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Distribution of *Candida* Species and Molecular Typing of *C. albicans* Isolates in a Mexico City Tertiary Care Hospital from 2011 to 2013

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Abstract

The incidence of yeast infections has increased significantly over the past few years, constituting a leading cause of morbidity and mortality among hospitalised patients. The rapid identification of candidiasis is important for the clinical management of patients and to facilitate tracing the sources of infections in hospitalized patients. Here, we report a retrospective, single-centre study of *Candida* spp. distribution and antifungal susceptibility from January 2011 to May 2013 at a hospital in México City, regarding the importance of elucidating the identity of the infection-causing *Candida* species in order to improve prophylactic measures and treatment. Clinical data were collected from patient medical records and the laboratory database. Isolates were initially identified using standard mycology techniques, and then confirmed by PCR-based system using amplification of intergenic spacers (rDNA ITS) and restriction length polymorphism of PCR products after sequence-specific enzymatic cleavage (PCR-RFLP). We observed no shift from *C. albicans* to non-albicans Candida species: *Candida albicans* (73.7%) was the most prevalent species isolated, while *C. dubliniensis* was not identified in this study. Antifungal susceptibility was determined using FUNGITEST®; 17.4% of *C. albicans* isolates were resistant to fluconazole and 21.7% to itraconazole. Multiplex PCR microsatellite analysis of the clinical *C. albicans* isolates using primers for the CAI, CAIII and CAVI loci identified 29 different alleles for CAI, 8 alleles for CAIII and 31 for CAVI. The combined discriminatory power of these three microsatellites was 0.98, which was considered reliable for molecular typing. Genetic analysis of these isolates revealed a clonal population with a total of 62 genotypes from the examined isolates.

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Keywords

Candidiasis, rDNA-ITS, RFLP, Microsatellites, Molecular Typing, CAI, *Candida* spp.

1. Introduction

The incidence of yeast infections in hospitals has greatly increased in recent decades; these infections are a major cause of morbidity and mortality in patients. Candidiasis is common among critically ill patients hospitalised in the intensive care unit (ICU) for over a week, especially in those with immune deficiency and severe underlying diseases. In addition, the incidence of candidiasis has increased with the use of broad-spectrum antibiotics treatment, chemotherapy and invasive medical devices [1]. Since several *Candida* spp. are present in the human-associated microbes colonisation develops in up to 80% of critically ill patients; however, invasive candidiasis is documented in only 5% - 10% of them [2].

Common diagnostic criteria of candidiasis include the observation of filamentous forms or clusters of budding yeasts in biological samples or during post mortem histopathological examination, a serum titre of β-1, 3-glucan and isolation of fungi from sterile samples [3]. *In vitro* studies have shown that the pseudo hyphae and hyphae of *Candida albicans* and *C. tropicalis* enable invasion of human and murine cells; these forms are considered as indicative of infection [4] [5] in most *Candida* species except for *C. glabrata*.

Although *C. albicans* is the most common candidiasis-causing species, approximately 45% of cases are caused by non-*albicans* species that have emerged as colonisers and pathogens [6]. The appearance of these non-*albicans* species is of special concern because of their intrinsic resistance to antifungal drugs. These features represent a clinical problem since inappropriate empirical antifungal therapy may result in adverse outcomes. Rapid diagnosis and species identification are required for early and accurate treatment that will eventually reduce the mortality rate of patients. Phenotypic identification is routinely used in clinical laboratories, but these methods can take 48 to 72 h to identify the pathogen to the species level, and the evaluation of these characteristics requires expertise [7].

Many attempts have been made over the last decade to develop techniques that can accurately identify species and distinguish between endemic and sporadic strains [8]-[10], such techniques would enable detection of the origin of infection and identification of strains involved in outbreaks. Transmission of *Candida* spp. occurs via direct contact; however, there have been worldwide reports of cross infection due to health care workers within hospitals.

Molecular methods are more reliable and have been used extensively for diagnosis and identification with high levels of sensitivity, specificity and reproducibility; this is advantageous for determining and optimising treatment early in the infection, and is important for elucidating the distribution of *Candida* species. For example, microsatellite analysis based on the amplification of highly polymorphic short tandem repeats can be used to generate characteristic profiles for different alleles of a specific locus; the high differentiation power (DP) of this technique permits studies of nosocomial transmission using the CAI, CAIII and CAVI loci [10].

In México, candidiasis surveillance programs have been sporadic, and data regarding the distribution of different *Candida* species in tertiary care hospitals are limited and these analyses have been important worldwide since *Candida*, is associated with almost 80% of all nosocomial fungal infections, representing the major cause of fungemia with high mortality rates (40%) [11].

In this study, we examined the distribution of *Candida* species in cases of nosocomial *Candida* infections. To achieve this, we analysed the clinical records from January 2011 to May 2013 of patients who had *Candida* infections and were treated at Hospital Juarez de México (HJM; representative of the public health system in México).The distribution and prevalence of the relevant yeast species were determined by phenotypic methods and corroborated by PCR-RFLP of the ribosomal DNA internal transcribed spacer (rDNA-ITS) region. Susceptibility patterns to amphotericin B (AMB), fluconazole (FLU) and itraconazole (ITR) were determined using the FUNGITEST® kit. The genetic relatedness of *C. albicans* isolates from hospitalized patients was determined using CAI, CAIII and CAVI multiplex PCR microsatellite assays [10].

Our study demonstrates that *C. albicans* is the prevalent *Candida* specie in HJM with antifungal resistance to

fluconazole and itraconazol. Genotypic analysis revealed some genotypes present in nearly all examined *C. albicans* isolates, suggesting they might represent endemic strains.

