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Genotyping and antifungal susceptibility of *Candida albicans* strains from patients with vulvovaginal and cutaneous candidiasis in Palestine

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The aims of this study was the genotyping of *Candida albicans* strains isolated from patients with invasive cutaneous and vulvovaginal infections and to investigate the fluconazole, flurocytosine (5-FC), and amphotericin B susceptibility of the strains genotypes. For this purpose, 151 *C. albicans* isolates [from 68 candidiasis (VVC) and 83 cutaneous (CC) infections] were genotyped by using specific PCR primers designed to spam the transposable group I of the 25S rDNA gene. Susceptibility of genotypes A, B, and C to the antifungal agents amphotericin B, flurocytosine and fluconazole was determined by disk-diffusion, and broth microdilution methods. Eighty-three of the 151 isolates were genotype C (83.55 %), 49 were genotype A (32.4%), and 19 were genotype B (12.6%). Genotypes D and E which represent *C. dubliniensis* were not found. Antifungal susceptibility testing showed that isolates of *C. albicans* genotype A were more resistant to flucanazole and flurocytosine than B and C genotypes (A>B>C), and also more resistant to amphotericin than C and B genotypes (A>C>B). These results indicate that there may be a relationship between *C. albicans* genotypes and resistance to antifungals. The presence or absence of the transposable group I intron in the 25S rDNA gene may be important in determining the resistance of *C. albicans* to antifungals. To our knowledge, this is the first study that reports *C. albicans* genotypes in Palestine and its differential resistance to flucanazol, flurocytosine, and amphotericin B.

Key words: *Candida albicans,* genotyping, antifungal susceptibility, Palestine.

INTRODUCTION

Candida albicans is the major etiologic species of candidiasis; it is responsible for 50-70% of all disseminated Candida infections (Dalle et al., 2000). In recent years, *C. albicans*, as a common opportunistic yeast, has caused an increasing number of human cutaneous (CC) as well as vaginal candidiasis (VVC) (Ri, 1988; Xiao-dong et al., 2008).

Since the pathogenicity and antifungal susceptibility of

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C. albicans often vary among strains, identification of the disease-causing strains is crucial for diagnosis, clinical treatment and epidemiological investigation. Furthermore, a few studies have indicated the presence of correlation of genetic diversity of *C. albicans* strains recovered from VVC and CC with antifungal susceptibility (Antonopoulou et al., 2009; Krawczyk et al., 2009; Costa et al., 2010).

A study conducted by Fan and colleagues (Fan et al., 2008) to determine the genotypic variations of *C. albicans* in patients with various conditions of VVC revealed that genotypes of *C. albicans* strains correlate with the severity of VVC. The study also shows that the strains with the dominant genotypes are more virulent than others in causing VVC, and that strain differences may play a significant role in the etiology of VVC. Zhu et al. (2011) and Emmanuel et al. (2012) also confirmed that susceptibility varies among the various genotypes of *C. albicans.* Hence in the management of candidiasis it is important to take into consideration the genotype of the Candida strain causing VVC or CC as genotypes show correlation to sensitivity.

To the best of our knowledge, this is the first study aimed at determining the genotypic variation among *C. albicans* causing VVC and CC in Palestine and testing whether genotypes correlate with patients' demographic factors.

Studying the genetic relatedness of clinical strains of Candida species that cause different CC locations and different VVC conditions may have significance in clinical management. Genotyping of strains may also help to distinguish candidiasis relapse due to inadequate treatment or from de novo infection by a new strain, or distinguish resistant or susceptible strains to antifungals.

Molecular typing of *C. albicans* is important for epidemiological studies and for the development of appropriate infection control strategies (McCullough et al., 1999). Using ribosomal sequences for genetic typing, McCullough et al. (1999) used a pair of PCR primers designed to span the region that includes the site of the transposable group I intron of the 25S rRNA gene (rDNA), and can classify *C. albicans* strains into three genotypes on the basis of the amplified PCR product length: genotype A (approx. 450 bp product), genotype B (approx. 840 bp product), and genotype C (approx. 450 and 840 bp products). The two other observed genotypes with this method, genotypes D (approx. 1080 bp product) and E (approx. 1400 bp product), are found to belong to the same taxon as *C. dubliniensis* (Tamura et al., 2001). This method has shown to be easy to perform, give clear and reliable results, be able to differentiate appropriate number of strains, and to be adapted to large number of isolates (Karahan et al., 2004).

