *Cryptococcus neoformans*. The most common infection site was urine (56%), followed by the bronchial aspirate (30%). Mostly the isolated fungi were susceptible to 5-flucytosine (98%) and to amphotericin B. Mainly *C. glabrata* followed by *C. krusei* and *C. tropicalis* were resistant to different concentrations of itraconazole, miconazole, and fluconazole. The present investigation contributes to the knowledge of non-*albicans Candida* species infections in patients and, opens the possibility for a better understanding and management in antifungal empirical therapy in Mexico.

Introduction

In Mexico, little is known about intrahospital fungal infections, their comorbidities, treatment, and antifungal susceptibility profiles [1,2]. However, a dramatic global increase in the incidence of fungal diseases has been documented [3], which represents a public health problem with unique challenges due to the lack of sensitivity of diagnostic tools and the high morbidity and mortality caused by these infections. [4]

In hospitalized patients, the main etiological agents causing mycoses belong to the genus *Candida* [6,5]. Mycoses caused by yeast infection, mainly candidemia and in a lesser proportion cryptococcosis, are one of the major causes of morbidity and mortality [7,8]. *Candida* and *Cryptococcus* infections are associated with high mortality, 38–75% and 70%, respectively [9,10]. Yeast infections are related to patients in intensive care units (ICU), prolonged hospitalization, and different comorbidities such as chronic renal insufficiency, diabetes, and hypertension [11]. Around 15 species of the genus *Candida* have been documented to cause invasive infections in humans [12]. Of these, about 90% of infections correspond to *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, with *C. albicans* and *C. glabrata* being the most prevalent species [13].

Candidiasis can present multifocal colonization in the human body, including cutaneous, pulmonary, gastrointestinal, and urogenital localization, and dissemination in the bloodstream among others [14]. The empirical treatment for yeast infections includes several antifungal drugs such as polyenes, azoles, and echinocandins, mainly amphotericin B and fluconazole [15].

However, the extensive use of antifungals in ICU patients for prophylaxis is the main cause of the increase in yeast infections [4], and drug resistance of *C. albicans* and non-*albicans Candida* species has been related to the extensive use of antifungals [16]. Several isolates of *Candida* and *Cryptococcus* have been described *in vivo* and *in vitro* as resistant to conventional antifungals such as polyenes (amphotericin B), azoles (fluconazole and itraconazole), and echinocandins (caspofungin) [7,18]. Conversely, fluconazole is one of the most used antifungals in empirical therapy in hospitals in Mexico (regardless of the origin or type of mycosis) against disseminated and localized infection by *Candida* or any fungi [2]. The aim of the present study was to screen *Candida* infections in ICU patients at the Hospital General de Querétaro (HGQ) over a period of one year, to identify cultivable isolates, and to characterize their resistance to the six most used antifungals in Mexico.

Materials And Methods

Sample collection and identification

The data were collected in a public secondary referral hospital in Mexico. The HGQ covers different medical and surgical specialties and has an ICU; it is an 85-bed public hospital serving a population of 1,405,992 inhabitants in the metropolitan area of Querétaro city, Mexico. A prospective study was aimed on *Candida* infection in ICU. The authors confirm that the ethical policies have been conducted according to the World Medical Association Declaration of Helsinki and adhered to Ethical and Investigation Committee of Servicios de Salud del Estado de Queretaro which reviewed and approved the implementation of this study (09-28-2019/ 11110/UNIVERSIDAD AUTONOMA DE QUERETARO) in the HGQ and Official Mexican Standard NOM-024-SSA3-2012 was followed.

