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RESEARCH ARTICLE

DETECTION OF SECRETED ASPARTYL PROTEINASE (SAP) IN DIFFERENT *CANDIDA* SPECIES ISOLATED FROM URINARY TRACT INFECTION

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ABSTRACT

Candiduria is an increasingly difficult problem to recognize and manage. It is associated with high mortality, especially in patients with comorbidities. Secretory aspartyl proteinases (SAPs) have been recognized as virulence factors and are considered to be key enzymes that contribute to *Candida* infection by promoting damage to the host mucosa. In the present study we investigated the extent of *Candida* urinary tract infection (UTI) among hospitalized patients and the presence of *Sap* genes as virulence factors. A total of 50 *Candida* species were isolated from urine samples of UTI patients, processed at the routine Laboratory in Medical Microbiology Department, from different departments. Out of the 50 *Candida* species isolated, 52% were *Candida tropicalis* followed by *Candida glabrata* (30%), *Candida albicans* (14%) and *Candida krusei* (4%) identified by both conventional methods and MALDI-TOF. This study revealed the predominance of *Non- albicans Candida* species as causative agents of *Candida* UTI with different antifungal susceptibility patterns, where *Candida tropicalis* reported the highest sensitivity to amphotericin B (23/26=88%), fluconazole (19/26=73%) and finally voriconazole (17/26=65%). Again amphotericin B and voriconazole had equal effect (14/15=93%) on *Candida glabrata*, while fluconazole was less effective (12/15=80%). All *Candida albicans* strains were sensitive to fluconazole, followed by voriconazole and amphotericin B (6/7=86%). Finally both *Candida krusei* isolates were sensitive to amphotericin B and voriconazole. *Sap* genes were detected in 6 *Candida albicans* and *Candida tropicalis* isolates, 3 were SAP 1, 3 in *Candida albicans* and 2 were SAPT4 in *Candida tropicalis* and 1 was SAPT 1 in *Candida tropicalis*.

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INTRODUCTION

Candida species exist as an element of normal flora in the skin, mucous membranes, and gastrointestinal tract of humans. The extent and severity of *Candida* infections depend on the immune status of the host (Milan, 2004; Blanco and Garcia, 2008). Secretory aspartyl proteinases (SAPs) have been recognized as virulence factors since their discovery in 1965. Moreover, SAPs are considered to be key enzymes that contribute to *candida* infection by promoting damage to the host mucosa, thereby facilitating the invasion of the organism into the epithelium by digestion of proteins and providing nitrogen to aid the survival of fungal cells (Hube and Naglik, 2001).

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However, SAPs may have developed other functions related to virulence such as degrading structural proteins and proteins of the immune system (Staniszewska *et al*., 2016). In *candida albicans*, the production of Sap is encoded by a family of 10 SAP genes that are grouped into six subfamilies:SAP 1-3, SAP 4-6, SAP 7, SAP8, SAP9 and SAP 10(Staniszewska *et al*., 2014). According to genetic identity, Sap1-Sap3 share 67% genetic identity and Sap4-Sap6 share as much as 89% identity. Sap1 - Sap 3 and Sap4-Sap6 are closely clustered. Sap 7 only shares 20-27% identity with the other Sap proteins. Sap 9 and Sap 10 differ from the other Sap 1-8 isoenzymes and constitute a distinct group(Gilfillan *et al*., 1998), (Naglik *et al*., 2003). In the genome of *candidakrusei* and *candida kefyr* SAP genes have not been found (Albrecht *et al*., 2006). Candiduria is an increasingly difficult problem for modern physicians to recognize and manage. It is also associated with high crude mortality, especially in patients with comorbidities (Kauffman

et al., 2000; Magill, 2006). The presence of *Candida* spp. may lead to *Candida* cystitis, which is known as asymptomatic lower urinary tract infection (UTI). Sometimes, *Candida* cystitis may lead to symptomatic candiduria. *Candida* cystitis is identified via symptoms of high urination frequency, dysuria and hematuria (Kauffman, 2014). *Candida* pyelonephritis is a severe upper UTI which may lead to candidemia and sepsis. The most predominant primary symptoms pertaining to *candida* pyelonephritis is reported as fever and candiduria (Kauffman, 2014). Generally, *Candida* urinary tract infection is more common in females and among catheterized patients (Kim *et al*., 2016). There is an increasing need for various methods for precise identification of the *Candida* species like the newly approved method; MALDI-TOF MS. All *Candida* species have been shown to cause a similar spectrum of diseases, yet differences in disease severity and susceptibility to different antifungal agents have been reported (Fisher *et al*., 2011). The aim of our study is to estimate the prevalence of *Candida* species as a causative agent of urinary tract infection, investigate the susceptibility pattern of isolated *Candida* species towards different antifungal drugs and detect the presence of (SAP) as a virulence factor among the isolated *Candida* spp.

