Molecular identification of SAP9, SAP10 genes with evaluation virulence factors and antifungal susceptibility profile among Candida Species isolated from women with urinary tract infections in Thi-Qar Province, Iraq

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ABSTRACT

Background. In recent years, there has been a rise in the occurrence of urinary tract infections (UTIs) caused by Candida pathogens. This increase is attributed to the growing resistance of these pathogens to antifungal drugs often used for their treatment. Many virulence factors encoded by virulent genes, which enable opportunistic *Candida* to invade host tissues and cause infection, for survey the existence of *SAP9 andSAP10* genes with determine some virulence factors and antifungal susceptibility profiles among *Candida species* isolated from women suffering from urinary tract infections.

Methods. For this investigation, urine specimens were gathered from female individuals residing in Thi-Qar Province, located in southern Iraq, over the period spanning from December 2022 to March 2023. The identification of all isolates was performed using several laboratory techniques, including assessment of colony characteristics on culture media, Gramme staining, germ tube formation, and analysis of morphological chromatic features on chromogenic agar. The identification was further validated using PCR analysis. All isolates were assessed for their production of extracellular secretory proteinase and hemolysin. Additionally, they were tested for antifungal activity using the disc diffusion method. The DNA was isolated, and the PCR technique was employed to identify the presence of virulence genes (SAP9 and SAP10).

Results. A total of 50 Candida species isolates were collected from 150 urine samples. These isolates belonged to four different species within the Candida genus. The most found species was *C. albicans*, accounting for 54% of the isolates. This was followed by *C. krusei* at 24%, *C. glabrata* at 16%, and *C. tropicalis* at 6%. Proteinase activity was observed in 84% of Candida isolates. All Candida isolates exhibit positive hemolysin production with varying degrees of intensity. Among Candida species showed high resistance to Itraconazole (86%) and Clotrimazole (76%). The prevalence of SAP9 and SAP10 genes among *Candida albicans* was 62.69% and 100%, respectively.

Conclusions. The present study highlights the prevalence of *SAP* genes and virulence factors with antifungal susceptibility which reflect high pathogenicity of *Candida species*.

Keywords: *Candida*, Proteinase, *SAP* genes, Hemolysin, Antifungal

Introduction

Candida species are an infrequent cause of urinary tract infections (UTIs) in healthy persons but are common in hospital settings or among patients with predisposing illnesses and anatomical abnormalities of the kidney and collecting system [1,2]. Urinary tract infections are more prevalent in women than in males due to the genitourinary system's anatomical structure and closeness to the anus [3]. The clinical appearance of a urinary tract infection depends on the type of causative agent, the severity of the disease, and the immune response of the infected person [4].

The Candida genus is a diploid fungus that exhibits several forms and is recognized as a constituent of the human microbiome [5]. Under specific pathogenic circumstances such as

elevated temperature, high moisture levels, inadequate nutrition, the use of broad-spectrum antibiotics, excessive sugar consumption, and a compromised immune system, the likelihood of developing pathogenic infections increases. These infections can manifest as superficial skin and mucous membrane infections or more severe systemic invasive infections [6, 7]. The pathogenicity of Candida species and their ability to cause infection is due to many virulence characteristics, such as evading host immune responses, phenotypic switching, adherence, biofilm formation, and production of hydrolytic enzymes [8, 9]. The production of hydrolytic enzymes during infection boosts Candida's ability to adhere, invade, destroy immunological components in the host, and acquire nutrition [10]. The exoenzymes necessary for colonization and attack of host organs include secreted aspartyl proteinases (Saps), phospholipases, lipase, esterase, and hemolysins [11]. Secreted aspartyl proteinases (SAPs) are external proteolytic enzymes that are crucial for the successful ability of opportunistic Candida to cause disease [44]. SAPs perform a range of specialized roles during the infectious process, enabling the elongation of filaments and facilitating their adherence and penetration into deeper layers of tissue [12,13]. Secreted aspartic proteases (Saps) are produced by a group of 10 SAP genes (SAP1- SAP 10) that play a critical role in the ability of Candida to cause disease by breaking down proteins found in host tissues, including hemoglobin, albumin, keratin, collagen, laminin, fibronectin, and mucus. Additionally, these proteases help the Candida cells attach to the epithelial tissues of the host [14]. The opportunistic fungus evades the initial host defenses by breaking down proteins involved in immunological defense, such as antibodies, complement, and cytokines [15]. The secretion of sap enzymes is associated with several other mechanisms that contribute to virulence, including hyphal growth, adhesion, phenotypic switching, and dimorphism [16]. Sap1 to Sap8 are fully excreted into the extracellular milieu, whereas Sap9 and Sap10 are non-excreted and stay tethered to the cell wall through a glycosylphosphatidylinositol anchor [17]. The Sap9 and Sap10 enzymes play a crucial role in preserving the structural integrity of the Candida cell wall through their regulating proteinase activity. Additionally, these enzymes are highly implicated in the production of biofilms [14,18]. Candida biofilms exhibit decreased responsiveness to antifungal treatment and diminished vulnerability to the host immune system's killing mechanisms [19]. The pathogenicity of Candida has been enhanced by the existence of virulence genes and the growing prevalence of resistance to antifungal medicines [20]. The virulence factors and virulence genes of Candida have varying patterns of expression at different stages and locations of infection [21]. Hence, comprehending the role of SAP genes with virulence factors is crucial in devising novel approaches to elucidate the pathophysiology of Candida [6]. Furthermore, it is of utmost importance to accurately determine the Candida strains at the species level due to the variations in antifungal susceptibility patterns [22,23].

