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ORIGINAL PAPER

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, or the antifungal susceptibility of such strains without performing antifungal tests; fluconazole is one of the most used treatments in empirical therapy. In the present study, we included patients from the intensive care unit of one hospital in Mexico (2019–2020) with yeast infection and positive cultures. Yeasts obtained from cultivable isolates were identified using an automated identification instrument and by PCR (nrDNA ITS), and their susceptibilities to six antifungals were characterized across a range of concentrations. Yeast cultures from 105 patients which were suspected etiological agents of primary diagnosis were recovered and identified as mainly non-*albicans Candida* species (57.2%). 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%). Most isolated fungi were susceptible to 5-flucytosine (98%) and amphotericin B. However, *C. glabrata*, *C. krusei*, and *C. tropicalis* demonstrated to be resistant to 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.

Keywords Candidemia · Intensive care unit patients · Fungal identification · Antifungal resistance

Introduction

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

In hospitalized patients, the main etiological agents causing mycoses belong to the genus *Candida* [5, 6]. Mycoses caused by yeasts, 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, with rates of 38–75% and 70%, respectively [9, 10]. Yeast infections are related to patients in intensive care units (ICUs), prolonged hospitalization, and comorbidities such as immune suppressed status, 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 often involves multifocal colonization in the human body, including cutaneous, pulmonary, gastrointestinal, and urogenital localization, and dissemination in the

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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 during therapy and for prophylaxis may cause an increase of infections and drug resistance by *C. albicans* and non-*albicans Candida* species [16]. Several isolates of *Candida* and *Cryptococcus* have been described as resistant to conventional antifungals such as polyenes (amphotericin B), azoles (fluconazole and itraconazole), and echinocandins (caspofungin) in countries such as Spain and France [7, 17]. Concerningly, fluconazole is one of the most used antifungals in empirical therapy in hospitals in Mexico against disseminated and localized infection by *Candida* or any fungi, regardless of the origin or type of mycosis [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 1 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 study adhered to the policies of the World Medical Association Declaration of Helsinki and the Ethical and Investigation Committee of Servicios de Salud del Estado de Querétaro which reviewed and approved the implementation of this study (09-28-2019/11110/UNIVERSIDAD AUTONOMA DE QUERETARO) in the HGQ, and that Official Mexican Standard NOM-024-SSA3-2012 was followed.

Patients admitted to ICU for > 24 h that required intensive post-operative monitoring or underwent therapy for one or more acute organ system failures from January 2019 through January 2020 were included. Clinical and demographic data were recorded prospectively and analyzed (age, gender, comorbidities, and risk factors). Patients with positive cultures obtained from different clinical samples (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 using the Vitek 2k system (bioMérieux, Lyon, France), based on the manufacturer's instructions [18], and by PCR based on method described by Calvillo-Medina et al. [19]. Briefly, DNA was obtained using cetyl-trimethylammonium bromide (CTAB)-based method (Sigma®), and the fungal barcode nrDNA internal transcribed spacer (ITS) sequence 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 of *Candida* species and genera-related sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/genbank/).

Antifungal Susceptibility Assay

A panel of six antifungals (Sigma Aldrich®, USA), commonly used in systemic or localized infections therapy in Mexico, were used for susceptibility testing. Concentration ranges for the assays were for amphotericin B (AMB) 1 to 8 mg/mL, 5-flucytosine (FCZ) 1 to 32 mg/mL, fluconazole (FLC) 8 to 64 mg/mL, itraconazole (ITR) 0.5 to 4 mg/mL, ketoconazole (KTC) 0.5 to 4 mg/mL, and miconazole (MCN) 0.5 to 8 mg/mL. Each antifungal stock solution was prepared in sterile dimethyl sulfoxide (DMSO) and sterilized by 0.22 µm filtration (Millipore®, USA) and stored at – 70 °C until their use [21]. 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 broth microdilution M27-A3 Clinical and Laboratory Standards Institute (CLSI) [22, 23].

Each isolate grew for 24 h in SDA, inoculum concentration was calculated, adjusted to 1×10^3 to 5×10^3 CFU/mL in RPMI 1640 medium (Sigma®, Germany), and 100 µL was placed into each well with the antifungal agent. Antifungal susceptibility assays were done in quadruplicate. The microplates were incubated at 35 °C and read after 48 h. Plates were measured with a microplate spectrophotometer (Multiskan Ascent Thermo Labsystems, USA) at an optical density (OD) of 595 nm and were compared with drug-free controls. Strains from the American Type Culture Collection (ATCC) were used as quality control, *Candida krusei* (ATCC 6258), and *C. parapsilosis* (ATCC 22019). MIC breakpoints were interpreted according to the CLSI M27-S3. [22, 24].