2. Materials and Methods

2.1. Study Design

This was a retrospective, observational and transversal study conducted in HJM by the Medical Mycology Laboratory from January 2011 to May 2013 evaluating 167 clinical samples from patients being treated at the HJM. This hospital mainly provides primary and tertiary medical services not only to patients from Distrito Federal and Mexico State, but also to patients originating from different hospitals from the states of Hidalgo, Michoacán, Veracruz and Guerrero. The study was approved by the hospital ethics committee; however, informed consent was not requested from the patients because of the observational nature of the study. The investigators did not intervene in the standard of care, and patient's records and all information was kept confidential. The clinical data (age, gender, underlying diseases, prior use of antimicrobial and antifungal agents, malignancies, mechanical ventilation, diabetes mellitus, HIV/AIDS, invasive procedures and prematurity or low weight at birth) of patients with a clinical and laboratory diagnosis of candidiasis were collected.

Patients whose biological samples fulfilled the following inclusion criteria were considered positive for *Candida* infection:

- Positive *Candida* spp. cultures from systemic and peripheral blood subsequent to 3 days hospitalisation.
- Bronchoalveolar lavage (BAL): Respiratory tract infection is difficult to diagnose. Therefore, a combination of clinical, laboratory and radiological indicators was used to select isolates from patients who had severe respiratory diseases, exhibited symptoms such as fever, tachypnoea, dyspnoea, and chest pain, and had negative results for bacterial infection and robust positive results for yeast microscopic examination (presence of budding yeast clusters and/or filamentous morphologies).
- Urine samples, sterilely collected from patients diagnosed with a urinary tract infection (based on symptoms), with a positive microscopic exam for budding yeast clusters and/or filamentous morphologies and concomitant pyuria with counts >100,000 CFU/mL urine. It should be noted that the fungal burden could be relevant, since a statistically significant correlation has been established between heavy candiduria (>10^4 CFU/ml urine) and a high Pittet *Candida* colonisation index (>0.5) [12].
- Medical devices such as central venous catheter (CVC) tips and tracheal tube tips with culture counts > 20 CFU per plate.
- Oral cavity swabs taken from creamy white, slightly raised and membranous lesions present in the mouth, gums, tonsils, soft or hard palate, and acute atrophic erythematous lesions with positive microscopic exam for budding yeast clusters and/or filamentous morphologies.
- Skin and nail scrapings and genital mucosa and pharynx swabs with positive microscopic exam for budding yeast clusters and/or filamentous morphologies as well as symptoms indicating onychomycosis, skin or mucosa lesions with erythematous borders and satellite lesions or thrush.

2.2. Isolation and Phenotypic and Molecular Identification of Candida Isolates

Yeast colonies were isolated from culture on Sabouraud agar (Difco, USA) or Mycosel agar (Difco, USA) incubated at 37˚C. Phenotypic species identification was based on the production of germ-tube, chlamydoconidium and hyphae morphology on cornmeal-Tween 80 agar, colony colour on CHRO Magar *Candida* and carbohydrate assimilation profiles generated using API 20C AUX kits (bioMérieux, France). Identification was confirmed by molecular analysis (PCR-RFLP) of the ITS1-5.8S-ITS2 rDNA fragment from all isolates. DNA extraction was performed as follows: cells grown on Sabouraud agar for 48 h were mechanically disrupted with glass beads in lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]), purified by chloroform/isopropanol extraction and finally dissolved in 30 µl of double distilled water. Universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used as previously reported [13]. The control strains were *C. albicans* ATCC 90028, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. guilliermondii* ATCC 6260 and *C. dubliniensis* CD 36. The amplified products were digested with *Msp*I [13]; isolates identified as *C. albicans* were additionally digested with *Avr*II and then amplified with primers specific

for *C. dubliniensis*: DUB-F (5'-GTATTTGTCGTTCCCCTTTC-3') and DUB-R (5'-GTGTTGTGTGCACTAACGTC-3') [14].

2.3. In vitro Susceptibility Test

Susceptibility to antifungal drugs was tested using the FUNGITEST® Kit (Bio-Rad, France), a modified microtitre broth breakpoint test based on the NCCLS M27A standard. Each test plate contains six antifungal agents at two different concentrations, AMB (2 and 8 mg/mL), FLU (8 and 64 mg/ml), ITR (0.5 and 4 mg/ml), miconazole (0.5 and 8 mg/ml), ketoconazole (0.5 and 4 mg/ml) and 5-fluorocytosine (5FC, 2 and 32 mg/ml) in individual wells. Growth assessment is based on the colour change of a redox indicator. FUNGITEST results are known to correlate well with those of the NCCLS microdilution plate method; the kit has the advantage of being easy to read (coloured indicator) and use [15].

2.4. Multiplex PCR Microsatellite Assay

Microsatellite genotyping was performed by combining PCR buffer (20 mMTris-HCl, pH 8.4, 50 mMKCl), 0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, 50 ng of genomic DNA and 0.5 U of *Taq* polymerase (Applied Biosystems) in a final volume of 25 µl. The forward primers used in the PCR amplification were labelled with the following fluorochromes: CAI-FAM 6-carboxyfluorescein (CAA)2CTG(CAA)n), CAIII-FAM 6-carboxyfluorescein (GAA)*n* and CAVI-HEX hexachlorofluorescein (TAAA)n, as previously described [10]. The PCR products were run on an ABI 310 Genetic Analyser (Applied Biosystems) together with the GeneScan-500 (TAMRA) size standard (Applied Biosystems). Fragment sizes were determined automatically using the Gene Scan 3.7 Analysis software. The alleles observed for each locus were sequenced on an ABI PRISM 310 DNA automatic sequencer (Applied Biosystems). PCR product fragment sizes and allele sizes were determined automatically with Gene Scan 3.7 analysis software. Alleles were designated by the number of repeated units determined. The DP was calculated for each marker according to the Simpson index of diversity.

$$\mathrm{DP} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} xj\left(xj-1\right)$$

where *N* is the number of strains, *s* is the total number of different genotypes and *nj* is the number of genotype *j* strains [16]. Genetic distance between genotyped isolates was calculated using the Cavalli-Sforza and Edwards genetic distance index [17] with the Populations 1.2.32 software. A dendrogram was constructed using the unweighted pair group analysis with arithmetic mean (UPGMA) model using MEGA 5.2 software. Clonality and recombination in *C. albicans* isolates were verified using the Hardy-Weinberg equilibrium test and F_{IS} value were calculated with the web version of GENEPOP (http://genepop.curtin.edu.au/) and GraphPad Prism 6.