Patients hospitalized medical/post-operative admitted to ICU for >24 h that requiring intensive post-operative monitoring or undergoing therapy for one or more acute organ system failure was included from January 2019 through January 2020. Clinical and demographic data were recorded prospectively and analyzed (age, gender, comorbidities, and risk factors). Patients with clinical evidence of yeast infection and with positive cultures obtained from different clinical samples as: bloodstream, bronchial aspirate, catheter tip, cerebrospinal fluid, peritoneal fluid, skin and soft tissue and urine were studied. *All samples were collected and cultured on Sabouraud Dextrose Agar (SDA)* (Bioxon®, Mexico) supplemented with chloramphenicol (50 mg/L) (Sigma®) to prevent Bacteria growth, *for 24-72 h at 37°C. Positive cultures were cultured in CHROMagar Candida™ (BD®, US) for 24 h at 37°C. Macroscopic characteristics of the cultures were observed directly on solid medium. Axenic isolates were identified by two methods. Using Vitek 2k system (bioMérieux, Lyon, France), based on the manufacturer's instructions [17] and by molecular identification based on method described by Calvillo-Medina et al [19]. Briefly DNA was obtained using cetyl-trimethylammonium bromide (CTAB)-based method (Sigma®), the fungal barcode nrDNA ITS (ITS), was amplified using primers ITS1f and ITS4r [20] in a GeneAmp PCR System 9700 (Thermo Fisher Scientific). PCR products were*

sent to Macrogen Inc. (Seoul, Korea) for standard- seq single DNA sequencing (Sanger sequencing). Sequences were compared via BLAST with ITS *Candida* species and genera related sequences from NCBI database (<http://www.ncbi.nlm.nih.gov/genbank/>).

Antifungal susceptibility assay

Isolates were screened to determine the minimum inhibitory concentration (MIC) *in vitro*, defined as the minimum concentration of antifungal that inhibited the growth of isolates after the incubation period, based on Clinical and Laboratory Standards Institute (CLSI) document M38-A. A panel of six antifungals (Sigma Aldrich®, USA), commonly used in systemic or localized infections therapy in Mexico, were tested: Each antifungal stock solutions were prepared in sterile dimethyl sulfoxide (DMSO) at two different concentration: amphotericin B (AMB) 2 and 8 mg/mL; 5-flucytosine (FCZ) 2 and 32 mg/mL, fluconazole (FLC) 8 and 64 mg/mL, itraconazole (ITR) 0.5 and 4 mg/mL, ketoconazole (KTC) 0.5 and 4 mg/mL and miconazole (MCN) 0.5 and 8 mg/mL. They were sterilized by 0.22 mm filtration (Millipore®, USA) and stored at -70°C until their use. Microdilution method was done according to Sánchez-Vargas et al. [21] based on composed of microplates. The inoculum concentration was calculated using a Neubauer hemocytometer (Lo-Laboroptik Ldh®, UK) under a light microscope and adjusted to 1×10^3 conidia/mL in 200 µL of RPMI 1640 medium (Sigma®, Germany). The inoculum (100 µL) was placed into each well with the antifungal agent. Controls of isolates were grown without antifungals and with or without DMSO in microtiter plates with 200 µL of RPMI 1640 medium. Antifungal susceptibility assays were done in quadruplicate. The microplates were incubated at 37°C and read after 72 h. Plates were measured with a microplate spectrophotometer (Multiskan Ascent Thermo Labsystems, USA) at an optical density (OD) of 595 nm.

Statistical analysis

Differences between continuous variables were evaluated statistically by means of two-tailed Student's t-test for differences in means and percentages. Differences between categorical variables were evaluated by chi-square test. Values of $p < 0.01$ (***) were considered statistically significant. Graphs and tests were performed with GraphPad Prism 7.0 (San Diego, CA, USA).

Results

Fungal isolation and identification

The study included 105 patients that tested positive. The age range at which the fungal infections presented the highest incidence was 41–60 years. The average age was 54.5 years (± 18.13); 58/105 (62%) were males and 47/105 (38%) were females. Principal risk factors were chronic renal failure (CRF) (55/105; 47.3%), diabetes (32/105; 30.4%), hypertension (28/105; 26.6%), and metabolic acidosis (18/105; 17.1%) (Table I).

Identification of the 105 isolates were mainly non-*albicans* *Candida* species (60/105; 57.2%); the most prevalent was *C. glabrata* (44/105; 41.9%), followed by *C. albicans* (43/105; 40.9%), *C. tropicalis* (9/105; 8.6%), *Candida* sp. (5/105; 4.7%), *C. parapsilosis* (1/105; 0.9%), *C. krusei* (1/105; 0.9%), and *Cryptococcus neoformans* (2/105; 1.9%). Of all the isolates, 5% could not be identified to species level and were classified as *Candida* sp. (Fig. 1 and Table I). Considering the relation between fungal species and chronic diseases, all patients with *C. albicans* infection had CRF, of whom 16 presented diabetes (37.2%). *C. glabrata* fungemia was related to hypertension (12/44; 27.3%) and CRF (12/44; 27.3%), and *C. tropicalis* infections to hypertension (8/9; 89%) and diabetes (5/9; 55.5%). All patients with *Candida* sp. infections had diabetes, and 60% had metabolic acidosis (3/5). The one patient that had *C. parapsilosis* infection presented hypertension, obesity, and metabolic acidosis. The patient with *C. krusei* presented diabetes; finally, the two patients with *Cry. neoformans* infection presented diabetes (100%) and 1 of them had AIDS (50%) (Table I).

