INVESTIGATION OF VIRULENCE FACTORS AND DETERMINATION OF ANTIFUNGAL SUSCEPTIBILITIES OF CANDIDA STRAINS ISOLATED AT SULEYMAN DEMIREL UNIVERSITY HOSPITAL

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ABSTRACT

***Objective**: The aim of this study was to evaluate the virulence factors and antifungal susceptibilities of Candida strains, which were isolated from various clinical specimens.*

***Methods**: One-hundred Candida strains were identified. Bovine serum albumin agar was used to investigate proteinase production and egg yolk agar was used to test phospholipase production. Modified tube adherence method was used for investigation of slime activity. The antifungal susceptibilities of the strains for amphotericin B, fluconazole, voriconazole and caspofungin were evaluated by using reference broth microdilution method.*

***Results**: Forty-eight percent (n=48) of the Candida strains were identified as C. albicans which represented the most common species. Both proteinase and phospholipase production rates were significantly higher among C. albicans isolates (79%; 66%) than non–albicans Candida strains (11.5%; 0%) (r=33.22, p<0.001 and r=206.8, p<0.001 repectively). On the other hand, slime activity was significantly higher among non-albicans strains (30%) when compered with C. albicans (0%) (r=0.228, p<0.001). MIC ranges were found as 0.03-1 μg/mL, 0.125 - ≥64 μg/L, 0.03-2 μg/mL and 0.015-2 μg/mL for amphotericin B, fluconazole, voriconazole and caspofungin respectively.*

***Conclusion**: Proteinase and phospholipase activities can be suggested as important virulence factors in C. albicans infections, while the slime factor production seems to be more important in non-albicans Candida infections. In addition, amphotericin-B, voriconazole and caspofungin can be suggested to be effective on Candida strains and may be used as alternative drugs for treatment of infections caused by fluconazole-resistant strains.*

***Key words**: Candida, C. albicans, virulence factors, antifungal susceptibility.*

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Introduction

Mycetes lead to severe life-threatening infections particularly in immunocompromised individuals due to severe side effects and absence of an approved vaccine. Host phagocytes may phagocyte yeasts. Hyphae require extracellular killing mechanisms. Some pathogenic mycetes require two phases as they are dimorphic and cause various problems in terms of virulence factors' diversities[1, 2]. These latter are microbial products formed in order to prevent elimination by host defense mechanisms. They may be classified as those that "initiate and proceed colonization" and "harm the host"[3]. The increased frequency and severity of fungal infections have led to a higher use of antifungals. The importance of antifungal susceptibility methods has increased due to the problem of resistance. Antifungal susceptibility tests are used to determine in vitro effects of a spectrum of drugs obtain data about resistance rates and predict clinical response[4].

The aim of this study was to investigate virulence factors like proteinase, phospholipase and slime factor production that play a role in the pathogenesis of fungal infections and to determine their antifungal susceptibilities.

Materials and methods

One hundred Candida strains isolated from various clinical specimens and accepted as infectious agents at the Microbiology Laboratory of Suleyman

Demirel University Medical School, were analysed. Clinical specimens were cultured on Sabouraud dextrose agar medium and incubated at 37°C for 24-48 hours. Identifications of the isolates were performed by looking for the presence of germ tube formation, microscopic appearances at corn meal tween 80 agar with Dalmau technique, colony colours in Candida ID2 agar (bioMérieux, Marcy l'Étoile, France) and by using the API ID 32C (bioMérieux, Marcy l'Étoile, France) assimilation kit.

A 1% bovine serum albumin-containing solid medium was prepared in order to determine excretory acid proteinase activity. The width of enzyme zones showing degradation of the protein in the medium was measured and the level of proteinase activity determined. Extractions without a melting zone were evaluated as negative for acid protease activity, isolates with a melting zone were evaluated as positive.

Egg yolk medium was used for phospholipase activity. The ratio of colony diameter to the sum of colony diameter and diameter of precipitation zone (Pz) formed around the colony was evaluated as Pz. According to this calculation, Pz=1 was accepted as negative phospholipase activity, and Pz <1.00 was accepted as positive. A modified tube adherence method was used for biofilm production.