In this study, the 25S ribosomal DNA (rDNA) based PCR method (Hattori et al., 2006), which is easy and quick to be performed with low cost (Karahan et al., 2004) was used to characterize the genotypic distribution of *C. albicans* affecting women with VVC and infants with CC in Northern West Bank (Palestine). We also examined whether there was an association between these genotypes and susceptibility to antifungals.

MATERIALS AND METHODS

Patients and clinical specimens

The study population comprised 120 infant patients (62 males, 58 females; aged 1 day- 18 months) with cutaneous candidiasis (lesion locations: groin, armpit, perianal, hip, neck and back), and 126 pregnant women (aged 17-44 years) with vulvo-vaginal candidiasis (underlying conditions: intense vulval pruritus, erythema, burning and dyspareunia associated with a creamy discharge). Scales and macerated skin of infected lesions, and vaginal secretions were collected from CC, and VVC patients, respectively, as clinical specimens by sterile swabs.

All clinical specimens were submitted to direct microscopy with 20% KOH and cultured on yeast extract-peptone-dextrose medium (YPD) agar plates containing 50 µg/ml chloramphenicol, and incubated at 37°C for 48 h. Each colony was identified as *C. albicans* by colony morphology, Chromogenic agar (CHROMagar Candida; Oxoid Co., UK) and germ tube test. A total of 151 strains of *C. albicans* (83 from CC specimens, and 68 from VVC specimens), were recovered and subsequently used for genotyping.

Genomic DNA isolation from Candida

Genomic DNA extraction from Candida isolates was carried as described by Harju et al. (2004). Breifly, a single colony of *C. albicans* was added to 2 ml of YPD broth medium and grown in a shaking incubator for approximately 48 h at 30°C. The overnight cultures were pelleted in a microcentrifuge tube and the pellets were resuspended in 200 µl of lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] (Hoffman and Winston, 1987). The tubes were then placed in a -80°C freezer until completely frozen, then immersed in a 95°C hotplate for 1 minto thaw quickly. The process was repeated once, and the tubes were vortexed vigorously for 30 s. Chloroform (200 µl) was added and the tubes were vortexed for 2 minand then centrifuged 3 min at room temperature at 20,000 × *g*. The aqueous layer was transferred to a tube containing 400 µl of ice-cold 100% ethanol. The samples were allowed to precipitate 5 min at room temperature and centrifuged. DNA pellets were washed with 0.5 ml of 70% ethanol, dried and resuspended in 20 µl TE [10 mM Tris, 1 mM EDTA (pH 8.0)] (Harju et al., 2004).

Genotyping of *C. albicans* isolates using PCR primers for the 25S rDNA (P-I)

For genotype determination of *C. albicans* on the basis of 25S rDNA, primers CA-INT-L (5'ATAAGGGAAGTCGGCAAAATAGATCCGTAA3') / CA-INT-R (5'CCTTGGCTGTGGTTTCGCTAGATAGTAGAT3') (referred to as P-I) were used (Hattori et al., 2006). *C. albicans* was grouped into five genotypes (A, B, C, D, and E) on the basis of the sizes of PCR products. Genotype D corresponds to *C. dubliniensis* (Hattori et al., 2006; McCullough et al., 1999).

PCR conditions and agarose gel electrophoresis

Genomic DNAs were amplified in a reaction mixture (25 µl) containing

genomic DNA (10-100 ng), forward primer and reverse primer (Hylabs, Israel), each (10 µmol/L), 10× buffer , $MgSO_4$, dNTPs (10 mM) , Taq DNA polymerase 0.2 µl (2.5 U/ml; Hylab, Ltd., Israel), and sterile distilled water. The PCR cycle parameters were as follows: preheating at 96°C for 120 s; followed by 35 cycles of 96°C for 30 s, 60°C or 65 C for 30 s, and 72°C for 60 s, and final extension at 72°C for 5 min. The annealing temperature of the primers was 65°C. All reaction mixtures were amplified using a thermal cycler (TC-Plus, Techne, UK).