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

Demographic Data of Patients

The study included 105 patients that showed a positive yeast culture. The age range at which the fungal infections presented the highest incidence was 41–60 years, and the average age was 54.5 years (± 18.13). Of the 105 patients, 58 (62%) were males and 47 (38%) were females. Principal risk factors observed included 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 1).

Fungal Isolation and Identification

Identification of the 105 isolates using the Vitek 2k and PCR revealed that most were 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; Table 1). The results between fungal species and chronic diseases were all patients with *C. albicans* infection had CRF ($p < 0.01$), 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 ($p < 0.01$), and 60% had metabolic acidosis (3/5). The single patient that had *C. parapsilosis* infection presented hypertension, obesity, and metabolic acidosis ($p < 0.01$), and the patient with *C. krusei* presented diabetes ($p < 0.01$). Finally, the two patients with *C. neoformans* infection presented diabetes ($p < 0.01$) and one of them had AIDS (50%) (Table 1).

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).

Table 1 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	CAN	COPD	CRF	Dia	Hyp	MA	Obe	Sep	
C. albicans ($n = 43$)	2	9	16	13	3	21	22	0	1	0	2	43***	16	6	7	0	9	**10**
C. glabrata ($n = 44$)	1	7	20	12	4	22	22	0	1	4	2	11	3	12	7	3	10	9
C krusei ($n = 1$)	0	0	1***	0	0	1***	0	0	0	0	0	0	1***	0	0	0	1***	0
C. parapsilosis ($n = 1$)	0	1***	0	0	0	1***	0	0	0	0	0	0	0	1***	1***	1***	1***	1**
C tropicalis ($n = 9$)	1	3	3	2	0	1	8	0	2	0	0	1	5	8	0	1	3	4
Candida sp. ($n = 5$)	0	0	4	1	0	0	5***	0	1	0	0	0	5***	0	3	2	1	1
C. neoformans ($n = 2$)	0	1	1	0	0	1	1	1	0	0	0	0	2***	1	0	1	0	2***
Total ($n = 105$)	4	21	**45**	28	7	47	**58**	1	5	0	4	**55**	32	28	18	8	25	27

In bold are the most prevalent period of age, gender, comorbidities, and mortality

F female, *M* male, *Mor* mortality, *AA* antecedent to accidents, *AIDS* acquired immune deficiency syndrome, *CAN* cancer, *COPD* chronic obstructive pulmonary disease, *CRF* chronic renal failure, *Dia* diabetes, *H* hypertension. *MA* metabolic acidosis, *Obe* obesity, *Sep* sepsis

***$p < 0.01$

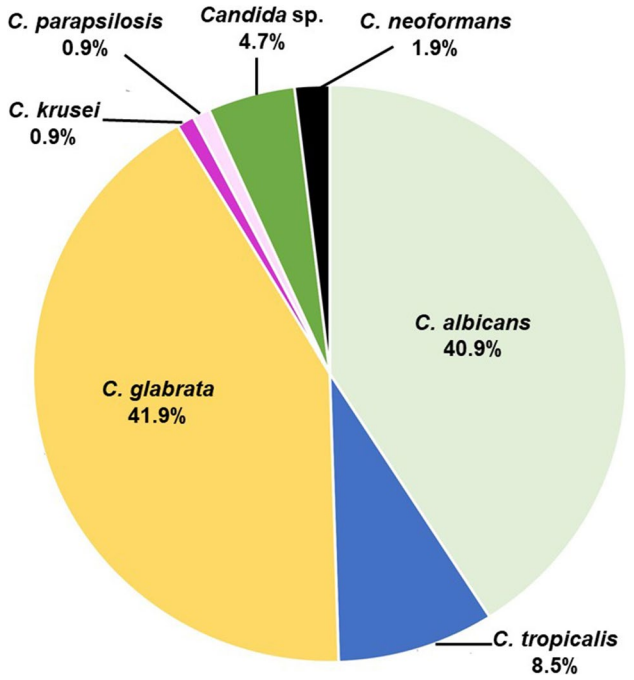


Fig. 1 Distribution of *Candida and Cryptococcus* species. Species distribution of 105 *Candida* (including *C. glabrata*, *C. albicans*, *C. tropicalis*, *Candida* sp., *C. parapsilosis*, and *C krusei*) and *C. neoformans* human isolates from Querétaro, Mexico, over one year (2019–2020)

Analysis of fungal infection with respect to isolation site revealed 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 single isolate of *C. parapsilosis* was found in a bloodstream. Finally, *C. 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 *C. 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 1).