Methods

Sample Collection

A total of 150 urine samples were obtained from women, consisting of 108 patients and 42 healthy individuals, with ages ranging from 15 to 60 years. These samples were collected between December 2022 and March 2023 from the Al Nasiriyah Teaching Hospital, Bint Al Huda Hospital, and private clinics for women in Thi-Qar Province, southern Iraq. The urine

samples were delivered to the Microbiology Laboratory in sterile containers and cultivated within three hours of collection.

Isolation and Identification of *Candida species*

The urine samples were cultured on Sabouraud Dextrose agar plates and Malt extract agar (MEA) supplemented with chloramphenicol. Subsequently, the colonies of Candida species were isolated through subculture on Sabouraud Dextrose agar and incubated at a temperature of 30°C for a period of 48-72 hours. The Candida isolates were identified based on their colony morphology, Gramme staining, germ tube test in serum, and further confirmation through culturing on differential medium (CHROM Agar Candida, CONDA, Spain) to observe morphological colony colors. This medium was made in accordance with the directions provided by the manufacturer. The Candida colonies were identified based on their distinct coloration, which was compared to the standard color photos provided by the manufacturer. This coloration was observed after 72 hours of incubation [24].

Virulence Factors of *Candida spp*

The virulence factors evaluated were exoenzymatic activity, proteinase, and hemolysin production.

Detection of proteinase production

Candida species were introduced onto proteinase agar and thereafter subjected to incubation at a temperature of 37°C for a duration of 48 to 72 hours. A beneficial outcome was observed in the form of distinct zones around the colonies, indicating their diameter. The proteinase activity score (Prz) was determined and computed using the methodology outlined by those [25], with measurements of both the colony and the proteinase area expressed in mm [26].

Detection of Hemolysins activity

The hemolytic activity of Candida species was assessed on a Sabouraud dextrose agar plate containing 3% glucose and sheep blood. Approximately 10 μL of a standard inoculum (108 Candida cells/mL) was carefully introduced onto the medium in a sterile manner. For 48 hours, the culture plates were kept at a temperature of 37◦ C. Hemostasin production was confirmed by the presence of a hemolysis zone surrounding the colony. This is the formula that was used to determine the hemolytic activity (Hz) results: Colony diameter divided by the sum of colony diameter and precipitation zone yields the Hz value [26].

Antifungal susceptibility test by disk diffusion method

Each Candida species isolate was tested for its resistance to seven different antifungal agents, including Amphotercin-B, Clotrimazole, Itraconazole, Voriconazoles, Fluconazole, Ketoconazole, and Nystatin, using the disc diffusion method on Mueller Hinton agar supplemented with 2% glucose and 0.5 µg/ml of methylene blue. The procedure described in the Clinical and Laboratory Standard and the susceptibility Profiles was determined by using CLSI-2019 guidelines and Himedia Laboratories Limited [27].

Molecular Study for Isolated *Candida albicans*.