3. Results

3.1. Demographics, Clinical Characteristics and Risk Factors

A total of 167 *Candida* spp. isolates were evaluated, each representing an individual infectious episode. Each case was considered to be caused by a single *Candida* species; three cases in which different or several yeast species were isolated were discarded from the study. Samples originated from different medical wards: internal medicine (60.5%; cardiology, endocrinology, haematology, infectology, pneumology), intensive care unit (34.1%; surgery, adults and paediatrics), emergency room (1.8%) and others (3.6%; geriatrics and dermatology). The number of isolates referred for testing each year was as follows: 65 in the first year, 62 in the second year and 40 in the 5 months of the third year. The samples were obtained from 95 male (56.9%) and 72 (43.1%) female patients. Patient clinical data and *Candida* species distribution are summarised in **Table 1**.

In most infection cases (141 cases, 84.4%) the *Candida* isolates were identified in the BAL (40.7%) and urine (37.1%); only eight cases had positive blood cultures (4.8%) and three were isolated from medical devices (1.8%; one CVC tip and two tracheal tube tips). Isolates from superficial infections (26, 15.6%) were identified in the oral cavity (6.0%), pharynx swabs (6.6%) and skin and nail scrapings (3.0%).

Patient age ranged from 2 days to 84 years with a mean of 41 years; most cases involved patients aged 19 to 40 years (47, 28.1%) and 41 to 60 years (60, 35.9%).

Table 1. Clinical data of patients with nosocomial candidiasis and *Candida* species distribution.

Sex (n= 167)		(%)									
Male	95	(56.9)									
Female	72	(43.1)									
Age (years)	**Total**		***C. albicans***		***C. tropicalis***		***C. parapsilosis***		***C. glabrata***		***Candida* spp.**
	(%)		(73.7)		(15.0)		(5.4)		(3.6)		(2.4)	
<1	5	(3.0)	5	(3.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
1 to 18	25	(15.0)	15	(9.0)	7	(4.2)	3	(1.8)	0	(0.0)	0	(0.0)
19 to 40	47	(28.1)	38	(22.8)	4	(2.4)	1	(0.6)	4	(2.4)	0	(0.0)
41 to 60	60	(35.9)	43	(25.7)	9	(5.4)	4	(2.4)	1	(0.6)	3	(1.8)
>60	30	(18.0)	22	(13.2)	5	(3.0)	1	(0.6)	1	(0.6)	1	(0.6)
Mean age	41											
Isolation sample												
Deep infections												
BAL	68	(40.7)	56	(33.5)	7	(4.2)	2	(1.2)	2	(1.2)	1	(0.6)
Urine	62	(37.1)	40	(24.0)	13	(7.8)	4	(2.4)	3	(1.8)	2	(1.2)
Blood	8	(4.8)	7	(4.2)	0	(0.0)	1	(0.6)	0	(0.0)	0	(0.0)
Medical devices	3	(1.8)	1	(0.6)	1	(0.6)	1	(0.6)	0	(0.0)	0	(0.0)
Superficial infections												
Swabing (genital and pharynx)	11	(6.6)	6	(3.6)	4	(2.4)	0	(0.0)	0	(0.0)	1	(0.6)
Oral cavity	10	(6.0)	9	(5.4)	0	(0.0)	0	(0.6)	1	(0.6)	0	(0.0)
Skin and nail scrapping	5	(3.0)	4	(2.4)	0	(0.0)	1	(0.6)	0	(0.0)	0	(0.0)
Impatient units												
Internal Medicine	101	(60.5)	71	(42.5)	14	(8.4)	7	(4.2)	5	(3.0)	4	(2.4)
ICU	57	(34.1)	44	(26.3)	11	(6.6)	1	(0.6)	1	(0.6)	0	(0.0)
Other wards	6	(3.6)	5	(3.0)	0	(0.0)	1	(0.6)	0	(0.0)	0	(0.0)
ER	3	(1.8)	3	(1.8)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Underlying diseases												
Respiratory diseases	55	(32.9)	43	(25.7)	9	(5.4)	0	(0.0)	2	(1.2)	1	(0.6)
Malignancies	20	(12.0)	13	(7.8)	4	(2.4)	1	(0.6)	1	(0.6)	1	(0.6)
Tuberculosis	10	(6.0)	8	(4.8)	0	(0.0)	0	(0.0)	2	(1.2)	0	(0.0)
Diabetes mellitus	7	(4.2)	6	(3.6)	0	(0.0)	1	(0.6)	0	(0.0)	0	(0.0)
UTI	7	(4.2)	5	(3.0)	0	(0.0)	0	(0.0)	1	(0.6)	1	(0.6)
Risk factors												
CVC	79	(47.3)	61	(36.5)	11	(6.6)	2	(1.2)	3	(1.8)	2	(1.2)
broad-spectrum antimicrobial	33	(19.8)	25	(15.0)	5	(3.0)	1	(0.6)	1	(0.6)	1	(0.6)
Mechanical ventilation	44	(26.3)	35	(21.0)	7	(4.2)	0	(0.0)	1	(0.6)	1	(0.6)
sepsis	43	(25.7)	36	(21.6)	5	(3.0)	1	(0.6)	0	(0.0)	1	(0.6)
previous use of antifungal drugs	21	(12.6)	16	(9.6)	4	(2.4)	1	(0.6)	0	(0.0)	0	(0.0)
HIV/AIDS	10	(6.0)	7	(4.2)	2	(1.2)	0	(0.0)	1	(0.6)	0	(0.0)
Invasive procedures	8	(4.8)	8	(4.8)	1	(0.6)	0	(0.0)	1	(0.6)	0	(0.0)
autoimmunity disorders	7	(4.2)	5	(3.0)	1	(0.6)	1	(0.6)	0	(0.0)	0	(0.0)

BAL: Broncho Alveolar Lavage; ICU: Intensive Care Unit; ER: Emergency Room; UTI: Urinary Tract Infection; CVC: Central Venous Catheter.

