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Distribution of *Candida* species in oral candidiasis patients: Association between sites of isolation, ability to form biofilm, and susceptibility to antifungal drugs

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ABSTRACT

Background: The oral cavity is a complex structure. Differences in oral mucosa surfaces are keratinized epithelium (KE) lined on the gingival, palate, and tongue surface, while non-keratinized epithelium (NKE) lined on the buccal surface and lips. In denture wearer, denture surface is also exposed in the oral cavity. Clinical manifestations of oral candidiasis vary depending on the type of infection. The ability to form biofilm which is the virulent factor of *Candida* spp. may affects by these mucosa and abiotic surfaces and leading to drug resistant strains.

Objectives: To compare the distribution of *Candida* spp. by site of infection, its ability to form biofilm, and susceptibility to antifungal agents.

Materials and methods: The samples were collected from lesions in the oral cavity by using the imprint culture technique, and yeast species were identified by conventional and PCR methods. Biofilm formation was measured by crystal violet (CV) assay. Susceptibility to amphotericin B and azoles was performed in according to CLSI guideline (M27 A3).

Results: One hundred and fifty-two isolates were identified from 99 patients. A majority of isolates were 50% isolated from KE surface (gingiva, palate, and tongue), followed by 34.9% from NKE surface (buccal mucosa and lip), and 15.1% from surface of denture. *Candida albican*s was the most common species (80.9%) frequently isolated from the tongue and buccal surface, followed by *C. tropicalis* (7.2%) frequently isolated from the tongue and palate, and *C. glabrata* (5.3%) was frequently isolated from dentures. In consideration to site of infections, yeast isolated from denture surface showed a significant lower biofilm production compared to the NKE surface (p=0.029). The percentage of drug resistant strains in *Candida* spp. isolated from denture was 17.4%, NKE surface 14.6% and KE surface 8.1%.

Conclusion: This data indicate that site of infection; KE and NKE surfaces in the oral cavity had not affected to biofilm formation of *Candida* spp., except in denture wearer. Drug resistant in clinical isolates involved in high biofilm former strains and the species *C. glabrata*.

Introduction

Candida infection of the oral cavity (oral candidiasis) can occur either as an opportunistic fungal infection or an infection in healthy individuals that impact the quality of life. This infection is common among the elderly, immunocompromised hosts, denture wearers, and even infants.[1, 2] Distribution of *Candida albican*s in the oral cavity was different in children of different nationalities[3] and in kidney transplant recipients of different geographic regions.[4] In denture-wearers, the numbers of *Candida* spp. were high on the tongue and

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palate, followed by buccal mocusoa.[5] The most frequently isolated species is *C. albicans*, followed by non-albicans *Candida* spp. (NAC), such as *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, and *C. krusei*.[4] Over the last few decades, the percentage of patients infected by NAC has increased from around 10% to 60%.[6, 7] Susceptibility of these NAC species to antifungal drugs has decreased, which has correlated with an increase in use of azoles as empirical treatment and ability to form biofilm.[6, 8]

In the oral cavity of who has oral candidiasis, lesions can be found at various sites—even if it is lined with a different epithelium layer.[9] The gingival mucosa, palatal mucosa and the dorsal surface of the tongue are covered by highly keratinized epithelium (KE) whereas the inner lip, buccal mucosa and the floor of the mouth are covered by non-keratinized epithelium (NKE).[9] The manifestations include erythematous, pseudomembranous, hyperplastic, or angular cheilitis forms.[10] Denture-associated erythematous stomatitis is also a common type of oral candidiasis among denture wearers; in this stomatitis, inflammation of the oral mucous membrane occurs underneath dentures, where the areas of erythematous are observed.[10, 11]

Keratin production was purposed to involve in pathogenicity of *C. albicans* in keratinized tissue by inhibiting the transition of yeast to hyphal form.[12] This may reduce the ability of biofilm formation. Moreover, mucosal biofilm on the tongue by *C. albicans* is a complex structure composed of yeast, hyphae, commensal bacterial flora, and the host component—ketratin.[13] Most common infected area of oral candidiasis in diabetis mellitus was at the palate (KE) followed by the buccal mucosa (NKE), and tongue (KE) with psedomembranous form.[14] In patients with AIDS, the most frequent clinical manifestation was the pseudomembranous form; the common site of infection was at the tongue (KE).[2] However, there is no report in comparison of phenotypic expression of *Candida* spp. isolates from KE surface (gingiva, palate, and tongue), NKE surface (buccal mucosa and lip) and abiotic (denture) surface in the oral cavity.