MATERIALS AND METHODS

Suspected *Candida* colonies isolated on (Sabouraud Dextrose Agar) SDA from urine specimens delivered to the Routine Laboratory in Medical Microbiology Department, Faculty of Medicine, Alexandria University from different departments of Alexandria Main University Hospital (AMUH) over a period of 12 months from August 2014 to July 2015 constituted materials of this study.

Candida identification by conventional techniques

This was performed using Gram's stain, germ tube and Chlamydospore production tests (Forbes, 2013).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using Formic Acid Extraction

Isolated *Candida* was cultured on SDA plates overnight at 37^{o}C. A loop-full of fresh *Candida* culture was mixed in 300 µL sterile distilled water and briefly vortexed; then 900 µL absolute ethanol was mixed into this suspension followed by centrifugation at 13 000 rpm for 2 minutes. The supernatant was discarded, and the pellet was air dried. It was resuspended in 50 µL of 70% formic acid and 50 µL acetonitrile. The suspension was recentrifuged at 13 000 rpm for 2 minutes. One microlitre of supernatant was placed on a MALDI polished steel plate and air dried. Finally, for every extraction method, 1 µL of matrix solution (100 µL of matrix solution containing: 50 µL acetonitrile, 2.5 µL trifluoroacetic acid, 1 mg α-cyano-4-hydroxycinnamic acid and 47.4 µL sterile distilled water) was added on each spot and allowed to air dry prior to MALDI-TOF MS analysis. MALDI-TOF MS UltraFlex system (BrükerDaltonik) for the MALDI MS identification was used. It was operated in the positive linear mode (mass over charge ratio (m/z) ranging from 2,000 to 20,000) under control of FlexControl software. Each spectrum was obtained by averaging 240 laser shots in 40 shot steps acquired in automatic mode at the minimum laser power being necessary for ionization of the samples according to the manufacturer's suggested recommendations. MALDI-TOF MS interpretation: Spectra were compared to fingerprint database by using the Bruker Biotyper 3.1 software and library of 5,623 entries.

Antifungal susceptibility testing using disc diffusion method

This was performed and interpreted according to CLSI guidelines 2003 and 2004 antifungal sensitivity using disc diffusion method (CLSI, 2004). Briefly, Mueller-Hinton with 2% glucose and 0.5 µg/ml methylene blue was prepared. An inoculum equivalent to 0.5 McFarland standard was plated. Antifungal discs containing Fluconazole (25 µg/disc), Voriconazole (1µg/disc) and Amphotericin B (10µg/disc**)** were applied.

Detection of SAP genes in different Candida species by conventional PCR (Giolo, 2010; Pinto *et al*., 2011)

Genomic JET Genomic DNA Purification Kit (Thermo Scientific) was used for DNA extractionaccording to the manufacturer instructions**.** Oligonucleotide primers used to amplify SAP genes are shown in Table 1 (Parra-Ortega *et al*., 2009). Family I (SAP1-SAP3 from *C. albicans* and SAPT4 from *C. tropicalis*), familyII (SAP4-SAP6 from *C. albicans*), family III (SAP8 from *C. albicans* and SAPT1 from *C. tropicalis*). PCRs were performed by Maxima Hot Start Green PCR Master Mix (Thermo Fisher Scientific) in 25 µL volumes containing: 12.5ul Maxima Hot Start Green PCR Master Mix, 0.5 µM total forward and reverse primers, 3µL template DNA. PCR reaction conditions included an initial denaturation step for 3 min at 94°C, followed by 38 amplification cycles consisting of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C. A final extension step was performed for 7 min at 72°C using (2720 Thermal Cycler applied biosystems). Amplified products were visualized using 1% agarose gel electrophoresis**.**

Statistical analysis of the data (Kotz, 2006)

Data were fed to the computer and analyzed using IBM SPSS (Statistical Package for the Social Sciences manufactured by International Business Machines Corporation) software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum).