The most frequent localizations of candidemia were urine (56/105; 53.3%), bronchial aspirate (30/105; 28.5%), and skin and soft tissue (6/105; 5.7%) followed by bloodstream (4/105; 3.8%) and tip catheter (4/105; 3.8%) (Fig. 2). Considering the relation of fungal infection and isolation site, 19 of the 56 (33.9%) urine samples presented *C. albicans*, 31 (55.3%) *C. glabrata*, 5 (9%) *C. tropicalis*, and 1 *C. krusei* (1.8%). From bronchial aspirate, *C. albicans* was the most frequently isolated (19/30; 63.3%) followed by *C. glabrata* (6/30; 20%) and *Candida* sp. (5/30; 16.7%). The isolate of *C. parapsilosis* was found in a bloodstream; finally, *Cry. neoformans* infections were found in cerebrospinal fluid (1/2; 50%) and peritoneal fluid (1/2; 50%). The outcome of fungal infections was that 78 (74.2%) patients survived and 27 (25.8%) died within 30 days after diagnosis. The highest mortality by species was found for *Cry. neoformans* (100%) and *C. parapsilosis* (100%), followed by *C. tropicalis* (4/9; 44.4%), *C. albicans* (10/43; 23.25%), and *C. glabrata* (9/44; 20.4%). The least pathogenic fungus was *C. krusei* with 0% mortality (Table I).

Antifungal assay

In patients who died, the most important comorbidity was sepsis (23/27; 85%) followed by hypertension (21/27; 77.7%), CRF (20/27; 74%), and diabetes (20/27; 74%). All patients diagnosed with mycoses were given FLC treatment and voriconazole was used only in 3 patients. The results (Table II) showed that most effective antifungals were AMB and FCZ. The fungi were resistant mainly to ITR (60%), MCN (39%), and FLC (38%) and susceptible to FCZ (98%) and AMB (97%), with significant differences ($p < 0.01$) for all times and concentrations.

In particular, the two most prevalent fungi, *C. glabrata* and *C. albicans*, were both susceptible to AMB > FCZ and resistant to ITR > FLC > MCN. Isolates of *C. krusei* and *C. parapsilosis* showed susceptibility to all antifungals tested, and *C. tropicalis* isolates were susceptible to FCZ (100%), FLC (78%), AMB (67%) and KTC (67%), and moderately resistant to ITR > MCN. *Cry. neoformans* isolates presented sensitivity to all the antifungals tested except FCZ (50%). However, it is important to recall that both cases of cryptococcosis were fatal. According to *in vitro* results, the antifungal tests suggest that

the MIC of the resistant antifungals were: ITR > 4 mg/mL, FLC > 64 mg/mL, and MCN > 8 mg/mL after 72 h of growth. Comparison of all isolate controls against antifungal treatments showed a significant difference ($p < 0.01$) in all experiments (Table II).

Discussion

There is a lack of data about *Candida* infections in Mexico despite their frequency and the severe infections that they can cause. Mainly information about yeast infections is from Mexico City and a few investigations have been conducted for other parts of the country [2, 22]. Our study address, in one year 105 isolates from different human sites and tested for antifungal effects. Here, the results showed a correlation between risk factors (diabetes mellitus, hypertension, renal failure, and obesity) and mycoses. The relation between these comorbidities predisposes patients as more vulnerable to fungal infection, mainly candidiasis and cryptococcosis. [2, 22] The mortality rate found in HGQ was 25.8% lower than the Brazilian rate ranging from 54–72.2% and similar to that found in studies in China (23.3%) [23].