Underlying diseases included respiratory diseases (32.9%; community acquired pneumonia, mechanic ventilator associated pneumonia, atypical pneumonia, obstructive chronic pulmonary disease), malignancies (12.0%; leukaemia, lymphoma, myeloma), tuberculosis (6.0%), type II diabetes mellitus (4.2%) and urinary tract bacterial infections (4.2%). The principal risk factors observed were the presence of CVC (47.3%), use of broad-spectrum antibiotics (19.8%), mechanical ventilation (26.3%), sepsis (25.7%) and previous use of antifungal drugs (12.6%).

Phenotypic and molecular analyses revealed that the most common *Candida* species was *C. albicans* (73.7%), followed by *C. tropicalis* (15.0%), *C. glabrata* (3.6%) and *C. parapsilosis* (5.4%) (**Table 1**). Other identified species (2.4%) included the following: *C. lusitaniae* (two isolates), *C. krusei* and *C. guilliermondii* (one isolate each) **Table 1**). The phenotypic identity of all isolates (data not shown) was confirmed by comparing the resulting patterns of MspIwith the patterns of ITS-PCR RFLP for reference yeast strains after digestion with Msp I. These analyses showed that 12 of the isolates (9.3%) had been misidentified by phenotypic methods (data not shown). Further analysis of all samples identified as *C. albicans* by PCR-RFLP with *Avr*II or by PCR using the specific primers dubF-dubRdemonstrated that *C. dubliniensis* was not present in any of the samples.

The distribution of *Candida* spp. did not vary significantly across the age groups, although the 41 - 60 years group exhibited the highest number of non-*albicans* species: *C. tropicalis* (5.4%), *C. parapsilosis* (2.4%), *C. glabrata* (0.6%) and other *Candida* spp. (1.8%). Younger age groups were dominated by other species; in patients younger than 1 year, only *C. albicans* was isolated, while in the 1 - 18 years group, *C. albicans* (9.0%) was the most abundant isolate followed by *C. tropicalis* (4.2%) and *C. parapsilosis* (1.8%).

3.2. Susceptibility to Antifungal Drugs

The *in vitro* susceptibility of isolates to AMB, FLU, ITR and 5FC is summarised in **Table 2**. The 5-fluorocytosine miconazole and ketoconazole data were omitted from further analysis since these drugs are no longer used for inpatient antifungal therapy in our hospital. Resistance tended to be more frequent among the *C. albicans* isolates (35%) than the non-*albicans* isolates (12.5%); however, this difference was not statistically significant.

Overall, 87.5% of the tested isolates were susceptible to AMB, 84.3% to FLU and 80% to ITR. AMB resistance was observed mainly in *C. albicans* (7.8%), *C. parapsilosis* (3.1%) and *C. guilliermondii* (1.5%) isolates. Of the five AMB resistant *C. albicans* strains, four originated from internal medicine patients (three were isolated from BAL and one from urine) and one from the BAL of an ICU patient. Patients with BAL-isolated strains (age 18 to 54 years) exhibited severe sepsis, CVC, had received mechanical ventilation and had been hospitalised for an extended period of time; a single patient had been diagnosed with AIDS. Unexpectedly, the five AMB resistant strains were also resistant to FLU and ITR. In addition, two AMB resistant *C. parapsilosis* strains were identified, one from a septic patient and the other from nail scrapings. One *C. guilliermondii* strain isolated from nail scrapings was also AMB resistant.

Resistance to FLU was observed in *C. albicans* (12.5%), *C. glabrata*(1.5%) and *C. krusei* (1.5%) strains; *C. krusei* is thought to be intrinsically resistant to FLU. As mentioned, five *C. albicans* strains were resistant to AMB, FLU and ITR; the remaining three strains were resistant to FLU and ITR and originated from ICU, inter-

Table 2. Resistance of *Candida* isolates to antifungal agents.

		Frequency of resistant isolates (%)							
Species	**Isolates tested**	**Fluconazole**		**Itraconazole**		**Flucytosine**		**Amphotericine B**	
C. albicans	46	8	(17.4)	10	(21.7)	0	(0)	5	(10.9)
C. tropicalis	7	0	(0)	1	(14.3)	0	(0)	0	(0)
C. parapsilosis	3	0	(0)	1	(33.3)	0	(0)	2	(66.7)
C. glabrata	5	1	(20.0)	0	(0)	0	(0)	0	(0)
C. guilliermondii	1	0	(0)	0	(0)	0	(0)	1	(100)
C. lusitaniae	1	0	(0)	1	(100)	0	(0)	0	(0)
C. krusei	1	1	(100)	0	(0)	0	(0)	0	(0)

nal medicine and emergency room patients. These strains were isolated from the blood of a male patient with disseminated tuberculosis and meningitis, the urine of a female patient with pneumonia and from the tracheal tube tip of a male patient diagnosed with pneumonia, respectively. The FLU resistant *C. glabrata* strain was isolated from the urine of a kidney transplant recipient who had not received previous antifungal prophylaxis with FLU. Resistance to ITR was observed in 10 *C. albicans* isolates (two were resistant only to ITR), and one strain of *C. tropicalis and C. parapsilosis.*

3.3. Strain Differentiation of *Candida albicans* Isolates

The microsatellite analysis results are summarised in **Table 3**. Since *C. albicans* is diploid, each resulting PCR fragment was defined as an allele; strains with two PCR products were considered as heterozygous, while those with a single product as homozygous. Microsatellite multiplex PCR yielded one or two PCR fragments per locus for each of the isolates. The discriminatory power (DP) of the primers was 0.95 for CAI, 0.70 for CAIII and 0.81 for CAVII. The combined DP value for the three loci is 0.98, which is considered a reliable DP value for molecular typing studies. Genotyping analysis showed that most of the isolates were genetically related and that there was a considerable level of similarity between individual strains. Using a separate microsatellite analysis, we were able to verify the presence of 28 different alleles with the CAI primer, five alleles with CAIII and 31 with CAVI. Analysis conducted with only the CAI microsatellite (higher DP) grouped together several independent or unrelated isolates (data not shown). Moreover, when the three loci were simultaneously analysed, isolates the same CAI allele corresponded to different strains or strains with a minimal variation in CAIII or CAVI alleles.