RESULTS

The 50 *Candida* isolates were collected from 1110 urine samples diagnosed as significant bacteruria/Candiduria referred to Routine laboratory, Medical Microbiology Department, Faculty of Medicine, over a period of 12 months from September 2014 through August 2015, constituted the material for this study (Table 2) *Candida* urinary tract infection constituted 14.5% (162/1110) of all cases of UTI. The most isolated pathogen was *E.coli* 33% (366/1110), followed by *Klebsiella sp*p 19% (215/1110), *Acinetobacter spp*10.5 % (118/1110), *Pseudomonas spp* 10% (109/1110), *Enterococcus spp* 8% (92/1110) and the least isolated was *Staphylococcus spp* 5% (48/1110). The 50 *Candida* spp. were isolated from 28 males (56%) and 22 females (44%). They were most commonly isolated from patients with acute renal failure (24%), diabetes (18%) and chronic renal failure (10%). Patients with urinary catheters provided 16% of the isolates.

Table 1. Primers used to amplify SAP genes

Primer	Sequence 5′ - 3′	Product (bp)
Family I	5′TACTGGATCATCTGATTTATGGG-3′	C.tropicalis:846
F	5′GCTGATCTTAAAAAGTTATTACCAAGAAT-3′	C.albicans: 843
R		
Family II	5′ -GTTTGTTACCTTAGACTTTAATGTC-3′	
F	5′ -TTGTCACCAAGAATATTATCTTCAC-3′	C.albicans :1077
R		
Family III	5′ -TTGCTTTGTTTGCTCAAGGT-3′	C.tropicalis:1109
F	5′ -AGTGTACTTGACTTGAGCCA-3′	C.albicans: 1142
R		

Table 2. The prevalence of different *Candida* species by MALDI-TOFMS

Identification	No.	%
Candida albicans	7	14.0
Candida tropicalis	26	52.0
Candida glabràta	15	30.0
Candida krusei	2	4.0
Total	50	100

Table 3. Antifungal susceptibility pattern of different *Candida* species

Candida species	Fluconazole (Diflucan) (25 µg g/disc)			Voriconazole (1µg g/disc)(V-fend)			Amphotericine B (10µg g/disc (Fungizone)		
Candida tropicalis(n=26)	S ≥19mm n%	SDD 15-18mm n%	R ≤14mm n%	S≥17 mm n%	SDD 16- 14mm n%	R≤13mm n%	S≥15 mm n%	SDD14 – 10mm n%	R < 10mm n%
	19(73%)	0	7(27%)	17(65%)	2(8%)	7(27%)	23(88%)	0	3(12%)
Candida glabràta (n=15)	12(80%)	1(7%)	2(13%)	14(93%)	0	1 (7%)	14(93%)	0	1 (7%)
Candida albicans (n=7)	7(100%)	0	0	6 (86%)	0	1(14%)	6(86%)	0	1(14%)
Candida krusei (n=2)	NT	NT	NT	2(100%)			2(100 %)		
Total	38	1	9	37	3	8	44	0	6

NT: Not Tested because *Candida krusei* is intrinsically resistant to fluconazole

Table 4. Distribution of the different SAP genes in studied *C. albicans* and *tropicalis* species

Gene	*Candida tropicalis*	*Candida albicans*	%	Product size(bp)
SAP1,3 SAPT4				
Negative	24	4	85.7	*C.tropicalis*:846 *C.albicans*: 843
Positive	2	3	14.3	
SAP8,SAPT1				*C.tropicalis*:1109 *C.albicans*: 1142
Negative	25	7	97.1	
Positive	1	0	2.9	
SAP4,6				*C.albicans:* 1077
Negative	-----	7	100	
Total = 33	26	7		

Candida identification (Table 2)

When identified using standard microbiological techniques, there was predominance of *non-albicans* spp. 43/50 (86%) over *albicans* spp. 7/50 (14%), however these methods were unable to determine the species of *the non-albicans* isolates. By MALDI MS, out of the studied 50 *Candida* species, *C.albicans* constituted 7/50 (14%), while the 43 *non albicans* species comprised *C.tropicalis* 26/50 (52%), *C.glabrata* 15/50 (30%) and *C.krusei 2/50* (4%) (Figure 1).

Antifungal sensitivitytesting (Table 3)

By analyzing the susceptibility pattern of the studied isolates to the different antifungals agents, *C.tropicalis* reported the highest sensitivity to amphotericin B (23/26=88%) followed by fluconazole (19/26=73%) and finally voriconazole (17/26=65%).