We described the distribution of the most frequent species of the genus *Candida*: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* [12] and *Cry. neoformans* (1.9%). Our report highlight, the predominance of Non-*albicans Candida* species (57.2%), *C. glabrata* being the most frequent which may a cause of concern in the management of candidiasis. According to de la Torres-Saldaña et al. [24] and Reyes-Montes et al. [2] *C. albicans* is the most frequent etiological agent isolated from Mexican patients. However, González et al. [] found a prevalence of non-*albicans Candida* species in hospitals in Monterrey, Mexico similar to that described here. In tropical countries such as Brazil and India, common causes of nosocomial candidemia are *C. parapsilosis* and *C. tropicalis*, respectively [12, 14]. According to Manzano-Gayosso et al. [25], the etiology of *Candida* infections in Mexico has changed in the last 20 years, with non-*albicans Candida* species being more prevalent.

Regardless of *Candida* infection etiology, the antifungal most used in public hospitals in Mexico is FLC [2] which has been broadly used in empirical therapy against disseminated mycoses [26]. However, indiscriminate use of FLC (for example prophylaxis) can produce a great economic and ecological impact [2, 17]. As well as generating resistant yeast, its efficacy rate has decreased, and it offers variable protection against non-*albicans Candida* species [12]. Based on differences in susceptibility to FLC, Jenks et al. [27] recommended the implementation of newer triazoles with antifungal activity such as voriconazole [28]. Therefore, *Candida* and *Cryptococcus* antifungal tolerance and the appropriate treatment of patients with proven infection, started as early as possible and selected through strategies, must be taken into consideration [12, 29].

In general, resistance to azoles, singularly to FLC, has been reported in *Candida* and *Cryptococcus* [2, 17]. Moreover, from the data generated here, ITR, FLC, and MCN were the most resistant antifungals, which contrasts with the use of FLC in therapy. The findings for *C. glabrata* resistance to azoles shown in this study are similar to those previously published [1, 30]. In contrast with Corzo-Leon et al. [17] and González et al. [1] we found high resistance of *C. tropicalis* and *C. albicans* to azoles and AMB. Also, *C. parapsilosis* was susceptible to all antifungal agents tested. According to González et al. [1], Pfaller and Diekema [30] and Corzo-Leon et al. [17], and based on our results, the use of FLC should not be continued for prophylaxis or in patients with recurrent yeast infections.

The limitations our study are, first some epidemiological data (such as previous infection, transfers between hospitals or previous exposure to antifungals) was not always available. Second the isolates were collected and analyzed for one year, the duration of the study could be extended. Third we did not collected samples from the hospital environment or health care workers, information that will help to understand the source and spread of infections. Finally, we could not be allowed to implement the therapy with the susceptible antifungals to each patient.

In conclusion is important to improve the identification and antifungal test (identification by PCR or susceptibility to echinocandins) of no *albicans Candida* species in ICU patients in Mexico. To ensure a specific identification, diagnosis, and selection of appropriate therapeutic strategies which should be based on antifungal susceptibility patterns and control measures for risk factors. Nevertheless, the use of standardized technology in diagnostics and research remains a major global challenge, especially in countries such as Mexico. All the aforementioned will allow a better understanding of the epidemiology and susceptibility patterns of fungal infection in Mexico and in other countries with limited resources.

Declarations

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Conflicting interests, The Authors declares that there is no conflict of interest.

Availability of data material Not applicable

Code availability Not applicable

Author Contributions

Rosa Paulina Calvillo-Medina designed this project, carried out fungal identification contributed in the data and statistical analysis, and wrote the first manuscript. Rocio Alejandrina Mejía-Romero carried out isolates collection, performed the antifungal susceptibility data analysis and revised the manuscript. Magda Martínez-Neria participated in the data and statistical analysis and contributed to the discussion and revision of the manuscript.

Juan José Olalde-Elias, and Fernando Domínguez-Márquez participated in the design of the study, manuscript revision and review. All the authors contribute, read and approved the manuscript

Ethics approval Authors confirm that this material is an original study of our authorship and has not been published in whole or in part elsewhere. It is not currently under consideration for publication by another journal simultaneously.

Consent to participate The Ethical and Investigation Committee of Servicios de Salud del Estado de Querétaro approved the implementation of this study (09-28-2019/11110/UNIVERSIDAD AUTONOMA DE QUERETARO) in the General Hospital of Querétaro and Official Mexican Standard NOM-024-SSA3-2012 was followed.