DNA Extraction for *Candida*

Genomic DNA was extracted from the isolated Candida albicans using the Geneaid Genomic DNA extraction Kit (Taiwan) following the manufacturer's instructions (Geneaid, Twain). Following this, the samples were run on a 1.5% gel agarose to confirm DNA extraction and purity.

Molecular identification

Conventional PCR test was the most well-developed molecular technique to amplify the extracted DNA using specific primer pairs the internal transcribed spacer (ITS1, ITS4) of rDNA regions for molecular identification of *Candida albicans*. Primers utilized in the amplification processes are detailed in Table 1, according to the methods outlined in [28].

Table 1: The sequences of the primers and the molecular size of the PCR product used for molecular study of isolated Candida albicans.

Product size (bp)	Oligonucleotide sequence (primers) (5′-3′)		Reference
500 bp	ITS 1	F-TCCGTAGGTGAACCTGCGG	[29]
	ITS 4	R-TCCTCCGCTTATTGATATGC	
102 bp	SAP10-F	CAAAGTCCTGACACCATCCA	[8]
	SAP10-R	TTCGAACCGATCTCCAATTC	
213 bp	SAP9-F	TGGGTGTTATGCAACAATCG	
	SAP9-R	TGGTAGAGGTGCCAGATGAA	

Detection of *SAP9* and *SAP10* genes by PCR

The DNA that was obtained was utilized to amplify the SAP9 and SAP10 virulence genes by the PCR technique, employing oligonucleotide primers as indicated in Table 1. The DNA samples were amplified in a reaction mixture consisting of 2 µl genomic DNA, 2 µl forward primer, 2 µl reverse primer, 2 µl template DNA, 5 µl Master Mix, and 14 µl Nuclease free dH2O, with a total volume of 25 µl. The DNA was amplified using a PCR thermal cycler. The amplification process involved performing one cycle at a temperature of 95°C for a duration of 5 minutes, followed by 35 cycles according to the following protocol: The denaturation step lasts for 30 seconds at a temperature of 95°C, followed by an annealing step lasting 30 seconds at a temperature range of 50-55°C. Finally, there is a primer extension step lasting 10 seconds at a temperature of 72°C. After the previous cycle, a further incubation of 10 minutes at 72°C was performed to assure the full polymerization of any residual PCR products. The PCR amplification products were observed by electrophoresis on 1.5% agarose gels for a duration of 45 minutes at a voltage of 70 volts. The size of the amplicons was assessed by comparing them to the 100-3000 bp DNA ladder from Bioneer / Korea.

DNA sequencing of *ITS* and *SAP9* genes

The PCR products amplified with two primers were sequenced by Macrogen Company (South Korea). NCBI BLAST tool was used to check the results of this program, and to detect any DNA alterations in the sequences of the gene.

Statistical Analysis

Statistical analysis was performed on the present data using the SPSS (Statistical Package for the Social Sciences) software program with a p-value of less than 0.05, utilizing both descriptive and non-parametric and Descriptive Chi-Square.

Results

Isolation of *Candida species*

The results of samples culture on SDA and Malt Extract agar showed that 50 *Candida species* isolates were obtained from specimens of urine. The *Candida Spp.* isolated from patients where 49 urine samples of 108 patients were positive for culture with rate of 45.37%. In comparison, out of 42 samples (2.38% of the total) in the control group, just one tested positive for culture. to the extent shown in figure 1.

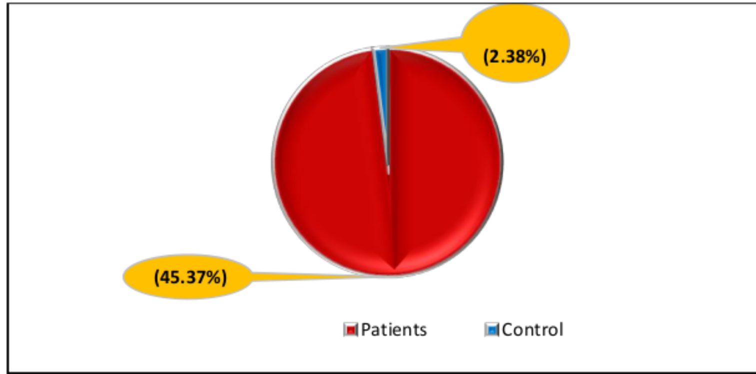

Figure 1: isolation of *Candida species* from patients and control groups.