The microsatellite analysis revealed that most of the 84 isolates share the same or similar (only one different allele) genotype (**Table 3**). The most common alleles in CAI were 30, 41, 35 and 28, in CAIII, 5 and 4 and CAVI 06, 09 and 03. The expected heterozygosity varied from 0.934 to 0.705 and the observed genotypes of each isolated showed significant departures from the Hardy-Weinberg equilibrium in all loci ($p < 0.05$). F_{IS} value for CAI and CAVI loci indicates inbreeding in the population, while negative F_{IS} value for CAIII loci points to out breeding (**Table 4**). Interestingly most of this isolates have heterozygous CAI alleles. Almost half of the isolate share the heterozygous CAIII 04:05, 05:10 and 05:08 genotypes while CAVI homozygous 06:06 and 09:09 genotypes were common. UPGMA dendrogram using Cavalli-Sforza &Edwards genetic distance [17] shows that isolates are close related and those with the same or similar genotype cluster together (**Figure 1**).

4. Discussion

To the best of our knowledge, this is the first surveillance study of *Candida* species obtained from patients hospitalised in Hospital Juarez de México (HJM). In this study we sought to determine the relative prevalence of *C. albicans* and *non-albicans Candida* spp. in different patient specimens. All isolates were identified using a highly sensitive and specific molecular method; this precise identification ensured the reliability of our distribution data.

The biological specimens from which *Candida* spp. were most commonly isolated were BAL (40.7%) and urine (37.1%) samples originating from anatomical locations that are colonised by yeast. Only samples with a positive microscopic examination and clinical evidence of infection were included in this study as opposed to other studies that have used culturing techniques for verification, while discarding infection due to incapacity to determine if its colonization or infection growth. Most of the virulence factors of *Candida* species are expressed in filamentous morphologies [18], therefore the microscopic examination could help in discrimination and diagnosis. Further studies may determine the relationship of expression of virulence factor genes in *Candida* yeast and filamentous morphologies biological samples (*in vivo*).

This study shows that *C. albicans* was the most prevalent isolate (73.7%), in accordance with previous reports from various countries [19]. However, the abundance of this species does not agree with other global studies demonstrating a significant increase in the prevalence of non-*albicans* species over the past three decades. Other abundant species identified in this study were *C. tropicalis* (15%) followed by C. *parapsilosis* (5.4%). There was a clear predominance of *C. albicans* in children while non-*albicans* species were present mainly in adults aged 19 - 60 years. No significant differences were found between male and female patients.

Two previous studies conducted in Mexico describe species distribution and antifungal susceptibilities among *Candida* isolates obtained from blood cultures collected in different tertiary hospitals: two hospitals in the south

Table 3. Microsatellite typing of *C. albicans* isolates from Hospital Juarez de Mexico. Isolates with the same genotype or with a single allele difference are in bold with grey background.

Microsatellite alleles				Sample's information		
Isolate	**CAI**	**CAIII**	**CAIV**	**Ward**	**Diagnosis**	**Sample**
150-13	48:48	05:10	09:09	IM	Diabetes mellitus II	Urine
223-11	**37:52**	**05:10**	**09:20**	**IM**	**HIV+**	**BAL**
223-12	**37:52**	**05:10**	**09:20**	**IM**	**CAP**	**Urine**
094-13	37:46	05:10	08:08	IM	ALL	Swab
462-12	36:36	05:10	07:17	IM	Diabetes mellitus II	Urine
391-11	**35:47**	**05:10**	**09:09**	**IM**	**Cystic fibrosis**	**BAL**
393-11	**35:47**	**05:10**	**09:09**	**IM**	**HIV+**	**Blood**
069-12	**35:47**	**10:10**	**09:09**	**IM**	**Lymphoid Leukemia**	**Urine**
045-13	35:43	10:10	09:14	ICU	Septic shock	Urine
430-12	**35:41**	**05:08**	**06:06**	**Others**	**Onychomycosis**	**Skin scrapping**
469-12	**35:41**	**05:08**	**06:06**	**ICU**	**Septic shock**	**Urine**
963-10	35:40	04:04	09:09	IM	Tb, meningitis	Blood
483-10	35:38	10:10	19:19	IM	Sepsis	BAL
043-11	**35:35**	**05:10**	**09:09**	**Ped. ICU**	**Fungal sepsis**	**Urine**
047-13	**35:35**	**10:10**	**09:09**	**IM**	**Tb**	**BAL**
167-12	**35:35**	**05:10**	**09:09**	**Ped. ICU**	**HIV+, Balanitis, Onychomycosis**	**Skin scrapping**
478-12	**34:52**	**05:10**	**09:09**	**IM**	**Diabetes mellitus II**	**Urine**
493-11	**33:41**	**05:08**	**06:06**	**IM**	**SLE, VAP**	**BAL**
398-12	**34:41**	**05:08**	**06:06**	**ICU**	**Septic shock**	**BAL**
483-09	**34:39**	**05:10**	**09:12**	**IM**	**Sepsis**	**BAL**
492-09	**33:41**	**08:08**	**06:06**	**IM**	**Atypical CAP**	**BAL**
922-10	**33:41**	**04:04**	**06:06**	**Others**	**septic shock**	**Urine**
059-12	**33:41**	**04:05**	**05:06**	**IM**	**CAP**	**BAL**
141-12	**33:41**	**04:05**	**06:06**	**ICU**	**Pneumonia**	**BAL**
394-12	**33:41**	**05:08**	**06:06**	**ICU**	**SLE**	**BAL**
457-12	**33:41**	**04:05**	**06:06**	**ICU**	**ARDS**	**Urine**
216-13	**33:41**	**04:05**	**06:06**	**IM**	**Septic shock**	**Urine**
219-13	**33:41**	**04:05**	**06:06**	**IM**	**Sepsis**	**Urine**
120-11	**33:40**	**04:05**	**09:09**	**IM**	**ALL Pre B**	**BAL**
225-12S	**33:33**	**04:05**	**05:06**	**IM**	**Bacterial endocarditis**	**Swab**
225-12U	**33:33**	**04:05**	**06:06**	**IM**	**Bacterial endocarditis**	**Urine**
027-11	**32:42**	**04:05**	**06:06**	**ICU**	**Tb**	**BAL**
119-11	**32:41**	**05:08**	**06:06**	**ICU**	**Pneumonia**	**BAL**
016-12	**32:41**	**05:08**	**06:06**	**IM**	**Sepsis**	**BAL**
161-12	**32:41**	**05:08**	**06:06**	**IM**	**Pneumonia, SLE**	**BAL**
162-12	**32:41**	**05:10**	**06:06**	**IM**	**Pneumonia**	**Urine**
164-12	**32:41**	**05:08**	**06:06**	**IM**	**Ascites**	**Urine**
166-12	**32:42**	**04:05**	**06:06**	**ICU**	**Lung sepsis**	**BAL**
334-12	**32:42**	**04:05**	**06:06**	**IM**	**Tb**	**BAL**
396-12	**32:41**	**05:08**	**06:06**	**IM**	**Tb**	**BAL**
439-12	**32:42**	**04:05**	**06:06**	**ICU**	**Fungal sepsis**	**BAL**