Consent for publication All authors contribute, approved the submission of the manuscript for publication and the written consent for publication has been obtained.

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Tables

Table I. Hospital admission data, primary comorbidities, and risk factors from patients in ICU.

Fungal species	Age					Gender		Comorbidities/ Risk factors									Mor	
	18–20	21–40	41–60	61–80	>80	F	M	AIDS	AA	Cancer	COPD	CRF	Dia	Hyp	MA	Obe		Sep
<i>C. albicans</i> (43)	2	9	16	13	3	21	22	0	1	0	2	43	16	6	7	0	9	10
<i>C. glabrata</i> (44)	1	7	20	12	4	22	22	0	1	4	2	11	3	12	7	3	10	9
<i>C. krusei</i> (1)	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0
<i>C. parapsilosis</i> (1)	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1
<i>C. tropicalis</i> (9)	1	3	3	2	0	1	8	0	2	0	0	1	5	8	0	1	3	4
<i>Candida</i> sp (5)	0	0	4	1	0	0	5	0	1	0	0	0	5	0	3	2	1	1
<i>Cry. neoformans</i> (2)	0	1	1	0	0	1	1	1	0	0	0	0	2	1	0	1	0	2
Total (105)	4	21	45	28	7	47	58	1	5	0	4	55	32	28	18	8	25	27

In bold the most prevalent period of age, gender, comorbidities, and risk factors for each yeast. F: Female, M: Male, Mor: Mortality, AA: Antecedent to accidents, AIDS: Acquired *Immune Deficiency Syndrome*, COPD: Chronic Obstructive Pulmonary Disease, CRI: Chronic Renal Failure, Dia: Diabetes, H: Hypertension. MA: Metabolic Acidosis, Obe: Obesity, Sep Sepsis.

Table II. *In vitro* susceptibilities of *Candida albicans* and non-albicans *Candida* species against six antifungal agents from clinic isolates.

Fungi	Antifungal	Range($\mu\text{g/mL}$)	Susceptible	Resistant
			MIC($\mu\text{g/mL}$)	
<i>C. albicans</i> (43)	<u>AMB</u>	2-8	***43 (100%)	-
	FCZ	8-64	***42 (98%)	**<64 1 (2%)
	FLC	2-32	***41 (95%)	**<32 2 (5%)
	ITC	0.5-4	***28 (65%)	**<4 15 (35%)
	KTC	0.5-4	***39 (91%)	**<4 4 (9%)
	MCZ	0.5-8	***39 (91%)	**<8 5 (9%)
<i>C. glabrata</i> (44)	<u>AMB</u>	2-8	***44 (100%)	-
	<u>FCZ</u>	8-64	***44 (100%)	-
	FLC	2-32	***10 (23%)	**<32 34 (77%)
	ITC	0.5-4	**7 (16%)	**<4 37 (84%)
	KTC	0.5-4	***19 (43%)	**<4 25 (56%)
	MCZ	0.5-8	**15 (34%)	**<8 27 (66%)
<i>C. krusei</i> (1)	<u>AMB</u>	2-8	***1 (100%)	-
	<u>FCZ</u>	8-64	***1 (100%)	-
	FLC	2-32	***1 (100%)	-
	ITC	0.5-4	-	**<4 1 (100%)
	KTC	0.5-4	1 (100%)	-
	MCZ	0.5-8	***1 (100%)	-
<i>C. parapsilosis</i> (1)	<u>AMB</u>	2-8	***1 (100%)	-
	<u>FCZ</u>	8-64	***1 (100%)	-
	FLC	2-32	***1 (100%)	-
	ITC	0.5-4	***1 (100%)	-
	KTC	0.5-4	***1 (100%)	-
	MCZ	0.5-8	***1 (100%)	-
<i>C. tropicalis</i> (9)	AMB	2-8	***6 (67%)	**<8 3 (33%)
	<u>FCZ</u>	8-64	***9 (100%)	-
	FLC	2-32	***7 (78%)	**<32 2 (22%)
	ITC	0.5-4	***2 (22%)	**<4 7 (78%)
	KTC	0.5-4	***6 (67%)	**<4 3 (33%)
	MCZ	0.5-8	***3 (33%)	**<8 6 (67%)
<i>Cry. neoformans</i> (2)	<u>AMB</u>	2-8	***2 (100%)	-
	FCZ	8-64	***1 (50%)	**<64 1 (50%)
	FLC	2-32	***2 (100%)	-
	ITC	0.5-4	***2 (100%)	-
	KTC	0.5-4	***2 (100%)	-
	MCZ	0.5-8	***2 (100%)	-

