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SAP(1-3) Gene Expression in High Proteinase Producer *Candida* Species Strains Isolated from Iranian Patients with Different Candidosis

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Candida species are one of the commensal organisms that produce diseases in immune deficiency or disrupted normal flora. Several virulence factors such as proteinase production have an aggressive role in Candida species. Aspartyl proteinase gene expressed some molecular proteins that have an important role in virulence of Candida species. In this study 73 isolates from outpatient of Iranian Pasteur institute from various site of origin were analyzed. These species identified up to species level by standard mycological techniques and tested for proteinase activity and *SAP1-3* expression by Real-time PCR. *SAP1-3* gene expression was assayed in Candida species that shown a high producer of proteinas activity and compared them with the source of infection. Our results was shown the *SAP1-3* protein in vaginal *C. albicans* was detected in 80% and *SAP2* in 100% of studied strain. In sputum all three saps detected but not in all strain. It is concluded that *C. albicans* have a high ability to proteinase production. This ability result in more virulence and pathogenic effect. In other hand, *SAP* expression in Candida species is strain and source dependent and plays an important role in candidosis infections.

Keywords: *Candida* spp., Candidosis, Proteinase activity, *SAP (1-3)* expression, Real-time PCR.

Although *C. albicans* and some non-*C. albicans* is a normal commensal inhabitant of mucosal surfaces, it frequently causes surface infections when certain host factors are imbalanced. Under certain circumstances, these superficial infections may disseminate and cause serious systemic infections. The incidence of Candida spp. Infection has increased steadily over the past three decades[1]. Mainly due to recent advances in medical and surgical intervention and increasing population of immunocompromised patients with diseases such as AIDS, tissue transplantation, antibiotic therapy and malignancy[2]. The yeasts of the genus Candida are opportunistically invasive in individuals whose defense mechanisms are impaired. Pathogenic Candida species cause diseases ranging from superficial mycoses to disseminated and often fatal infections[3].

Some of the most important virulence factors are the secreted aspartyl proteinases (SAPs), which are encoded by 10 SAP genes[4]. The proteinases possess distinct differences in pH, with Sap1-Sap3 (yeast-associated) having optimum activity at lowers pH values and Sap4-Sap6 (hyphal-associated) having optimum activity at higher pH values, with a pH range of activity between 2.0 and 7.0[5].

Intensive research has been done to identify pathogenic factors in fungi, especially *Candida albicans* in order to facilitate the diagnosis, treatment and prevention of candidiasis. To date, phenotypic variability[6-8], adherence to host tissue[9-11], toxins and enzymes[12, 13] have been listed as candidates for virulence factors. Many factors like hyphal switching, surface recognition

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molecules, phenotypic switching and extracellular hydrolytic enzyme production such as proteinase and phospholipase have been suggested to be virulence attributes for Candida[14-16]. Extracellular hydrolytic enzymes seem to play an important role in candidal over growth, as these enzymes facilitate adherence and tissue penetration and hence invasion of the host. Among the most important hydrolytic enzymes produced by Candida are phospholipases and secreted aspartyl proteinases (SAPs).

Although the consequences of proteinase secretion during human infection are not precisely known, in vitro, animal, and human studies suggest that the proteinases may influence *C. albicans* virulence by a few probable mechanisms: 1) by facilitating adhesion through the proteolysis of host surface proteins, resulting in host tissue damage; 2) through defects in the host immune response due to deterioration of host proteins; 3) by increasing fungal nitrogen resources via peptide degradation products; 4) by damaging endothelial cells; and 5) by stimulating the host's proteolytic mechanisms. In normal conditions, candidal colonization of mucocutaneous surfaces is very rare. Adherence and persistence (colonization) of *Candida* spp. On mucosal surfaces is the first step in the process of candidiasis. The adherence capabilities of *C. albicans* and *C. tropicalis* are stronger than those of other *Candida* spp.[17, 18]. In hospitalized patients, mucosal colonization by *C. albicans* may reach 80%, but this rate is much lower in healthy adults, at 2%-37%[17, 19]. The main goal of this study is to confider the SAP1-3 gene experission in iranian patients based on the site of candidal infection.