This study was to investigate the distribution of yeast isolates from different mucosal surfaces; KE (gingiva, tongue, palate) and NKE (buccal mucosa, lip), and dentures surface as well as to determine the association in ability to form biofilm and drug resistance.

Materials and methods

Clinical isolates

A total of 152 clinical isolates were collected from 99 patients with oral candidiasis who attended the Dental Hospital at the Faculty of Dentistry, Chiang Mai University between July 2015 and February 2016. The samples were collected by imprint culture technique.[15] A sterilized foam pad (2.5x2.5 cm^2) was placed on a lesion in the oral cavity for 1 min and removed to place onto Sabouraud dextrose agar (SDA) plate for 6 h at 37 °C. The pad then was removed and the SDA plate was continually incubated at 37 °C for 24 to 48 hr. For the denture stomatitis cases, samples were collected from palatal mucosa or gingiva and the fitting surface of dentures if it was available. In non-denture wearers, samples were collected from wherever lesions were shown (gingiva, palate, tongue, buccal surface, and lip). Isolated yeast cells were stored in glycerol at -80 °C until used.

Identification of *Candida* spp.

All clinical isolates were identified by standard mycological methods and colour of colonies on CHORMAgar™ *Candida*. Cell morphology and chlamydospore formation were determined on rice agar at 25 °C after 24 to 48 hr. Ability to assimilate sugars[16] and utilization of urea were assessed. Pellicle in Sabouraud dextrose broth (SDB) was determined after 24 hr. *C. dubliniensis* was distinguished from *C. albicans* by PCR.[17]

DNA extraction

Genomic DNA (gDNA) was extracted by phenol/chloroform extraction method. A yeast pellet from overnight culture was suspended in lysis buffer (2% TritonX-100, 1%SDS, 100mM NaCl, 10mM Tris-HCl, 1 mM EDTA) and phenol/chloroform/isoamyl alcohol (25:24:1), and then was boiled at 100 °C for 5 min.[18] The gDNA in the supernatant was precipitated by 100% ethanol and 3 M sodium acetate. After centrifugation, the gDNA was reconstituted in sterile water and stored at -20 °C before being used.

PCR amplification

The internal transcribed spacer 2 (ITS2) region of *C. albicans* and *C. dubliniensis* ribosomal DNA was amplified with fungal-specific primers UNI2 (5'-TTCTTTTCCTCCGCTTATTG-3') and either Calb (5'-AGCTGCCGCCAGAGGTCTAA-3') or Cdub (5'-CTCAAACCCCTAGGGTTTGG-3').[17] PCR reaction was performed in a 20 µL reaction volume, consisting of 0.8x PCR buffer (200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl, pH 8.8), 3.5 mM $MgCl_2$, dNTP mixture (0.2 mM each), 0.55 µM UNI2, 0.15 µM Calb or 0.4 µM Cdub, 1 U Taq DNA polymerase and 500 ng gDNA. The remaining volumes consisted of sterilie water. PCR was performed in a thermal cycler (Sensoquest, Germany) under the following cycling conditions: initial period of denaturation at 94 °C for 10 min, 40 cycles of denaturation at 94 °C for 15 sec, annealing at 57 °C for 30 sec, extension at 65 °C for 45 sec, and final extension at 65 °C for 10 min. Ten microlitres of amplification product were separated on 2% agarose gel with 100 V and stained with ethidium bromide. The DNA fragments were visualized by a digital imaging system (Syngene, UK). The sizes of the DNA fragment by using primers UNI2 and Calb were 446 bp, and by using primers UNI2 and Cdub were 217 bp showed in Figure 1.