Underlining the most effective antifungal and in bold the less effective antifungal used *in vitro* experiments for each yeast. AMB: Amphotericin B, FCZ: 5-flucytosine, FLC: fluconazole, ITR: itraconazole, KTC: ketoconazole, and MCN: miconazole at different concentration at 37°C for 72h. *** $p < 0.01$; ** $p < 0.05$

Figures

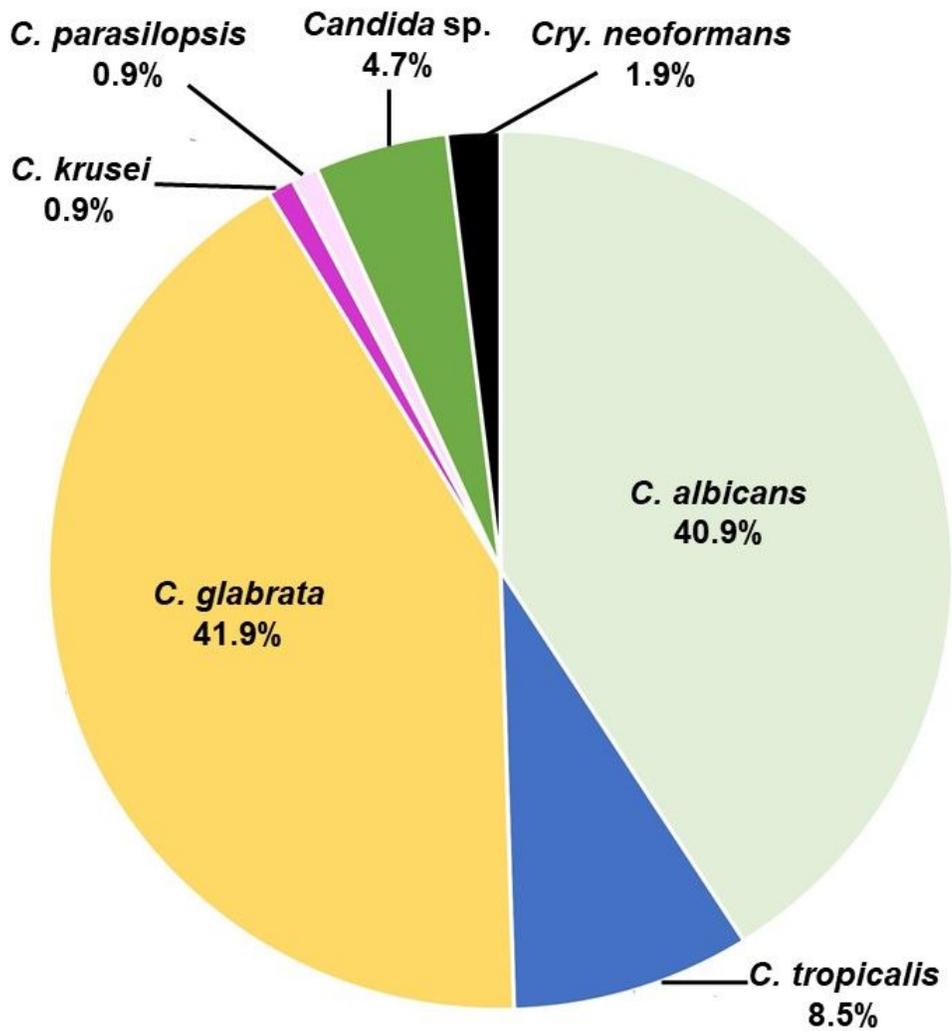


Figure 1

Distribution of *Candida* and *Cryptococcus* species. Species distribution of 105 *Candida* (including *C. glabrata*, *C. albicans*, *C. tropicalis*, *Candida sp.*, *C. parasilopsis*, and *C. krusei*) and *Cry. neoformans* human isolates from Querétaro, Mexico, over one year (2019-2020).