Continued

111-12	31:39	05:10	20:20	ICU	Lung sepsis	BAL
103-12	30:40	04:05	06:06	IM	Sepsis	BAL
498-11	30:30	04:05	27:27	IM	Oropharyngeal candidiasis	Swab
220-13	30:30	05:05	03:06	IM	Pneumonia	Urine
161-13	30:30	04:05	03:30	IM	Pneumonia	BAL
364-12	29:55	05:10	09:09	ICU	Atypical pneumonia, ARDS	Blood
498-09	**28:33**	**08:08**	**06:06**	**IM**	**Myeloma, oral candidosis**	**Swab**
217-11	**28:33**	**07:08**	**07:07**	**IM**	**Tb**	**BAL**
360-12	**28:33**	**07:08**	**07:10**	**IM**	**Pneumonia, scleroderma**	**BAL**
366-12	**28:33**	**07:08**	**07:07**	**IM**	**Sepsis**	**Urine**
108-13	**28:33**	**07:08**	**07:10**	**ICU**	**Fungemia**	**Blood**
198-12	28:28	05:05	09:15	ICU	CAP	BAL
175-13	28:28	07:08	07:07	ICU	Sepsis	Urine
401-12	27:27	05:10	09:09	IM	CAP, HIV+	BAL
390-12	26:30	05:10	07:07	IM	Tb	BAL
485-12	25:28	04:04	06:16	ICU	Sepsis	BAL
501-09	**24:30**	**04:05**	**37:37**	**IM**	**Wagner's granulomatosis**	**Swab**
451-09	24:30	05:05	03:17	IM	Oral candidiasis, pneumonia	Swab
274-11	**24:30**	**04:05**	**03:38**	**Ped. ICU**	**Sepsis, severe neutropenia**	**Urine**
318-12	**24:30**	**04:05**	**33:33**	**ICU**	**CAP**	**BAL**
423-12	**24:30**	**04:05**	**03:37**	**IM**	**CAP**	**BAL**
174-13	**24:30**	**04:05**	**03:26**	**ICU**	**Septic shock**	**Urine**
975-10	24:29	04:05	27:44	IM	ALL	Swab
355-12	24:29	04:05	37:39	IM	Tb	BAL
092-11	24:28	04:04	06:06	ICU	Sepsis	BAL
527-11	**23:30**	**04:05**	**03:03**	**IM**	**Tb**	**BAL**
105-12	**23:30**	**04:05**	**03:03**	**ICU**	**Leukemia**	**BAL**
375-12	**23:30**	**04:05**	**03:35**	**ICU**	**ARDS**	**Urine**
443-12	**23:30**	**04:05**	**03:36**	**IM**	**Nosocomial pneumonia**	**BAL**
503-12	**23:30**	**04:05**	**03:34**	**ICU**	**Septic shock**	**BAL**
203-13	**30:30**	**04:05**	**03:36**	**ICU**	**Septic shock, ARDS**	**Urine**
092-13	23:30	04:05	47:58	ICU	Pneumonia, ARDS	BAL
482-09	23:23	04:05	42:46	IM	Tb	BAL
402-12H	**19:26**	**04:05**	**06:06**	**Ped. ICU**	**Pneumonia, ARDS, gastroschisis**	**Blood**
402-12U	**18:26**	**04:05**	**06:06**	**Ped. ICU**	**Pneumonia, ARDS, gastroschisis**	**Urine**
854-10	**18:27**	**04:05**	**06:06**	**IM**	**Bacterial meningitis**	**Urine**
387-12	**18:28**	**04:05**	**06:06**	**ICU**	**Acute abdomen**	**BAL**
403-12	**18:27**	**04:05**	**06:06**	**ICU**	**Sepsis**	**BAL**
410-12	**18:26**	**05:10**	**06:06**	**Ped. ICU**	**Pneumonia, candidemia**	**Blood**
451-12	**18:27**	**04:05**	**06:06**	**ICU**	**Sepsis**	**BAL**
463-12	**18:26**	**04:05**	**06:06**	**EXT**	**Diabetes mellitus II, skin candidosis**	**Skin scrapping**
474-12	**18:28**	**04:05**	**06:06**	**IM**	**Sepsis, ARDS**	**BAL**
083-13	**18:18**	**04:05**	**06:06**	**IM**	**CAP**	**Urine**

*Number 1 and 2 next to the initials of each antifungal represent the lower and high concentration of the drug in the well of the FUNGITEST® plate. **0 represents that isolate didn't grow after 72 hours, 2 represents grow before 72 hours of incubation. –for isolates with missing antifungal sensitivity data. HIV: Human Immunodeficiency Virus; CAP: Community Acquired Pneumonia; Tb: Tuberculosis; ALL; Acute Lymphocytic Leukemia; SLE: Systemic Lupus Erythematosus; VAN: Ventilator Associated Pneumonia; ICU: Intensive Care Unit; Ped. ICU: Pediatric ICU; ARDS: Acute Respiratory Distress Syndrome; BAL: Broncho-Alveolar Lavage.