The PCR products were electrophoresed in a 1% agarose gel for identification and genotyping of *C. albicans* on the basis of 25S rDNA.

Antifungal susceptibility testing

A total of 11 *C. albicans* isolates, selected on the basis of their genotype, were tested, including 3 strains of each of genotype A (N43, M22, N96), B (N72, M97, M19), and C (N66, M35, M29). Type cultures *C. albicans* CBS9120, and *C. albicans* CBS6589 (Centraalbureau voor Schimmelcultures CBS, The Netherlands) were used as quality controlled strains in each run of the experiment.

The susceptibilities in vitro to fluconazole, flurocytosine (5-FC), and amphotericin B were carried out using disc diffusion and broth microdilution methods, described here.

Inoculum preparation

The inoculum was prepared using 24 h plate cultures of *C. albicans.* The colonies were suspended in 0.85% saline and the turbidity was compared with the 0.5 McFarland standard, to produce a yeast suspension of $1x10^6$ to $5x10^6$ cells/mL. The cell suspension was diluted 1:100 in the media to obtain a final concentration of 1×10^4 to 5×10^4 colony-forming units per milliliter (CFU/mL).

Disk diffusion method

The disk diffusion method was used as outlined in NCCLS document (M44-A) (NCCLS, 2004). Stock solutions (640 µg/ml) of antifungal agents were prepared in water (fluconazole, Pfizer; and flurocytosine, Alfa Aesar, A Johnson Matthey Company, GB) or dimethylsulphoxide (DMSO) for amphotericin B (Sigma Chemicals, St. Louis, MO). Inoculums (10^6 yeast cells/mL) were spread on Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/mL methylene blue dye (GMB) medium plates. The final concentrations of the antifungal agents ranged from 64 to 0.125 µg/ml. Filter paper discs (6 mm in diameter) were individually impregnated with 50 µL of each drug, and controls, placed onto the surface of inoculated Petri dishes, and incubated at 35°C (± 2 °C) within 15 min after the disks are applied. After 24 h incubation, the diameters (mm) of inhibition zones were measured. All the experiments were done in triplicates. DMSO or water served as a negative control.

Micro-dilution test

The broth microdilution method was performed according to the document M27-A3 of the Clinical and Laboratory Standards Institute (CLSI, 2008).

The antifungal agents used were amphotericin B, flurocytosine and fluconazole, and the susceptibility cut offs were in accordance to the parameters established by Yang et al. (2008) with MIC values ≤1 µg/mL considered susceptible and ≥2 µg/mL resistant to amphotericin, and by the supplement document M27-A3–M27-S3 (CLSI, supplement 2008) to fluconazole and flurocytosine.

Statistical analysis

All statistical analyses were conducted using SPSS. 16.0 statistical software. The chi-square test was performed to determine the differences between the *C. albicans* genotypes and associations with other variables such as site of infection, gender, age, residence and predisposing factors. $P < 0.05$ was considered as significant.

RESULTS

Genotype analysis of *C. albicans* strains

The genomic DNAs of the *C. albicans* strains obtained from different body locations of patients with CC and from patients with VVC were amplified by PCR using P-I to determine the genotypes based on variations in the 25S rDNA. The PCR profiles amplified with P-I defined DNA products of 450 bp for genotype A, 840 bp for genotype B, and 450, and 840 bp for genotype C (Figure 1). Of the one hundred and fifty one *C. albicans* strains, 83 (55%), 49 (32.4%), and 19 (12.6%) were recognized as genotypes C, A, and B, respectively (Table 1).