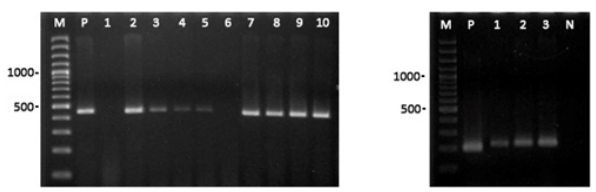

Figure 1. *Gel electrophoresis of PCR products from primers UNI2 and Calb (A), lane 2-5, 7-10 are C. albicans, lane 1 and 6 are not C. albicans and subject to do PCR with primers UNI2 and Cdub (B), lane 1-3 are C. dubliniensis. Lane M is DNA ladder, P is positive control (C. albicans ATCC 90028 and C. dubliniensis), N is negative control.*

Biofilm formation and biomass quantification

One hundred and forty-eight of *Candida* spp. was investigated the biofilm formation. The overnight culture of yeast cells in SDB was washed twice with PBS, and cells were suspended into RPMI-MOPS (RPMI1640 with 2% glucose and 0.165 M MOPS, pH 7) to McFarland No.3 (approx. $1x10^7$ cells/mL). One hundred microlitres of suspension was added into a 96-well plate and incubated at 37 °C at 75 rpm for 90 min. Non-adhering cells were washed once with 150 µL PBS and 100 µL RPMI-MOPS was added. The plate was incubated at 37°C at 75 rpm for 48 hr. Crystal violet (CV) assay was used to determine cell biomass. CV solution (0.5% [W/V]) was added into each well; after that, it was washed twice with PBS and incubated for 45 min at room temperature. The CV solution was discarded and wells were washed thrice with 200 µL sterile water, then 150 µL absolute ethanol was added into each well and left to stand for 45 min. The dissolved CV was transferred to a new flat 96-well plate and measured at OD_{595}. Biofilm producers were classified as high-, moderate-, or low-biofilm formers (HBF, MBF, or LBF) as done in a previous study.[19]

Susceptibility to antifungal drugs

Susceptibility of the yeast cells to antifungal agents (amphotericin B, AMB [BioChemica, UK], clotrimazole, CLT [Sigma, UK], fluconazole, FLC [Sigma, UK], itraconazole, ITC [Sigma, UK]) was investigated using the broth microdilution assay (CLSI M27 A3). Yeast cells ($1x10^3$ cells) were grown in RPMI-MOPS with diluted antifungal agents in a 96-well plate at 37 °C for 24 – 48 hr. The concentration of AMB and ITC ranged from 0.03-8 µg/mL, CLT ranged from 0.007-2 µg/mL, and FLC ranged from 0.5-128 µg/mL. The minimal inhibitory concentration (MIC) of azoles was determined as 50% inhibition of growth and the MIC of AMB was determined as 100% inhibition of growth compared to no drug control. The susceptibility breakpoints for FLC and ITC were followed by CLSI. Since there is no breakpoint for AMB and CLT, we use the breakpoint from the literature at MIC>1 µg/mL for AMB and >0.5 µg/mL for CLT.[20, 21]

Statistical analysis

The first analysis was distribution normality assessment using histogram evaluation. Because of the abnormal distribution, Mann-Whitney U test was used for the comparison between groups. P-values of less than 0.05 were considered to indicate statistical significance. All statistical analysis was performed using STATA™ version 10.1 software (Statacorp, College Station, TX).

Ethics statement

This study was approved by the Medical Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.

Results

Patients' characteristics

A total of ninety-nine patients with oral candidiasis lesions were in this study. Patients' ages ranged from 34 to 86 years (median 62 years); 33 were male and 66 were female. One-third of the patients (11 male and 21 female) were denture wearers. The lesions were found at various sites in the oral cavity: gingiva (KE), palate (KE), tongue (KE), buccal mucosa (NKE), and inner lip (NKE) (Table 1). In the non-denture wearers, the lesions were commonly shown at the tongue (48/142, 33.8%) and buccal surface (42/142, 29.6%). The erythematous was the majority of lesions on the tongue (26/48, 54.2%), and at the buccal surface the majority of lesions were pseudomembranous (26/42, 61.9%). In denture wearers, the lesions were found at the gingiva or palate, wherever the contact surface occurred.

Table 1 Characteristics of lesions in the oral cavity of patients.