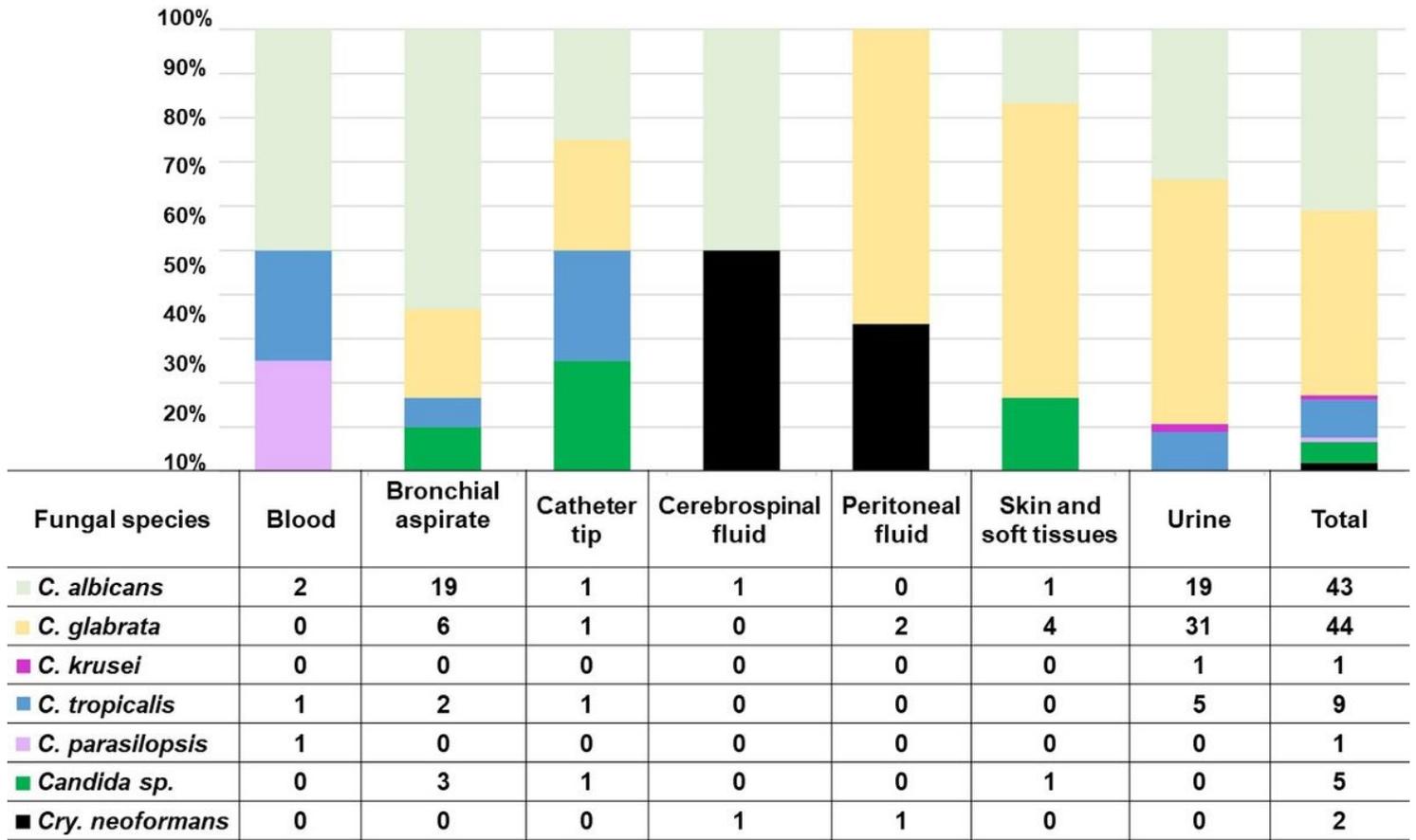


Figure 2
 Abundance of *Candida albicans* and non- albicans *Candida* culturable species isolated from different clinical samples. *Candida* and *Cry neoformans* identification from different clinical samples, which includes cultures from: bloodstream, bronchial aspirate, catheter tip, craniospinal fluid, peritoneal fluid, skin and soft tissues infections and urine.