Antifungal Assay

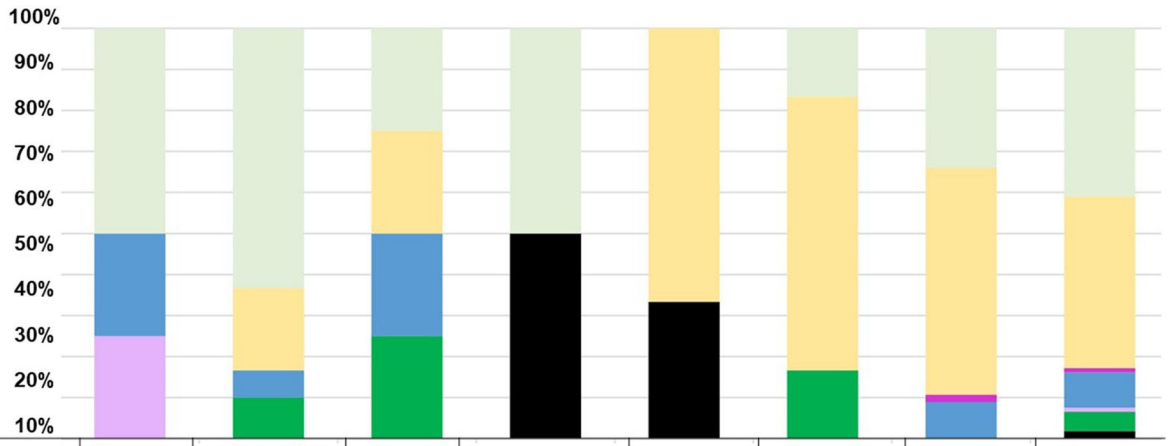
In patients who died, the most important comorbidity was sepsis (23/27; 85%) followed by hypertension (21/27; 77.7%), CRF (20/27; 74%), and diabetes (20/27; 74%). All patients diagnosed with mycoses were given FLC treatment, but voriconazole was used only in 3 patients. The results of antifungal assays testing each strain against the six most common treatments (Table 2) showed that the 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.

According to in vitro results and after 48 h of growth, the two most prevalent fungi, *C. glabrata* and *C. albicans*, were both susceptible (with no growth) to AMB (MIC < 1 mg/mL) and FCZ (MIC < 1 mg/mL) and resistant to ITR (MIC > 4 mg/mL), FLC (MIC > 64 mg/mL), and MCN (MIC >8 mg/mL). Isolates of *C. krusei* and *C. parapsilosis* showed susceptibility to all antifungals tested, but *C. tropicalis* isolates were susceptible to FCZ (100%), FLC (78%), AMB (67%), and KTC (67%) and only moderately resistant to ITR > MCN. *C. neoformans* isolates presented sensitivity to all the antifungals tested, except for FCZ (50%). However, it is important to recall that both cases of cryptococcosis were fatal. Comparison of all isolate controls against antifungal treatments showed a significant difference ($p < 0.01$) in all experiments (Table 2).

Discussion

There is a lack of data about *Candida* infections in Mexico despite the frequency and severity of the infections that they cause. Most information regarding yeast infections is from Mexico City, and a few investigations have been conducted for other parts of the country [2, 25]. Our study addresses this knowledge gap, by characterizing 105 fungal isolates from patients in HGQ in Querétaro, Mexico. These samples were isolated from different human sites across the span of 1 year, and each was tested for antifungal resistance. Here, the results showed a correlation between risk factors (diabetes mellitus, hypertension, renal failure, and obesity) and mycoses. These comorbidities predispose patients to fungal infection specifically candidiasis and cryptococcosis [2, 25]. The mortality rate found in HGQ was 25.8%, lower than the Brazilian rate which ranges from 54 to 72.2%, and similar to that found in China (23.3%) [26].