Distribution of *C. albicans* genotypes

Genotypic distribution of the strains isolated from different CC locations or different conditions of VVC is presented in Tables 2 and 3. The frequency and distribution of genotypes among subgroups (location or condition) of each sample group were variable. Analysis of genotype distribution of *C. albicans* in every subgroup showed no obvious association between the strains of a certain genotype colonizing a specific condition of VVC or cutaneous location, gender, age, predisposing factors, or residence (Tables 2 and 3). However, genotypes A, B, and C showed their highest frequency in infants with younger age (up to four months), in infants with recent administration of antibiotics, younger women, and presence of VVC at pregnancy. Also, genotype A seemed to have higher association with infant's mouth, genotype B with the perianal region, and intense vulval prutitis and burning sensation conditions, and C with groin and hips sites (Tables 2 and 3).

Relationship between genotypes and antifungal susceptibility

According to the analysis with a method of Multiway ANOVA, among the three antibiotics drugs of fluconazole, flurocytosine, and amphotericin B, the susceptibility of the tested isolates to flurocytosine and

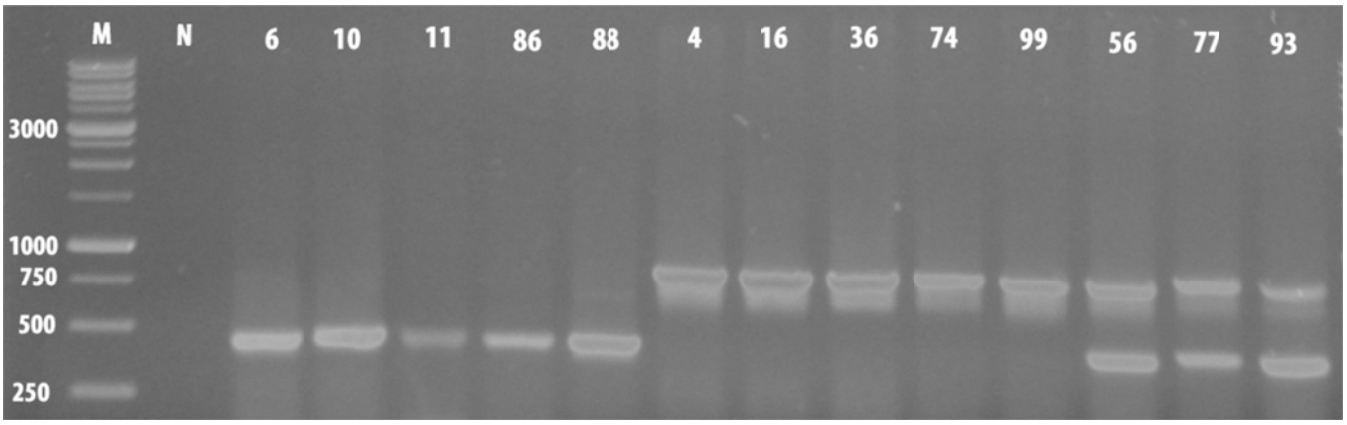

Figure 1. Ethedium bromide-stained UV transilluminated PCR products of the transposable group I intron of the 25S rDNA gene of C. albicans isolates. Molecular size marker is the lane marked M and corresponding sizes as base pairs are given on the left. Lanes 6, 10, 11, 86, and 88 are genotype A (approximately 450 bp PCR product), lanes 4, 16, 36, 74, and 99 are genotype B (approximately 840 bp PCR product), and lanes 56, 77, and 93 are genotype C (approximately 450 and 840 bp PCR products). N refers to negative control.

Table 1. The frequency and distribution of genotypes among cuteneous (CC) and vulvovaginal (VVC) candidiases groups.