Lesions	Sites of isolation, n						
	Keratinized epithelial (KE) surfaces			Non-keratinized epithelial (NKE) surfaces		Abiotic surface	Total number (%)
	Gingiva	Palate	Tongue	Buccal mucosa	Lips	Dentures*	
Erythematous	0	1	26	7	2	0	36 (25.4)
Hyperplastic	2	2	11	6	0	0	21 (14.8)
Psedomembranous	1	0	3	26	2	0	27 (19.0)
Denture-associated stomatitis	4	13	0	0	0	22	38 (26.8)
Others**	0	1	8	8	2	1	20 (14.1)
Total number (%)	7 (4.9)	17 (12.0)	48 (33.8)	42 (29.6)	6 (4.2)	22 (15.5)	142 (100)

**In cases of those who had denture-associated stomatitis, samples were collected from lesions and the fitting surface of dentures, if the dentures were available. **Others included mild anaemia, burning sensation, sores, dryness and non-identified.*

Distribution of isolated yeast

A total of 152 yeast isolated from 99 patients were identified by phenotypic methods. The differentiation between *C. dubliniensis* and *C. albicans* was done by PCR with specific primers (Figure 1). Yeast species distribution by sites of isolation was shown in Table 2. *C. albicans* was the most common species (80.9%) isolated from KE, NKE and dentures surface. The NAC included *C. tropicalis* (7.2%), *C. glabrata* (5.3%), *C. krusei* (2%), and *C. dubliniensis* (2%). It was observed that 75% (6 of 8) of the *C. glabrata* strains were isolated from denture-associated stomatitis which from gingival (1 isolate), palate (1 isolate), and from complete dentures (4 isolates).

Table 2 Distribution of yeast species isolated from the oral cavity of patients.

Yeast species, n (%)	Sites of isolation, n					
	Keratinized epithelial (KE) surfaces			Non-keratinized epithelial (NKE) surfaces		Abiotic surface
	Gingiva	Palate	Tongue	Buccal surface	Lip	Dentures*
C. albicans, 123 (80.9)	5	13	44	41	4	16
C. dubliniensis, 3 (2.0)	0	0	1	0	0	2
C. tropicalis, 11 (7.2)	1	3	3	2	1	1
C. glabrata, 8 (5.3)	1	1	0	2	0	4
C. krusei, 3 (2.0)	0	1	1	0	1	0
Trichosporon spp., 4 (2.6)	0	1	1	2	0	0
Total, 152 (100)	7 (4.6)	19 (12.5)	50 (32.9)	47 (30.9)	6 (4.0)	23 (15.1)

**In cases of those who had denture-associated stomatitis, samples were collected from lesions and the fitting surface of dentures, if dentures were available.*
***Others included mild anaemia, burning sensation, sores, dryness and non-identified.*

In this study, mixed *Candida* spp. were observed in 11 cases (11%) (Table 3). It is interesting that seven cases carried different *Candida* spp. which were isolated from different sites within the oral cavity. Additionally, different species of *Candida* were also isolated from the same site observed in four patients. The common species were *C. albicans* and *C. glabrata*. We observed that 6 of 8 of *C. glabrata* strains were mixed colonization with other *Candida* spp.

Table 3 Mixed colonization of *Candida* spp.

Species	Number of patients	Site of isolations[#]
C. albicans + *C. glabrata*	2	Dentures + Gingiva (1st patient)
		Buccal surface + Lip (2nd patient)
	1	Buccal surface
C. albicans + *C. tropicalis*	1	Tongue + Palate
C. albicans + *C. krusei*	2	Tongue (1st patient)
		Lower lip (2nd patient)
C. dubliniensis + *C. glabrata*	1	Dentures at upper left + dentures at upper right
C. glabrata + *C. tropicalis*	2	Dentures + Tongue (1st patient)
		Tongue + Gingiva (2nd patient)
C. dubliniensis + *C. glabrata* + *C. krusei*	1	Tongue + Dentures + Palate
C. albicans + (*C. tropicalis* + *Trichosporon* spp.)	1	Tongue + Palate

[#]The order of Candida species is corresponding to the site of isolation.

Biofilm formation of *Candida* spp. according to sites of infection

Biomass forming ability of *Candida* spp. (n=145) was determined by CV assay. Cut-off values were established by dividing the OD_{595} value into terciles to categorized high biofilm former (HBF) (OD_{595}>1.66), moderate biofilm former (MBF) (OD_{595} 0.74-1.66), and low biofilm former (LBF) (OD_{595} <0.74).[19] In Figure 2A, the biomass production had increased in *C. tropicalis* (p=0.0092, n=11), *C. glabrata* produced a lower quantity of biomass (p=0.015, n=8), and *C. dubliniensis* (n=3) showed no significant difference in biomass production (p=0.35), in comparison to *C. albicans* (n=120). The association between biofilm formation and sites of isolation was statistically analyzed and shown in Figure 2B. No significant difference (p=0.69) was found between the biomass production of yeast isolated from KE surfaces (gingiva, palate, and tongue (n=74)) and NKE surfaces (buccal surface and inner lip (n=48)). Markedly, yeast that was isolated from denture surfaces (n=23) had a significant decrease in biomass production (p=0.029) compared to yeast isolated from NKE surfaces.