Again amphotericin B and voriconazole had equal effect (14/15=93%) on *C.glabrata,* while fluconazole was less effective (12/15=80%).

All *C.albicans* were sensitive to fluconazole followed by voriconazole and amphotericin B (6/7=86%). Both *C.krusei* isolates were sensitive to amphotericin B and voriconazole.

SAP genes in *C.albicans* and *C.tropicalis* (Table 4)

Using specific primers for Family I (SAP1-SAP3from *C. albicans* and SAPT4 from *C. tropicalis*), familyII (SAP4-SAP6 from *C. albicans*) and family III (SAP8 from *C. albicans* and SAPT1 from *C. tropicalis*), SAP 1,3 genes were found in 3 *C.albicans* isolates, SAPT 4 in 2 *C.tropicalis* isolates and SAPT1 in one *C.tropicalis* isolate, while SAP 4.6 genes could not be detected (Figure 2).

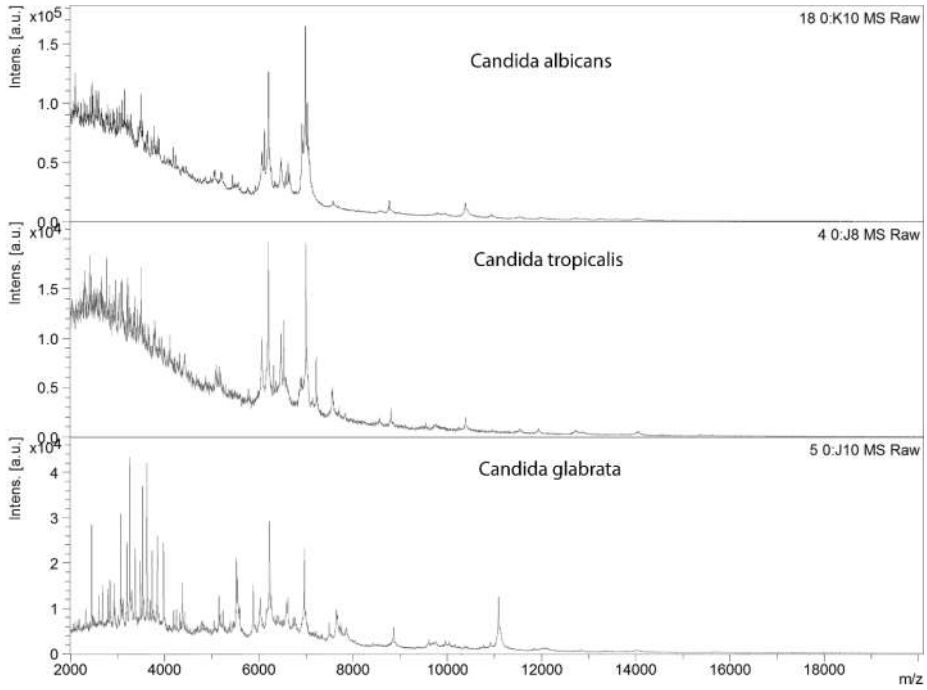

Figure 1. Mass spectra of various species isolated

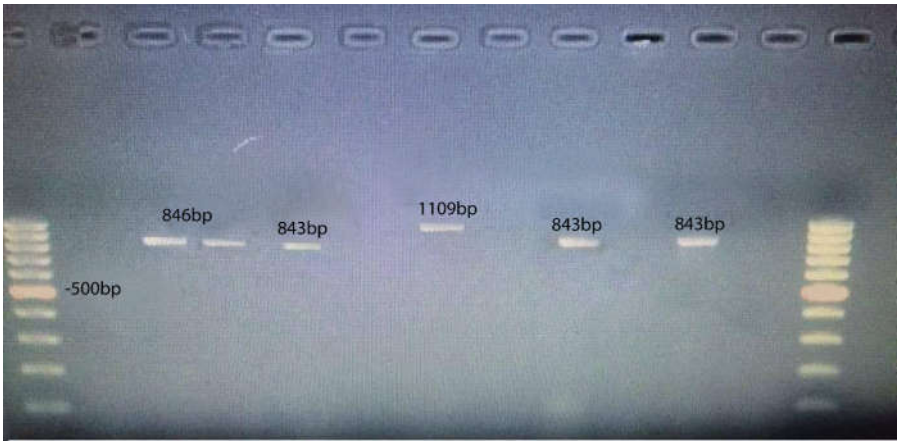

Figure 2. 1% agarose gel electrophoresis showing PCR products of different SAP genesLane 1 : negative control, Lane 2, 3 : 846 bp of Family I in *C.tropicalis* Lane 4,8,10 : 843 bp of Family I in *C.albicans*. Lane 6 :1109 bp of Family III in *C.tropicalis*, M: is Molecular marker (fermentas)