Primer	Genotype	Number of isolates			
		CC (%)	VVC (%)	All lesions (%)	Sig.
P-I	A	29 (34.9)	20 (29.4)	49 (32.5)	0.686
	B	11 (13.3)	8 (11.8)	19 (12.6)	
	C	43 (51.8)	40 (58.8)	83 (55)	
Total		83 (100)	68(100)	151	

Table 2. *C. albicans* 25S rDNA genotypes distribution among CC infant patients in relation to different cutaneous locations and demographic characteristics of study population

Variable	Genotype			Total	*P value**
	A Num (%)	B Num (%)	C Num (%)		
Site of infection					
Neck	4 (33.3)	3 (25.0)	5 (41.7)	12 (100)	
Groin	1(9.01)	1 (9.01)	9 (81.8)	11 (100)	
Mouth	8 (47.1)	2 (11.8)	7 (41.2)	17 (100)	
Hips	4 (28.6)	1 (7.1)	9 (64.3)	14 (100)	0.329
Perianal	4 (33.3)	3 (25.0)	5 (41.7)	12 (100)	
Armpit	2 (28.6)	0 (0.0)	5 (71.4)	07 (100)	
Back	6 (60.0)	1 (10.0)	3 (30.0)	10 (100)	
Gender					
Males	16 (37.2)	5 (11.6)	22 (51.2)	43 (100)	0.854
Females	13 (32.5)	6 (15.0)	21 (52.5)	40 (100)	
Age (months)					
(0-4)	15 (29.4)	8 (15.7)	28 (54.9)	51 (100)	
(5-9)	10 (43.5)	2 (8.7)	11 (47.8)	23 (100)	0.751
(10-14)	4 (50.0)	1 (12.5)	3 (37.5)	08 (100)	
(15-19)	0 (0.0)	0 (0.0)	1 (100)	01 (100)	

Table 2. Contd.

Predisposing factors					
Low weight birth	3 (33.3)	2 (22.2)	4 (24.4)	9 (100)	0.806
Recent administration of antibiotics	11 (32.4)	4 (11.8)	19 (55.9)	34 (100)	
Intravenous catheters	0 (0.0)	1 (50.0)	1 (50.0)	2 (100)	
Diaper	3 (30.0)	2 (20.0)	5 (50.0)	10 (100)	
Tight clothes	3 (60.0)	1 (20.0)	1 (20.0)	5 (100)	
Iatrogenic immune suppression	1 (33.3)	0 (0.0)	2 (66.7)	3 (100)	
No predisposing factors	8 (42.1)	1 (5.3)	10 (52.6)	19 (100)	
Residence					
City	29 (35.0)	11 (13.30)	43 (51.8)	83 (100)	
Village	0	0	0	0	
Camp	0	0	0	0	
Total				83	

*Statically significant at (α = 0.05).

Table 3. Frequency of *C. albicans* P-I genotypes by VVCs conditions, patients age, residence and predisposing factors.

Variable	Genotype			Total	p Value*
	A Num (%)	B Num (%)	C Num (%)		
Age					
17-23	8 (40.0)	2 (10.0)	10 (50.0)	20 (100)	0.854
24-30	11 (27.5)	5 (12.5)	24 (60.0)	40 (100)	
31-37	1 (14.3)	1 (14.3)	5 (71.4)	7 (100)	
38-45	0 (0.0)	0 (0.0)	1 (100)	1 (100)	
Residence					
Jenin	5 (25.0)	4 (20.0)	11 (55.0)	20 (100)	0.206
Tubas	6 (30.0)	3 (15.0)	11 (55.0)	20 (100)	
Tamon	4 (21.1)	1 (05.3)	14 (73.7)	19 (100)	
Aqaba	5 (71.4)	0 (0.0)	2 (28.6)	7 (100)	
Tayaser	0 (0.0)	0 (0.0)	2 (100)	2 (100)	
Predisposing factors					
Tight clothes	2 (22.2)	1 (11.1)	6 (66.6)	9 (100)	0.973
Prolonged administration of antibiotics	3 (23.1)	1 (7.7)	9 (69.2)	13 (100)	
Presence of vaginal candidiasis at pregnancy	12 (32.4)	5 (13.5)	20 (54.1)	37 (100)	
No predisposing factor	3 (33.3)	1 (11.1)	5 (55.6)	9 (100)	
Conditions (Symptoms)					
Intense vulval pruritis	6 (37.5)	3 (18.8)	7 (43.8)	16 (100)	0.110
Burning	2 (25.0)	3 (37.5)	3 (37.5)	8 (100)	
Erythema	3 (18.8)	1 (6.3)	12 (75.0)	16 (100)	
Dyspareunia	9 (32.1)	1 (3.6)	18 (64.3)	28 (100)	

fluconazole differed considerably (P>0.05); there were differences among the susceptibilities of the three genotypes of *C. albicans* to flurocytosine and fluconazole (Figure 2).