Susceptibility to antifungal drugs

Among the 120 strains of *C. albicans*, 4 (4.2%) were resistant to FLC; 2 (1.7%) were susceptible-dose dependent (SDD) to FLC; and 6 (5%) were resistant to ITC. No resistance to AMB or CLT was found. Regarding the 11 strains of *C. tropicalis*, 2 (9.1%) were resistant to AMB; 1 (9.1%) was SDD to ITC, and no resistance was found to CLT or ITC. All 8 strains of *C. glabrata* showed resistance: 1 (12.5%)

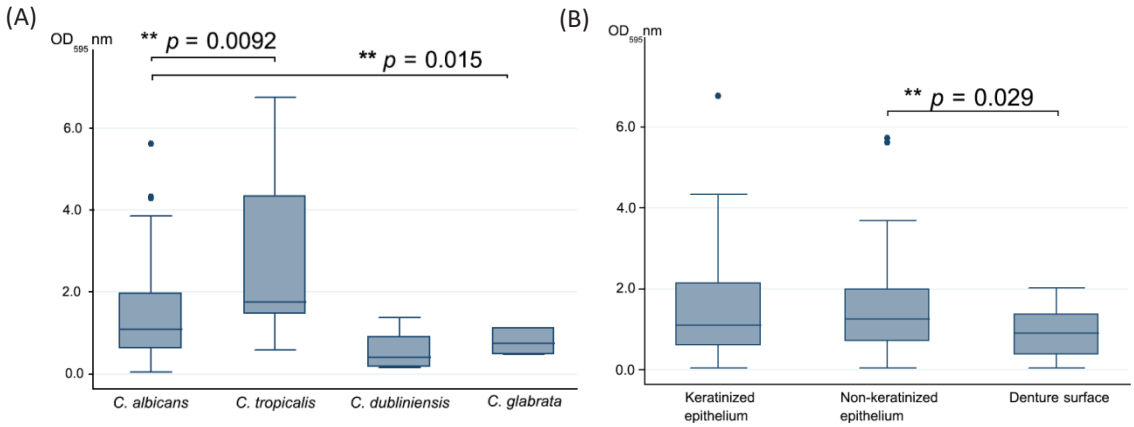

Figure 2. *Biofilm formation of Candida species at 48 hr. Production of biomass by C. albicans, C. tropicalis, C. dubliniensis and C. glabrata (A), by yeast isolated from different sites (B). *outlier data by Whisker plot, **significant difference at p<0.05.*

was resistant to AMB; 2 (25%) were resistant to FLC and 5 (62.5%) were SDD to FLC; and 7 (87.5%) were resistant to ITC. In the 3 strains of *C. krusei*, 2 (66.7%) were resistant to AMB. No drug resistance was found in *C. dubliniensis* (n=3).

In this study, 11.7% (17/145) of the *Candida* species demonstrated some resistance to the tested antifungal drugs. The distribution of resistant strains in consideration to sites of infection was different. The resistant rate of *Candida* spp. isolated from denture was 17.4% (4/23) which 3 isolates were *C. glabrata*. The rate of drug resistant in *Candida* spp. isolated from KE surface was 8.1% (6/74) which 4 isolates were HBF. The resistant rate of *Candida* spp. isolated from NKE surface was 14.6% (7/48), which 4 isolates were HBF and 2 were *C. glabrata*.

Discussion

In determination of *Candida* spp. isolated from oral candidiasis by phenotypic methods, four different *Candida* spp. were identified except *C. dubliniensis*. Furthermore, to distinguish *C. dubliniensis* from *C. albicans*, molecular analysis showed not exactly band size to *C. dubliniensis* reference strain (Fig. 1B). Therefore, those three isolates were confirmed by DNA sequencing with primer UNI2 and nucleotide blast using blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed 99-100% similarity to *C. dubliniensis* in the database (data not shown).