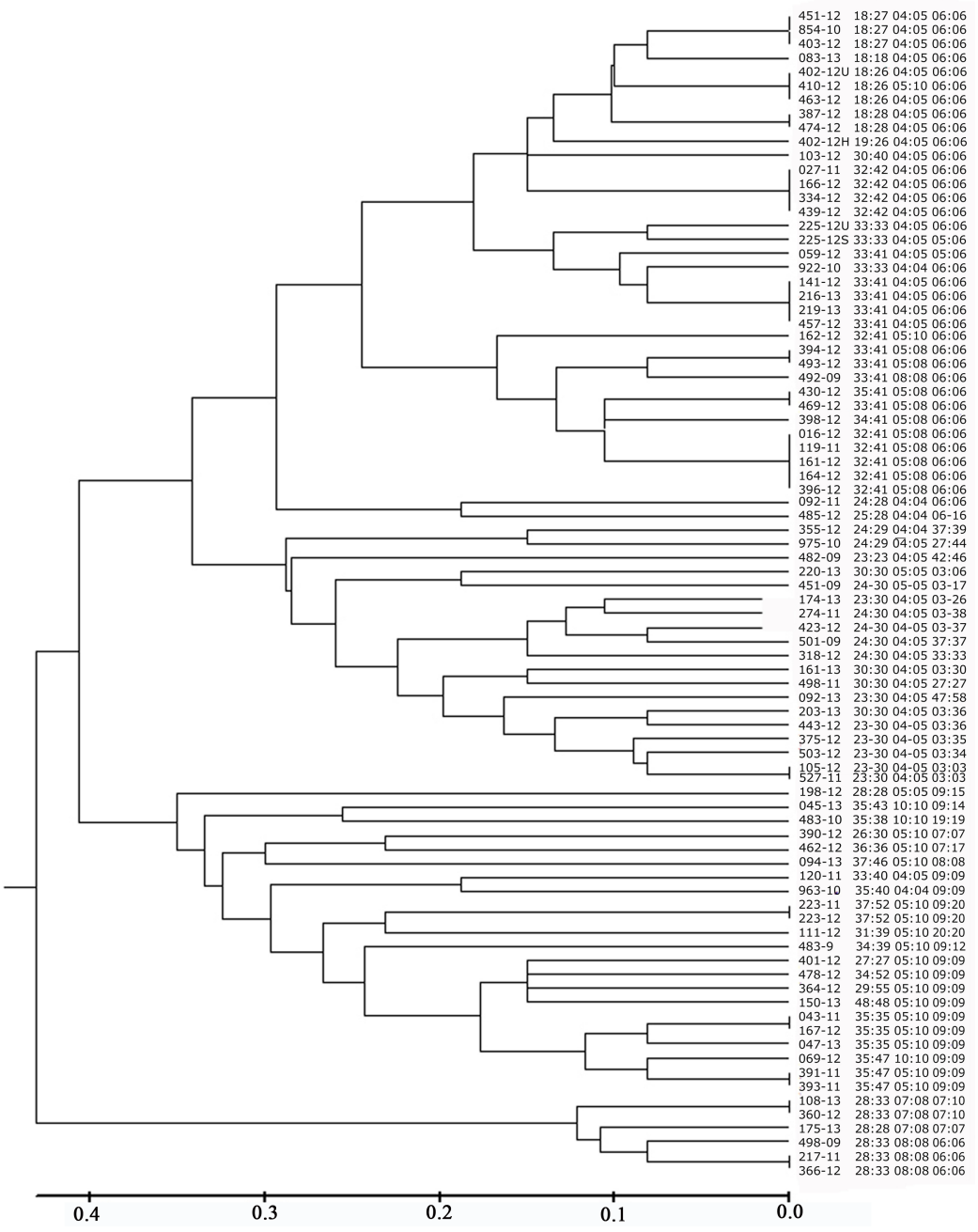

Figure 1. Dendrogram inferred using the UPGMA method in MEGA 5.2 software. The tree is drawn to scale, with branch lengths in the same units as those of the Cavalli, Sforza & Edwards (1987) genetic distance calculated by Populations 1.2.23. Scale represents genetic similitude between isolates. As can be seen, nodes of isolates with similar o some genotypes branch at values above 0.1 of genetic similitude scales.

Table 4. Genetic diversity (heterozygosity) for each locus and F_{IS} value in *C. albicans* isolates.

Locus	**CAI**		**CAIII**		**CAIV**	
	He	Ho	He	Ho	He	Ho
	0.934	0.809	0.705	0.845	0.775	0.298
F_{IS}	0.1340		−0.2007		0.6175	

of Mexico City (SMC) [20], and five tertiary-level teaching hospitals in Monterrey state (MS) [21]. These studies report frequency of *Candida* species from bloodstream infections; however they differ importantly in the distribution of *Candida* species. In Monterrey, *C. parapsilopsis* (37.9%) was observed as the prevalent specie followed by *C. albicans* (31.9%) while in SMC was *C. albicans* (46%) followed by *C. tropicalis* (26%).

It is important to emphasise that *C. tropicalis* infections are more common in patients with severe neutropenia and haematological cancer [22], this may explain the higher number of *C. tropicalis* isolates in the SMC study, which included samples from the National Cancer Institute, compared with our study, which contained a low number of samples from patients with haematological cancers.