Identification of *Candida Spp.*

Candida species were identified with microscopic examination as well as their characteristics on chromogenic agar. Most of them were *Candida albicans* (54.0%), *Candida krusei* (24.0%), *C. glabrata* (16.0%) and *C. tropicalis* (6.0%), the study recorded a significant difference between species of *Candida* isolated at p. value < 0.05 Figure 2.

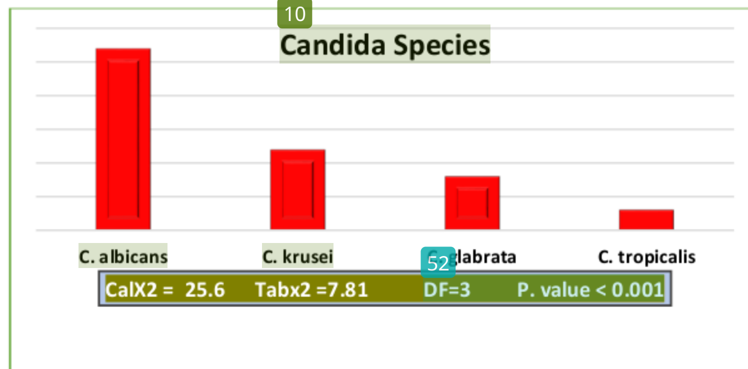

Figure 2: Identification of *Candida spp.* isolated from total urine samples in women with urinary tract infection and control group

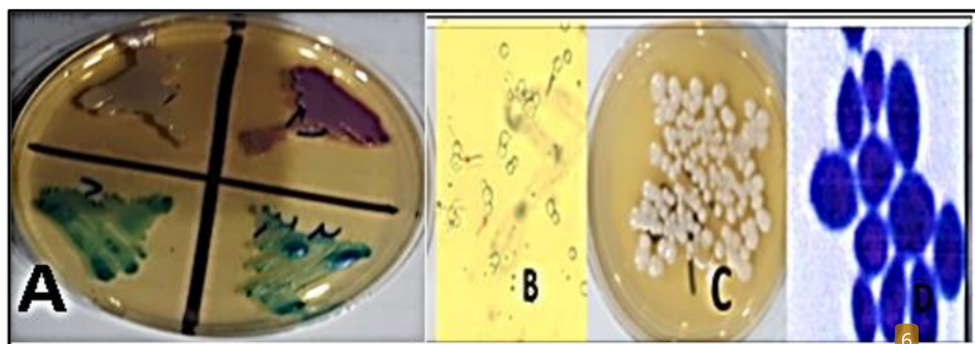

Figure 3: *Candida spp*. culture on chromogenic agar **A**, germ tube (40X) **B**, *Candida spp*. culture on Sabouraud dextrose agar **C**, *Candida spp*. isolates by using Gram stain (100 X) (D)

Virulence Factor Determination in *Candida species*

Ability of *Candida species* to Produce Protease Enzyme

This study recorded a significant difference at p. value < within same species of *Candida* to produce protease enzyme, were noted the 55.6% of *C. albicans* produce enzyme in strong statues, 50% of *C. krusei* produce enzyme in strong statues, 37.5% of *C. glabrata* produce enzyme in strong statues, while 33.3% of *C. tropicalis* produce enzyme, the results showed a significant difference at p. value < 0.05, between species of *Candida* and ability to produce enzyme as in table 2,figure 6.

Table 2: Ability of *Candida species* to produce protease enzyme.

Candida spp.	Negative 1		Weak 0.7- 0.99		Moderate 0.5-0.69		Strong < 0.5		Total		p. value
	No.	%	No.	%	No.	%	No.	%	No.	%	
C. albicans	1	3.7	0	0.0	11	40.7	15	55.6	27	54.0	< 0.001
C. krusei	2	16.7	1	8.3	3	25.0	6	50.0	12	24.0	< 0.001
C. glabrata	3	37.5	0	0.0	2	25.0	3	37.5	8	16.0	0.188
C. tropicalis	2	66.7	0	0.0	0	0.0	1	33.3	3	6.0	0.001
Total	8	16.0	1	2.0	16	32.0	25	50.0	50	100	
CalX^2 = 141.6 Tabx^2 =16.92 DF=9 P. value < 0.001											

Production of Hemolysin Enzyme

All isolates of *Candida species* produced hemolysin enzyme with the majority of isolates showed strong hemolysin activity isolates (82%), and (18%) revealed moderate activity, whereas no isolates showed weak and negative hemolytic activity respectively, the results indicated a significant difference at p. value < 0.05, within species and between species, with except *C. krusei* was not recorded a significant difference as Table 3, figure 6.