Our study in Northern Thai population showed that *C. albicans* was still the most common species (80.9%) with low rate of NAC among elderly (median 62 years old). This rate was not similar to the study of Muadcheingka in 2015, that oral candidiasis population was Central Thai, NAC had increased to nearly 40%.[7] The increasing of NAC from clinical specimens can cause the problem of treatment as *C. glabrata* and *C. krusei* are intrinsic resistance to azole drugs.[22, 23] This low rate of NAC in oral candidiasis in the northern Thai population is similar to prevalence of oral *Candida* carriage in Thai adolescents.[24] In this study, we observed that *C. glabrata* were common species isolated from dentures. It is consistent with the report that surface roughness affects the adhesion of organisms. *C. glabrata* had higher adhesive ability to denture acrylic surfaces than *C. albicans*.[25] This suggested that adequate cleaning of dentures is needed to remove the denture biofilm regularly. In addition, identification of the species of NAC is required for more effective therapy.

Mix colonisation of *Candida* spp. was normally reported, but sample collections were from oral rinsed or swab which could be not identified the origin of colonization.[26, 27] In this study, we found two *Candida* spp. were isolated from two sites in the oral cavity of denture wearers. In the case of *C. glabrata* isolated from the fitting surface of dentures

and *C. albicans* isolated from the surface of palatal mucosa (Table 3), it is possible that two *Candida* spp. co-localized when dentures was completely fitted in the oral. It has been reported that co-localized *C. albicans* and *C. glabrata* has a synergistic interaction and is associated with sever inflammation in denture wearers.[27, 28] Limited oxygen in between the palate and fitting surface of a complete denture could influence the virulence factors of *C. albicans*.[29] However, in the case of different *Candida* spp. isolated from other different anatomical sites (Table 3), more evidence is needed to support and investigate their specificity on different localization.

Distribution of *C. albicans* in the oral cavity, we found that the common sites of infection were at the tongue and the buccal mucosa which consistent to the report of Zahir.[5] In denture wearer *C. albicans* was common at the tongue and the palate[5] but in this study only lesions on the palate and gingiva had been investigated.

In biofilm formation study, *C. tropicalis* showed the highest biomass production, followed by *C. albicans* and *C. glabrata*. It is consistent with previous studies.[7, 19] In consideration to sites of infection, *Candida* spp. isolated from KE surface (i.e. tongue) were able to produce biofilm similar to *Candida* spp. isolated from NKE surface (i.e. buccal mucosa). It is not similar to the study of Zarkzewski that used engineered human oral epithelial mucosa (EHOM) to investigate the transition of *C. albicans* from yeast to hyphal form on keratinized (k) EHOM and non-keratinized (nk) EHOM.[12, 30] The number of *C. albicans* hyphal cells was significant higher on nkEHOM than on the kEHOM.[12] Since, the transition from yeast to hyphae is the initial process of biofilm formation of *Candida* spp. However, our results support the report of Dodd that the clinical manifestations either on tongue (KE) or on buccal mucosa (NKE) were strong indicator as severe infection.[31]

Susceptibility profile in this study showed high number of resistant strains to itraconazole, especially in *C. glabrata* compared to the other reported that showed resistant rate vary from 5-20%.[32] However, these results had been repeated. Recently epidemiological cutoff values (ECV) had been proposed to compare with CLSI cutoff values for determining the resistant strain. ECV of itraconazole for *C. glabrata* has been increased (>2μg/ml) for interpreting of resistance.[33] Therefore, the high number of itraconazole resistance may not thoroughly association in clinical treatment, as well as, clotrimazole is empirical treatment of oral candidiasis in this Dental hospital.

In this study, the local environment may affect to the drug resistant strains. The highest rate of drug resistance was strains isolated from dentures and associated to species of *C. glabrata*. In contrast, the drug resistant strains isolated from KE and NKE surface were majority of HBF strains. Therefore, awareness of treatment by azole drug should be considered on NAC and HBF strains. To determine the characteristic of HBF strains could be an indirect method to predict the outcome of treatment.

Conclusion

C. albicans was the most frequent species isolated from each site of infection. Frequency of NAC was not altered in this population. There was no association of ability to form biofilm and drug resistance with *Candida* spp. isolated from KE and NKE surface. Drug resistant strains involved in species *C. glabrata* and HBF of *C. albicans* and *C. tropicalis* strains. Finally, we proposed that phenotypic expressions of *Candida* spp. isolated from KE and NKE surface were similar except *Candida* spp. isolated from dentures.

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