Antifungal susceptibility tests were done with the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) criteria (5). Fluconazole (Sigma, Germany) was studied in dilutions of 64-0.125 µg/mL, amphotericin B (Sigma, Almanya) and voriconazole (Pfizer, USA) in 16-0.03 µg/mL, caspofungin (Merck, USA) in 8-0.015 µg/mL. An RPMI 1640 (Sigma, Germany) medium prepared with MOPS (3- [N-morfolino] propanesulphonic acid) (Sigma, Germany) was used. Minimal inhibitory concentration (MIC) values were determined according to CLSI criteria[6].

Statistical analysis of data was done using Fisher's exact test. A p level of <0.05 was accepted as statistically significant.

Results

Of 100 Candida isolates included in the study, C. albicans was the most frequently isolated species (48 isolates) and urine was the most frequent sample from which the strains were isolated (48 isolates) (Table 1).

A total of 44 strains (38/48 (79%) C. albicans; 6/52 (11.5%) non-albicans Candida) displayed proteinase activity and all of the non-albicans Candida strains which displayed proteinase activity were identified as C. parapsilosis.

A total of 32 (66%) strains were found to be positive for phospholipase activity all of which were C. albicans. On the other hand, slime activity was found to be positive among 16 isolates all of which were non-albicans Candida strains. Statistical analyses revealed that while the proteinase and phospholipase activities of C. albicans strains were found to be statistically significantly higher than that of non-albicans Candida strains ($r=33.22$, $p<0.001$); ($r=206.8$, $p<0.001$), slime activity was found to be higher among non-albicans isolates ($r=0.228$, $p<0.001$). The distribution of Candida species in terms of proteinase, phospholipase and slime activities are given in Table 2.

In the present study, no isolates were resistant to amphotericin B, caspofungin and voriconazole, and five (5%) isolates were resistant to fluconazole. In addition, two (2%) isolates were found to be dose-dependent susceptible to voriconazole (Table 3).

Discussion

In this study, the most commonly isolated strain was C. albicans (48%) and C. parapsilosis (16%) was the following in our study. This finding was in accordance with the study of Comert et al.[7] who have also reported that 65.6 % of 320 Candida species isolated from various clinical specimens were C. albicans, and 11.3% were C. parapsilosis.

In the study investigating proteinase activity made by Al-Hedaithy (8), proteinase activity was detected among all of 298 (100%) C. albicans strains and 22 of 200 (11%) non-albicans Candida strains. The proteinase activity of C. albicans strains was found to be statistically significantly higher than that of non-albicans Candida strains like it was in our study.

In our study, the phospholipase activity of C. albicans strains was found to be statistically significantly higher than that of non-albicans isolates. Oksuz et al.[9] investigated 122 Candida strains and detected that 53% of C. albicans strains and 17% of non-albicans Candida strains showed phospholipase activity. Kumar et al.[10] detected phospholipase activity in 100% of C. albicans strains, and in 29.6% of non-albicans ones, and they found the difference statistically significant in accordance with our study. In addition, Fotedar and Al-Hedaithy[11] detected phospholipase activity in all C. albicans strains similar to

Clinical Specimen	C.albicans(n)	C.parapsilosis(n)	C.tropicalis(n)	C.glabrata(n)	C.kefyr(n)	C. famata(n)	C.krusei(n)	C.lusitaniae(n)	C. inconspicua(n)	Total(n)
Urine	22	3	10	6	5	1	-	-	1	48
Blood	6	8	1	1	-	1	-	1	-	18
Wound	1	1	-	-	-	-	-	-	-	2
Sputum	11	-	-	1	1	-	-	-	-	13
Tracheal Aspirate	2	-	1	-	-	-	-	-	-	3
Pharyngeal Specimen	-	-	1	-	1	-	-	-	-	2
Abscess	-	1	-	-	-	-	-	-	-	1
Nail	1	-	-	1	-	-	-	-	-	2
Paracentesis	1	2	-	-	-	-	1	-	-	4
Vaginal Specimen	2	-	-	2	-	-	-	-	-	4
Catheter	1	1	-	-	-	-	-	-	-	2
Ear Specimens	1	-	-	-	-	-	-	-	-	1
Total	48	16	13	11	7	2	1	1	1	100

Table 1: The distribution of the isolated species according to clinical samples is shown (n).