The *C. albicans* genotype A was considerably less susceptible to flurocytosine and fluconazole than genotype B and C (both P < 0.05). However, to the susceptibility of genotype B and genotype C to flurocytosine and fluconazole, there was no statistical difference (P > 0.05). On the other hand, the susceptibility of genotypes A, B, and C to amphotericin B was similarly high.

The distribution of the MICs of the isolated *C. albicans* to the three antifungal agents was presented in Table 4.

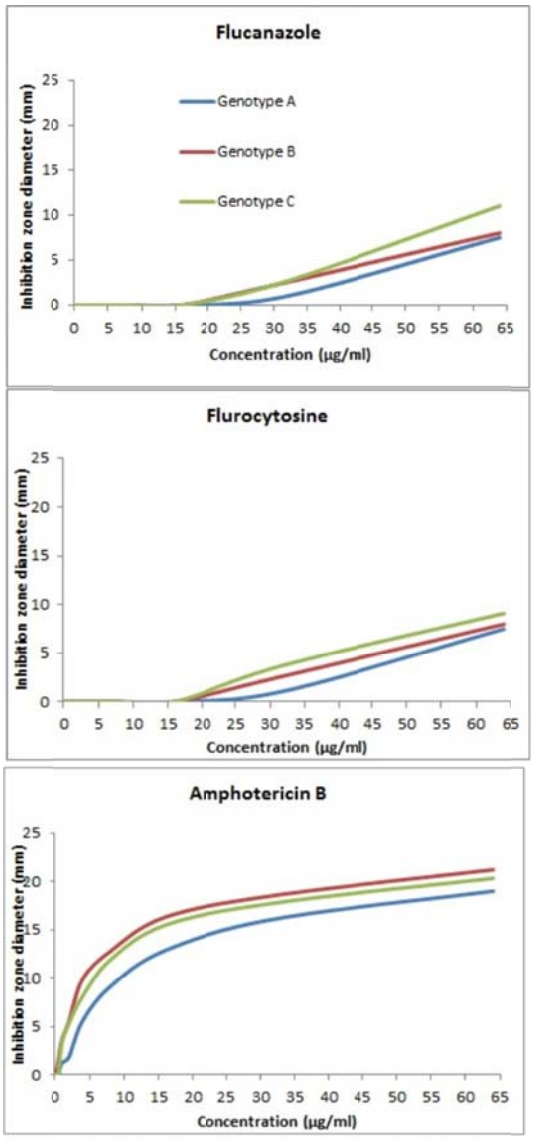

Figure 2. Susceptability of *Candida albicans* genotypes to antifungal drugs.

Table 4. Minimum inhibitory concentration (MIC) of antifungal drugs against *Candida albicans* genotypes.

Antifungal agent	Genotype (Number of strains)	MIC (µg/ml)
Flucanazole	Genotype A (4)	1
	Genotype B (4)	0.44
	Genotype C (3)	0.5
Flurocytosine	Genotype A (4)	3.5
	Genotype B (4)	1
	Genotype C (3)	1
Amphtericin B	Genotype A (4)	0.5
	Genotype B (4)	0.5
	Genotype C (3)	0.5

DISCUSSION

Molecular typing of *C. albicans* provides fundamental techniques for studying the epidemiology of this frequently isolated causative agent of candidiasis in humans, and for developing rational therapeutic strategies to combat the disease (Zhu et al., 2011; Redding et al., 1999).

In this study, the prevalence of *C. albicans* in the northern West Bank area of Palestine from 2012 to 2013 was investigated. Totally, 151 isolates of *C. albicans* in the northern West Bank area, Palestine was obtained from clinical specimen with a standard method.