MATERIALS AND METHODS

Sample collection and identification

A total of 73 Candida isolates obtained from Pathogenic Fungi Culture Collection of the Pasteur Institute of Iran. These isolates include of 6 species of Candida that obtained from various site of patient body. Speciation of Candida isolates were done by assessing of germ tube formation, chlamydospore formation on cornmeal agar plus twin 80, chromogenic assay on CHROM agar, carbohydrate assimilation in ID32C system (bioMerix,france). The standard strain *C. albicans* ATCC 10231, *C. glabrata* ATCC 90030 and *C. krusei* DSM 70079 were included in experiments.

Determination of proteinase activity

Enzyme activity was determined by colorimetric method, briefly proteinase activity was determined using serum bovine albumin as substrate (20, 21). Culture filtrate (0.2 ml) was incubated with 1ml of 0.1M sodium acetate buffer (PH=4) include of 5mg/ml serum bovine albumin. After 1h incubation at 37°C, 1ml Tricholoroacetic 10% was added to terminate of reaction. Then we centrifuged the solution, TCA soluble peptides were assayed by the Lowry method at 750 nm.

RNA extraction

RNA extraction was done by use of chloroform-phenol-isoamyl alcohol. Briefly three time washed Candida colonies was suspend in RNA buffer (Tris-Hcl, EDTA,Nacl) and then added chloroform-phenol-isoamyl alcohol and gently vortexes. After centrifuge, supernatant placed to micro tube and chloroform-phenol-isoamyl alcohol was added in. Supernatant of tube centrifuge mixed with ethanol and freeze in -20°C up to 1h. Then micro tube centrifuged and ethanol 70% added for precipitate. And finally centrifuged and supernatant discarded and precipitate mixed with distilled water[22-23].

RNA Purification and Reverse Transcription replace by cDNA preparation

The RNA concentration and purity were determined using a Nanodrop 2000 spectrophotometer. To obtain cDNA, a Fermentase Reverse transcription kit (thermo) was used according to the manufacturer's instructions. Figure 2 demonstrated RNA extraction.

Real-Time PCR Amplification of the *SAP* genes

The *SAP* primers that were used for the real-time PCR assay shown in Table 4. The ABI instrument with SYBR Green PCR method was used for the real-time PCR experiments. A final volume of 25 µl was used for each reaction and contained 12.5 µl of SYBR Green Master Mix, 1 µl of forward primer (1 µm), 1 µl of reverse primer (1 µm), 2 µl of cDNA (20 ng), and 8.5 µl of RNase-free water. The amplification conditions were 95°C for 5 minutes followed by 95°C for 5 seconds with an annealing/ extension combination step at 60°C for 10 seconds for 40 cycles. The same positive and negative

controls that were used for the endpoint PCR experiments were also used for the real-time PCR assay.

Statistical Analysis

Data were analyzed by the program spss version 22. Since these variables did not show normal pattern (P<0.05), Differences among the groups were analyzed in using the kruskal-wallis nonparametric variance test with significant value 5%.

RESULTS

This study aimed at determining of proteinase activity in 73 Candida species isolated, which were found in different site of patients who suffered from candidiasis. The distribution of isolated Candida spp according to the type of specimen, involved species are shown in table 1. Totally, 73 cases, contain 36 *C. albicans* (49.3%) and 5 *Candida glabrata* (6.8%) and 5 *C. guillermoundi* (6.8%) and 7 *C. krusei* (9.5%) and 15 *C. parapsilosis* (20.5%) and 5 *C. tropicalis* (6.8%) (Table1). Candida species were distributed as Sputum (18 cases; 24.6%), Skin and nail scrapping (33cases; 45.2%) Oral swap (4 cases; 5.4%). Skin and nail scraping include of toenail, Fingernail, Groin, Breast and Hand specimen. Oral swaps include of throat, pharynx and tongue.

Comparative Proteinase activity in Candida species shown in Fig.1. Also in table 2 shown that extra cellular proteinase activity was significantly higher in *Candida albicans*(p<0.05), followed by *C. parapsilosis* and *C. tropicalis*. Table 1 showed distribution of proteinase activity in different anatomical site. High proteinase activity belong to oral swaps (0.23±0.01) followed by Vagina (0.19±0.05) and sputum (0.19±0.03) and skin (0.14±0.07). Skin and nail scraping was a dominant specimen that Candida species isolated. High proteinase activity was seen in *C. albicans* at oral swaps (0.23±0.01) and lowest proteinase activity was seen in *C. glabrata* at oral swaps (0.01±0.0).