We described the distribution of the most frequent species of the genus *Candida*: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* [1, 12] and the presence of *C. neoformans* (1.9%). Our report highlights the predominance of non-*albicans Candida* species (57.2%), with *C. glabrata* being the most frequent which may be a cause of concern in the management of candidiasis. According to de la Torres-Saldaña et al. [27] and Reyes-Montes et al. [2], *C. albicans* is the most frequent etiological agent isolated from Mexican patients. However, González et al. [1] 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



Fungal species	Blood	Bronchial aspirate	Catheter tip	Cerebrospinal fluid	Peritoneal fluid	Skin and soft tissues	Urine	Total
C. albicans	2	19	1	1	0	1	19	43
C. glabrata	0	6	1	0	2	4	31	44
C. krusei	0	0	0	0	0	0	1	1
C. tropicalis	1	2	1	0	0	0	5	9
C. parapsilosis	1	0	0	0	0	0	0	1
Candida sp.	0	3	1	0	0	1	0	5
C. neoformans	0	0	0	1	1	0	0	2

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

[12, 14]. According to Manzano-Gayosso et al. [28], 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 [29]. However, indiscriminate use of FLC (for example prophylaxis) can produce a great economic and ecological impact [2, 18]. In addition to generating resistant yeast, excessive use of FLC has caused its efficacy rate to decrease. FLC offers only variable protection against non-*albicans Candida* species [12]. Based on differences in susceptibility to FLC, Jenks et al. [30] recommended the implementation of newer triazoles with antifungal activity such as voriconazole [31]. Therefore, to minimize the further development of resistance by *Candida* and *Cryptococcus*, appropriate antifungal treatments should be carefully selected, started as early as possible, and only used in patients with proven infection [12, 32].

General resistance to azoles, and specific resistance to FLC, have been reported in *Candida* and *Cryptococcus* [2, 18]. Moreover, the data generated from this study indicate that ITR, FLC, and MCN were the less effective 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, 33]. In contrast to Corzo-Leon et al. [18] and González et al. [1], we found high resistance of *C. tropicalis* and *C. albicans* to azoles and AMB. Additionally, *C. parapsilosis* was susceptible to all antifungal agents tested. According to González et al. [1], Pfaller and Diekema [30], and Corzo-Leon et al. [18], and based on our results, the use of FLC should not be continued for prophylaxis or in patients with recurrent yeast infections.

Our study had several limitations. First, some epidemiological data concerning (previous infection, transfers between hospitals, or previous exposure to antifungals) were not available. Second, the isolates were only collected and analyzed for 1 year. The duration of the study could be extended to provide a more comprehensive analysis of the state of fungal infections and treatments. Third, we did not collect samples from the hospital environment or health care workers, which could provide information to better understand the source and spread of infections. Finally, the decision on which treatments were administered was made by physicians at HGQ and was not based on the results of our antifungal assays.

Table 2 In vitro susceptibilities of *Candida albicans* and non-*albicans Candida* species against six antifungal agents from clinic isolates

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

In bold are the most effective antifungals (susceptible culture) and underlined the less effective (resistant yeast) used in vitro experiments for each yeast. At different concentration at 37 °C for 48 h. The concentrations of each antifungal in susceptible column are AMB < 1 mg/mL; FCZ < 1 mg/mL, FLC < 8 mg/mL, ITR < 0.5 mg/mL, KTC < 0.5 mg/mL and MCN < 0.5 mg/mL. In resistance column are AMB > 8 mg/mL; FCZ > 32 mg/mL, FLC > 64 mg/mL, ITR > 4 mg/mL, KTC 4 > mg/mL and MCN 8 > mg/mL

AMB amphotericin B, *FCZ* 5-flucytosine, *FLC* fluconazole, *ITR* itraconazole, *KTC* ketoconazole, and *MCN* miconazole

*** $p < 0.01$; ** $p < 0.05$

In conclusion, it is important to improve the identification and antifungal test (identification by PCR or susceptibility to echinocandins) of non-*albicans Candida* species in ICU patients in Mexico to ensure specific identification, diagnosis, and treatment. Selection of appropriate therapeutic strategies 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. Improving identification and antifungal testing strategies will allow a better understanding of the epidemiology and susceptibility

patterns of fungal infection in Mexico and in other countries with limited resources.

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Author Contribution Rosa Paulina Calvillo-Medina designed this project, carried out the fungal identification, contributed to statistical data 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 statistical data analysis and contributed to the revision of the manuscript. Juan José Olalde-Elias and Fernando Domínguez-Márquez participated in the design of the study and manuscript revision. All the authors contributed, read, and approved the manuscript.

Data availability Not applicable.

Code Availability Not applicable.

Declarations

Ethics Approval The 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 the authors contributed and approved the submission of the manuscript for publication, and the written consent for publication has been obtained.

Competing Interests The authors declare no competing interests.

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