DISCUSSION

Candida sppcan become one of the most significant causes of disabling and lethal infections (Sardi *et al*., 2013). Although *C.albicans* is the most prevalent species involved in both mucocutaneous and disseminated infections, the incidence of candidiasis due to *non-albicans Candida (NAC)* spp. is increasing.The problem is that several *NAC* spp. are inherently resistant or acquire resistance, or both, to commonly used antifungal drugs (Sullivan *et al*., 1996). The major problem is when *Candida* organisms are discovered in urine, the major decision that must be made is whether this signifies infection of either the upper or lower urinary tract, colonization of the bladder, or contamination of the urine sample. The physician should neither dismiss the finding out-of-hand nor begin antifungal treatment empirically (Fisher *et al*., 2011). The aim of the current study was to estimate the prevalence of *Candida* species as a causative agent of urinary tract infection, and to detect the presence of Secreted aspartyl proteinase (SAP) as a virulence factor among the isolated *candida* strains.

In the present study, *C.tropicalis* 52% constituted the majority of isolates, *C.glabrata* 30% followed by *C.albicans* 14%, *C.krusei*was the least species isolated 4%. *Candida* spp. identification using conventional methods like Germ-tube test and chlamydosporeproduction is considered simple, economical and efficient (Neppelenbroek *et al*., 2014). On the other hand some studies stated that these conventional methods are time consuming and sometimes inaccurate. While identification of *Candida* by these conventional methods requires 24–48 h of incubation, correct and fast identification of these pathogens is of major concern for optimal patient management and especially for the implementation of effective measures for disease control (Sanguinetti *et al*., 2007). In our study, the test procedure of MALDI TOF was completed in approximately 13 min**.** with the formic acid extraction method. Some studies stated that MALDI TOF identification using this method provides good-quality protein spectra and results in genus or species level identification for 92.5% to 98.2% of all yeast isolates and a correct identification rate of 99.3% to 100% (Dhiman *et al*., 2011). According to Pinto *et al*, the rapid, accurate identification of pathogenic yeasts by MALDI-

TOF MS is dependent on optimal protein extraction and upon robust reference spectra where *Candida* species were reliably identified, with superior performance of MALDI-TOF MS over conventional methods (Pinto *et al*., 2011). Another study performed by Stevenson *et al.* (van Veen *et al*., 2010) showed that MALDI TOF biotyper was able to correctly identify 99% of the 194 *Candida* isolates which were included in the spectral library while Van Veen *et al.* (Stevenson *et al*., 2010) evaluated the performance of MALDI-TOF biotyper for identification of 80 yeast isolates and reported that 97.5% of the isolates were correctly identified at the genus level and 87.5% at the species level with most of the non-identified organisms due to insufficient entries in the database in the spectral library and identification accepted as species level only if the spectral score was above 2.0. We detected the emergence of *NAC* species as a major cause of candiduria in the current work. Other studies have also documented a change in the trend from *C.albicans* to *NAC* linking that change toseveral factors like severe immunosuppression or illness, prematurity, use of broad spectrum antibiotics, and empirical use of antifungal drugs are reported to be associated with this change (Rani *et al*., 2002; Shivaprakasha *et al*., 2007). Our observation is similar to that of Alvarez-Lerma *et al.* where more than 50% of urinary *Candida* isolates belonged to *NAC* species, which are not only well adapted to the urinary tract but also more difficult to eradicate (Alvarez-Lerma *et al*., 2003).