To determine the SAP virulence marker, 17 clinical strains includes of 10 *C. albicans*, 5 *C. parapsilosis* and 2 *C. tropicalis* that have a high proteinase activity were analyzed for SAP1-3 expression. Table 3 shown that SAP3 were produced by most of the species but SAP1 and SAP2 produced in some species.

Table 1. Distribution of proteinase activity in Candida species base on source of infection

	N (%)	SPUTUM	N (%)	SKIN	N (%)	ORAL	N (%)	VAGINA	N (%)
C.albicans	36(49.3)	0.19±0.03	15(41.7)	0.14±0.07	4(9.9)	0.23±0.01	2(5.5)	0.19±0.05	15(41.7)
C. glabrata	5(6.8)			0.007±0.003	2(40)	0.01±0.0	1(20)	0.01±0.007	2(40)
C. guillermoundi	5(6.8)			0.02±0.005	4((80)	0.02±0.01	1(20)		
C. krusei	7(9.7)	0.02±0.01	2(28.6)	0.02±0.01	4(57.1)			0.03±0.0	1(14.3)
C. parapsilosis	15(20.6)			0.08±0.01	15(100)				
C. tropicalis	5(6.8)	0.09±0.01	1(20)	0.07±0.01	4(80)				
Total	73		18(24.6)		33(45.2)		4(5.48)		18(24.6)

DISCUSSION

The yeasts of the genus Candida are opportunistic pathogens associated with rising incidence of life-threatening infections in immunocompromised individuals. Proteinase activities are considered to play important roles in the pathogenesis of opportunistic fungi. The present study focused on extracellular proteinase in different Candida spp. isolated from several

Table 2. Average of proteinase activity in Candida species

Species	N (%)	Proteinase activity
C. albicans	36(49.3)	0.19±0.051
C. glabrata	5(6.9)	0.01±0.005
C. guillermoundi	5(6.9)	0.02±0.004
C. krusei	7(9.5)	0.02±0.012
C. parapsilosis	15(20.5)	0.08±0.014
C.tropicalis	5(6.9)	0.07±0.014
Total	73	0.11±0.08

Table 3. Frequency of the *SAP* genes and their expression in *Candida* species

Species	N	Source	SAP1 (%)	SAP2 (%)	SAP3 (%)
C. albicans	5	Vagina	4(80)	5(100)	4(80)
C. albicans	2	Oral	0(0.0)	2(100)	2(100)
C. albicans	3	Sputum	2(75)	2(75)	3(100)
C. parapsilosis	5	Skin	4(80)	1(20)	3(60)
C. tropicalis	1	Sputum	0(0.0)	0(0.0)	1(100)
C. tropicalis	1	Skin	0(0.0)	1(100)	1(100)

Table 4. Primer used in real time PCR assays in *C. albicans*, *C. parapsilosis* and *C. tropicalis*

Gene	Primer	Sequence (5”! 3’)	PCR product
SAP1	F	TGAGGCTGCTGGTGATTATG	224
	R	TGCCAACAGCTTTGAGAGAA	
SAPP1	F	AGTGGTCGTCAAACCACTCC	219
	R	GACGGAAGCAAGCGAAATAG	
SAPT1	F	GGAAGATCTGATGTGCCAACTACATTGA	1005
	R	CGTGCGGCCGCTCTACAAAGCCGAGATGTCT	
SAP2	F	ATCAGCTGGTTTCGTTGCTT	105
	R	GGGACAGCTTGTCTTTTGGA	
SAPP2	F	TTACTTGCCTGACAGCATCG	277
	R	CGCATAAGCGTGTCTCAAAA	
SAPT2	F	TTCTTCTAGTGGTACCTGGGTCAAAG	762
	R	CATAGATCTCTAAACAATAGTGACATTAGA	
SAP3	F	TGTTACTGGTCCCCAAGGTGAA	209
	R	CTTGTCCTTGACCAGCTTGACAT	
SAPP3	F	GCTCAAGGTGCTGCTATTCC	253
	R	TTGCATCAATGACCCAGAAA	
SAPT3	F	ACTTGGATTTCCAGCGAAGA	165
	R	AGCCCTTCCAATGCCTAAAT	

anatomically distinct sites of patients. In this study as shown in table 2, *C. albicans* was the most frequently isolated Candida species (49.3%). In anatomically distinct sites *C. albicans* have major result of vaginal and respiratory tract candidiasis (41.7%). Whereas oral infection has a low prevalence of candidiasis (5.5%).