Candida species	Quantitiy(n)	Proteinase Activity Positive (n)	Phospholipase Activity Positive (n)	Slime Activity Positive (n)
C. albicans	48	38	32	-
C. parapsilosis	16	6	-	2
C. tropicalis	13	-	-	9
C. glabrata	11	-	-	2
C. kefyr	7	-	-	1
C. famata	2	-	-	1
C. krusei	1	-	-	1
C. lusitaniae	1	-	-	-
C. inconspicua	1	-	-	-
Total(%)	100	44	32	16

Table 2: The distribution of Candida species in terms of proteinase activity, phospholipase activity and slime activity is given (n).

the results of our study. Slime activity of 16 out of 52 non-albicans isolates (30%) that we investigated with the modified tube adherence method was found positive for slime factor production; however, slime activity was encountered in no C. albicans strains. Shin et al.[12] detected biofilm activity in 11 out of

146 C. albicans strains (7.5%) and 130 out of 214 non-albicans strains (60.7%). In parallel with previous studies, slime activity among non-albicans isolates was found to be statistically significantly higher compared to C. albicans strains.

Antifungal	MIC (μg/ml)	R(n)	DD(n)	S(n)
Fluconazole	0,125-64	5*	3**	92
Voriconazole	0,03-2	-	2***	98
Caspofungin	0,015-2	-	-	100
Amphotericin B	0,03-1	-	-	100

Table 3: Distribution of antifungal MIC ranges and susceptibility of candida isolates.
**C. krusei (1), C. albicans (2), C. tropicalis (1) and C. glabrata (1), ** C. albicans (2), C. tropicalis (1), ***C. glabrata (2), MIC: Minimal inhibitory concentration, R: Resistant, DD: Dose-dependent susceptible, S: Susceptible*

None of the strains displayed all of proteinase, phospholipase and slime activities together. Thirtyfour isolates (3 C. albicans, 31 non-albicans Candida) were negative for all virulence factors investigated. In our study, amphotericin-B-resistance (≥2 μg/mL) was not detected among any strains and MIC values were between 0.03 and 1 μg/mL. Cuence-Estrella et al.[13] found MIC values between 0.03 and 0.5 μg/mL, and did not detect resistance to amphotericin B like our results. There are also studies reporting resistance to amphotericin B. Diekema et al.[14] reported that they have found MIC values between 0.25-2 μg/mL among 254 Candida strains, while 7 strains displayed MIC values of 2 μg/mL which shows resistance.

Fluconazole resistance rates show a large distribution in the studies done both in our country and abroad. In our study, the MIC values of fluconazole were 0.125-64 μg/mL, and 5 (5%) Candida strains were detected to be resistant to fluconazole (≥64 μg/mL). Skrodeniene et al.[15] reported 15.1% fluconazole resistance in 93 C. albicans strains.

In our study, the MIC values of voriconazole were 0.03-2 μg/mL, and resistant strains (≥4 μg/mL) were not encountered. Voriconazole resistance was not observed although fluconazole resistance was seen at a ratio of 5%. However, the voriconazole MIC value of the C. glabrata strain was 2 μg/mL, and this was evaluated as dose-dependent susceptible. In the study by Espinel-Ingroff et al.[16] investigating the voriconazole resistance of 90 Candida strains, while resistance was not observed in 20 C. albicans strains, it was found in 3 out of 70 non-albicans isolates (4.3%). In addition, fluconazole resistance was observed in 11/20 (55%) of C. albicans strains in this study.

Similarly to the results of previous studies, the MIC values of caspofungin were 0.015-2 μg/mL in our study, and resistant strains were not encountered. Pfaller et al.[17] did not encounter caspofungin-resistant strains in 8197 Candida isolates.

In another study, Pfaller et al. (18) detected that 99% of 351 fluconazole-resistant Candida strains were susceptible to caspofungin (MİK≤2 μg/mL), The MIC value of 8 isolates was ≥8 μg/mL.

In conclusion, our data showed that proteinase and phospholipase activities can be suggested as important virulence factors in C. albicans infections, while the slime factor production seems to be more important in non-albicans Candida infections. In addition, amphotericin-B, voriconazole and caspofungin can be suggested to be effective on Candida strains and may be used as alternative drugs for treatment of infections caused by fluconazole-resistant strains.

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