It was reported that the presence of indwelling urinary catheters, advanced age, diabetes mellitus, and pregnancy were major risk factors associated with candiduria. The incidence of candiduria was high among patients admitted to the ICU and among those who had a previous history of treatment with antibiotics (Alvarez-Lerma *et al*., 2003). In one study, of predisposing factors for community-acquired candiduria, several differences were noted when compared with nosocomial candiduria. Risk factors included diabetes mellitus (28.8%), bedridden status (46.4%) (Colodner *et al*., 2008). In our study *Candida* isolates were originated most commonly from acute renal failure patients (24%) followed by diabetic patients (18%) and chronic failure patients (10%) from non-catheterized patients which are conditions of immune suppression while catheterized patients provided 16% of the studied isolates. Although *C. albicans* is frequently reported as the most prevalent species infecting the urinary tract, reports from some institutions have identified *C. tropicalis* as the most prevalent fungal isolate, while others have identified *C. glabrata* as the dominant species (Paul *et al*., 2007).

This was in agreement with Sikora *et al.* in Poland 2011, where *Candida glabrata* constituted the majority of the isolated *Candida* species from urine samples (9 cases), while *C. albicans*, *C. krusei* and *C. lusitaniae*were detected as one case each. This supports our results concerning the emergence of *NAC* species as major pathogenic organisms (Sikora *et al*., 2011). This is different from Alhusseni *et al*. where *C.albicans* was detected in 27/50 (54%) of *Candida* strains isolated from urine specimens from renal failure patients while the remaining 23/50 (46%) isolates were identified as *NAC* species (Alhussaini *et al*., 2013). Similarly, an Egyptian study that investigated the virulence factors and susceptibility patterns of *Candida* species isolated from patients with obstructive uropathy and bladder cancer reported the predominance of *C. albicans* (34.5%) followed by *C. glabrata* (29.5%), then *C. tropicalis* and *C. krusei* each 18 % (Omar *et al*., 2008). Antifungal susceptibility results of this study agree with others who showed that all isolates of *C. albicans* were susceptible to fluconazole. Resistance to fluconazole was manifested in 20% of *C. glabrata* isolates compared to 13% resistance rate to fluconazole among our isolates (Dagi *et al*., 2016). As suggested by Bukhary (2008), fluconazole is the drug of choice if the organism isolated is not *C. glabrata*or *C.krusei*. Oral fluconazole has a more delayed but more lasting effect on candiduria than amphotericin-B bladder irrigation. Patients receiving amphotericin-B bladder irrigation had higher rates of eradication two days after the beginning of therapy than those receiving oral fluconazole but the cure rates were similar one month after the beginning of therapy (Bukhary, 2008).

In the present study 93% of *C.glabrata* were sensitive to amphotericin B and voriconazole and 80% sensitive to fluconazole in agreement with the findings of a study which described the low sensitivity of this species to triazol (Almeida *et al*., 2013) {Laverdiere M, 2007 #1}. When the presence of SAP genes was examined in all *C.albicans* and *C.tropicalis* isolates, SAP 1, 3 genes was found in3 of *C.albicans* isolates, SAPT 4 in 2 *C.tropicalis* isolates (14.3%) and SAPT1 in only one *C.tropicalis* isolate (2.9%), while SAP 4.6 genes could not be detected. This is matching the studyof Sikoraand coworkers,where *C. albicans* species isolated from urine samples had SAP1-3 genes and SAP 4 but lacking SAP6 denoting that isolated strains having SAP1-3 and lacking SAP 4-6 genes would cause mucosal infection and not systemic infection with milder course than those strains having both SAP1-3 and SAP 4-6 genes(Sikora *et al*., 2011). Again our results also agreed with a study on *Candida* species isolated from diabetic patients having vulvovaginal candidiasis. It reported that the most common detected SAP genes in isolated *C. albicans* species were SAP1 and SAP2, followed by SAP 5 then SAP3 (Bassyouni *et al*., 2015). In conclusion, candiduria is an insidious disease and its diagnosis is likely to be missed, because of a general lack of awareness and lack of characteristic features of the etiologic agent. MALDI TOF-MS proved that it is a rapid and accurate identification method especially for the most common *Candida* species. Implementation of MALDI TOF-MS to routine yeast identification in the clinical microbiology laboratory could help in improving the management of candiduria through accurate species identification and, consequently, very early administration of the most appropriate antifungal therapy. Voriconazole can be recommended as an alternative for treatment of candiduria particularly with *NAC* and *C.albicans* with fluconazole resistantstrains, especially it is not nephrotoxic and less infusion-related toxicity than amphotericin B and is also available as oral tablets. SAP gene detection showed low rates among *Candida* isolates, which could denotes that it is not the only virulence factor essential for *Candida* pathogenesis and highlights the importance of other virulence factors like phospholipases, hemolysins and biofilm formation in pathogenesis to be investigated.

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