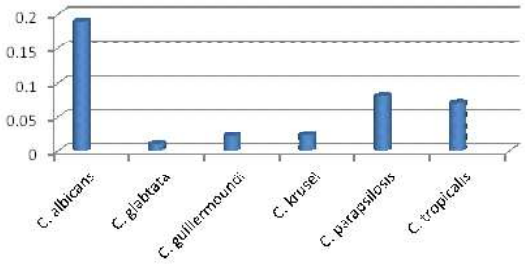


Fig. 1. Comparative proteinase production in Candida species(OD/750nm)

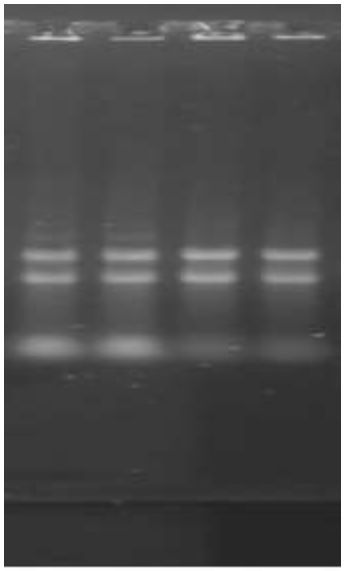


Fig. 2. RNA extraction in Candida species

In non-*C. albicans*, high candidiasis infection belong to skin infections (78.3%) and low belong to oral candidiasis (10.8%). The highest rate of proteinase production was found in *C. albicans* (0.19±0.05) and lowest in *C. glabrata* (0.01±0.005). (Table 2). This result is in agreement with previously reported frequencies for vaginal candidiasis, which occur in 20%-25% of vaginitis cases[22]. In another report from Singapore *C. albicans* cause of 54.6% respiratory tract candidiasis followed by *C. glabrata* (17.1%) and *C. tropicalis* (14.8%).

In our experiment SAP expression 17 Candida species which have high proteinase producer choose and SAP1-3 expression were studied (Table 3). The SAP1,3 protein in vaginal *C. albicans* was detected in 80% and SAP2 in 100% of studied strain. SAP2 can degrade many human proteins such as mucine, sIgA, keratin, collagen and vimentin. This ability results in dissemination of infection. In sputum all three saps detected but not in all strain. We conclude that SAP expression was strain dependent and except of SAP1 in *C. albicans* with oral source all sap proteins are produced by *C. albicans*. As shown in figure 3, SAPP1 was more frequency of expression (80%) followed by SAPP3 (60%) and SAPP2 (20%) in *C. parapsilosis*. SAPT1 was not detected but SAPT2,3 was expressed in *C. tropicalis*. Results presented in this study showed that SAP expression is strain and source dependent and play an important role in infections caused by *Candida* spp.

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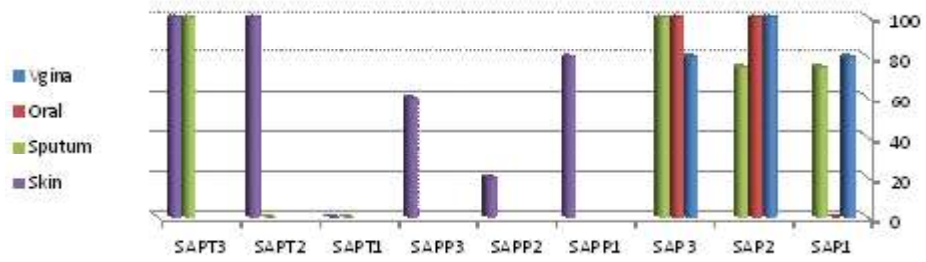


Fig. 3. Percentage of SAP expression in different anatomical site

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