Interestingly, there is significant geographic variation in the prevalence of non-albicans *Candida* NAC specie [23] [24] *C. glabrata* and *C. parapsilosis* are more frequent in North America compared with Latin America, while *C. tropicalis* is an emerging pathogen in hospitals in Latin America, certain Asian countries (Singapore and Hong Kong) [25] [26] and is the first non-*albicans* species identified in Pakistan [27].

One important finding of our study is that *C. dubliniensis* was not identified by molecular analysis. *C. albicans* and *C. dubliniensis* are closely related species that can be misidentified using phenotypic methods; however, both the PCR results using specific primers for *C. dubliniensis* (DUB) and the RFLP analysis with different enzymes clearly demonstrated the absence of *C. dubliniensis* in our *C. albicans* samples. This finding is surprising since a number of studies have reported infections due to *C. dubliniensis* in different geographic regions (Europe, North America, Argentina, Australia and Singapore) [28] [29].

Antifungal susceptibility analysis confirmed that resistance to AMB, FLU and ITR is an important characteristic of the HJM *C. albicans* isolates. Many studies have reported that resistant *C. albicans* and *C. parapsilosis* strains are rarely isolated [30] [31]; however, the rise in resistance against these important antifungal drugs has been increasingly associated with the use of these antifungal agents, resulting in the selection of specific resistant isolates. The widespread use of FLU as a prophylactic treatment and the preference for using AMB because of its relatively low cost, may promote the increase in the number of resistant isolates among these pathogens. Previous studies have demonstrated that resistance to AMB is not common in *Candida* spp., except for *C. lusitaniae* [32]; however, this specie was poorly represented in the current study. Our data indicate that 8 of 10 ITR resistant *C. albicans* strains were also resistant to FLU. A similar pattern has been observed in other studies, and it is known that resistance is mediated via specific ATP binding cassette (ABC) family transporters that afford cross resistance to azole antifungal drugs.

Microsatellites, or simple sequence repeats (SSRs), are tandem 1 - 6 bp nucleotide repeats dispersed throughout the genome and can be utilised for strain typing and epidemiological studies. Several polymorphic microsatellite loci have been identified for *C. albicans* including the *C. albicans* specific CAI, CAIII and CAVI. These loci are located in noncoding regions, and thus are under low selective pressures resulting in a high degree of polymorphism. These markers are stable and show high levels of robustness and specificity for *C. albicans* inter-strain differentiation; they have been used as a typing system for characterising clinical isolates and identifying nosocomial transmission of *C. albicans* [10] [33].

Our analysis with CAI, CAIII and CAVI of independent isolates (**Table 3**) revealed a high level of polymorphism; 56 isolates had identical CAI, CAIII and CAVI genotypes or had minor changes in the CAI or CAVI loci. Each of the isolates originated from a single patient, except for four isolates, 225-15S, 225-12U (two samples of the same patient) and , 402-12H and 402-12U (two samples of the same ICU patient), and most of these patients were hospitalised in the ICU and IM wards. It is important to emphasise that these same-genotype isolates were recovered from different samples and therefore different patients. Analysis of the 225-12 and 402-12 isolate pairs showed that they have almost identical genotypes, confirming that only one strain was present in each of the patients during *Candida* infection. Moreover, the presence of isolates with minimal variation in all microsatellite loci suggests the occurrence of an adaptation to environmental conditions as part of a microevolution process, as previously described [34].

Population analysis suggests that the genotyped *C. albicans* isolates represent a clonal population (according to the Hardy-Weinberg test). The F_{IS} values further confirm the CAI and CAVI loci results that indicate "inbreeding" and clonal expansion of conserved genotypes by mitotic reproduction (*C. albicans* lacks a conventional sexual cycle) [35]. However, the F_{IS} value for the CAIII locus seems somewhat inconsistent, and points to the prevalence of non-clonal *C. albicans* isolates. It is important to note, however, that the CAIII primer has a lower DP value, and therefore this marker may be not suitable for genotypic and genetic analysis. Moreover, the CAIII primer amplifies a microsatellite sequence in chromosome 5, and several studies have demonstrated that

loss of heterozygosity is common in this chromosome [16], resulting in homozygous genotypes for chromosome 5 genes (including the CAIII microsatellite). For these reasons, the CAIII F_{IS} value was subsequently discarded.

Exogenous nosocomial transmission of *Candida* spp. has been reported [36], however endogenous colonization is responsible for severe candidiasis [37]. The data reported in this study may represent endemic *Candida* strains in the hospital; however, the clinical data could not demonstrate that patients shared possible *Candida* transmission sources, including physical space, healthcare workers, caretakers or food. To address these variables, a prospective study should be conducted that would recover isolates from the same ward and time period and examine the prevalence of identical genotype isolates. It is possible that the *C. albicans* isolates identified in our current study may represent endemic isolates and not nosocomial strains; however, since this is the first study of its kind conducted in Mexico, further studies will be required to obtain additional epidemic data and to prevent for fungal drug resistance in the hospital environment.

5. Conclusions

C. albicans was the most prevalent species identified in patients hospitalised in the HJM, which was representative of conditions in the public health care system, in contrast with the decreasing worldwide prevalence of this pathogen. The distribution of *Candida* spp. isolates has been found to vary in different geographical areas, most likely due to the considerable effect of underlying conditions and prophylactic antifungal therapy agents on local epidemiology. Horizontal transmission may also affect species distribution.

The microsatellite multiplex PCR-based system enables high volume typing of isolates, and is therefore useful in epidemiological studies and in the identification of nosocomial and endemic *C. albicans* isolates. Genotypic analysis revealed the presence of a number of genotypes in nearly all examined *C. albicans* isolates, suggesting they might represent endemic strains. The current study is limited in that it is performed at a single México City hospital, and thus the results cannot be generalised. This study is very important in our hospital because further analysis of the *Candida* spp. will provide the changes in distribution of the different species due to prophylactic and antifungic treatments.

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Conflict of Interests

The authors declare that no conflict of interest exists regarding the publication of this paper.

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