PCR targeting 25S rDNA, which has frequently been used for genotype analysis of *C. albicans*, allows, on the basis of the length of the amplified PCR product, *C. albicans* isolates to be grouped into three main genotypes A, B, and C (Tamura et al., 2001; Hattori et al., 2006; McCullough et al., 1999; Iwata et al., 2006; Millar et al., 2002). In our study, the most prevalent *C. albicans* genotype among all isolates on the basis of amplification of 25S rDNA was genotype C (55%), followed by genotype A (32.4%), and B (12.6%). The ratios of genotypes A, B and C, of *C. albicans* varied among the reports, where the ratio of genotype B or C to genotype A *C. albicans* varied in each group of clinical specimens (Gurbuz and Kaleli, 2010; McCullough et al., 2004). These findings may be affected by the kinds of clinical specimens colonized by *C. albicans* (Iwata et al., 2006). However our results differed from those of previous researches carried out on VVC strains (Xiao-dong et al., 2008; Emmanuel et al., 2012; Zhu et al., 2011; Karahan and Akar, 2005), where it was shown that genotype A was represented with the highest frequency followed by genotype B and C. These variations of genotypes of *C. albicans* among these studies may be attributed to differences in type of strain, patient's age, and geographic locations (Clemons et al., 1997).

In this study, we could not detect a clear association between candidiasis strains and their invasive body sites, or predisposing factors, gender, age or conditions of infection of patients. This finding is in agreement with that of Xiao-dong et al. (2008) who also found no clear association between genotypes and the site of cutaneous infections, probably due to the fact that the influences of different skin surfaces in the cutaneous locations were minor and could not influence the genotype of strains. Our results are also in agreement with those of Xia-dong et al. (2008) who found no noticeable differences in genotypes of isolates from various VVC conditions, or significant association between genotypes of *C. albicans* and predisposing factor. The influences of residence and predisposing factors were minor and could not influence the genotype of strains.

Furthermore, based on the present findings, it was proposed that the influences of clearly different environments of cutaneous and vaginal mucous membranes, patients with different age and sex, different skin surfaces in CC, and the different conditions of the VVC were minor and could not significantly influence the genotype of strains.

In conclusion, no clear correlation was found between the genotypes of *C. albicans* and patient's gender, age, underlying conditions of VVC, location of the CC lesions, residence, or predisposing factors. In the same patient there was usually one genotype of *C. albicans* colonizing various CC locations.

Many genotyping methods mainly focused on the study of the cloning origin of *C. albicans* and the patterns of transmission. Little is focused on the relationship between the genotyping and virulence (Jain et al., 2001). Early reports demonstrated that there was correlation between the group I intron in the 25S rDNA of *C. albicans* and the susceptibility to flurocytosine (Mercure et al., 1997; Zhu et al., 2011).

Our results of the antifungal susceptibility testing showed that there were differences among the antifungal susceptibilities of genotype A, B and C to flurocytosine. Genotypes B and C of *C. albicans* were more susceptible to flurocytosine and fluconazole than genotype A, but no differences were found in the antifungal susceptibilities of genotype A, B and C to amphotericin. It has also been shown by other researchers that genotype A is significantly resistant to fluconazol and flurocytosine, than genotypes B and C (Emmanuel et al., 2012; Tamura et al., 2001; McCullough et al., 1999).

The classification of genotypes employing this PCR relies on the presence of group I intron of varying size in the 25S rDNA, and group I intron has self-splicing capability which is necessary for the formation of mature 25S rRNA (Mercure et al., 1993). The self-splicing capability can be inhibited by base analogs (Mercure et al., 1997), so the strains harboring group I intron will be more susceptible to base analogs, such as flurocytosine (Zhu et al., 2011).

The antifungal mechanisms of amphotericin B are different from flurocytosine, therefore there was no association between the presence of group I intron in the 25 S rDNA of *C. albicans* and strains susceptibility to amphotericin B, and reliable markers of drug sensitivity or resistance are needed. Thus, identifying *C. albicans* at the genotype level help in prescribing the suitable antifungal drugs by specialists, and help in the control of *C. albicans*.

Conflict of interests

The author(s) did not declare any conflict of interest.

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