Table 3: Ability of Candida species to produce hemolysin enzyme

Candida spp.	Negative 1		Weak 0.7- 0.99		Moderate 0.5-0.69		Strong < 0.5		Total		p. value
	No.	%	No.	%	No.	%	No.	%	No.	%	
C. albicans	0	0.0	0	0.0	2	7.4	25	92.6	27	54.0	< 0.001
C. krusei	0	0.0	0	0.0	5	41.7	7	58.3	12	24.0	0.110

C. glabrata	0	0.0	0	0.0	1	12.5	7	87.5	8	16.0	< 0.001
C. tropicalis	0	0.0	0	0.0	1	33.3	2	66.7	3	6.0	0.001
Total	0	0.0	0	0.0	9	18.0	41	82.0	50	100	
$CalX^2 = 45.2$ Tabx^2 =7.81 DF=3 P. value < 0.001											

Antifungal Resistance of *Candida species*

The resistance of the isolated *Candida species* against seven selected antifungal drugs were found to Itraconazole (8 6%), Clotrimazole (76%), Fluconazole (72%), Voriconzole (58%), Ketoconazole (56%), Amphotercin-B (46%) , and Nystatin (22%) . This study recorded a significant difference at p. value < 0.05 between activity of antibiotics and species of *Candida* Table 4, figure 5,6.

Table 4: Antifungal susceptibility profile of *Candida species* in present stud

Antifungal	Resistant		Sensitive		Intermediate	
	No.	%	No.	%	No.	%
Itraconazole (10mcg)	43	86	6	12	1	2
Clotrimazole(10mcg)	38	76	9	18	3	6
Fluconazole(25mcg)	36	72	13	26	1	2
Voriconazole(1mcg)	29	58	16	32	5	10
Ketoconazole(10mcg)	28	56	20	40	2	4
Amphotericin-B(100 unite)	23	46	20	40	7	14
Nystatin(100 unite)	11	22	32	64	7	14
Susceptibility %	59.43		33.14		7.43	
CalX²= 60.93 TabX²= 21.03 DF= 12 p. value < 0.001						

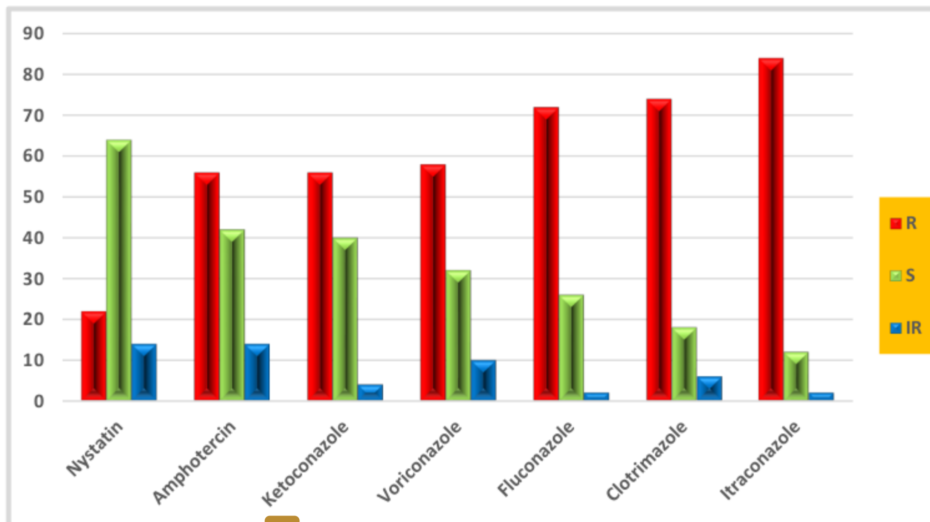

Figure5: **Percentage of antifungal susceptibility pattern of *Candida species* to seven antifungal**

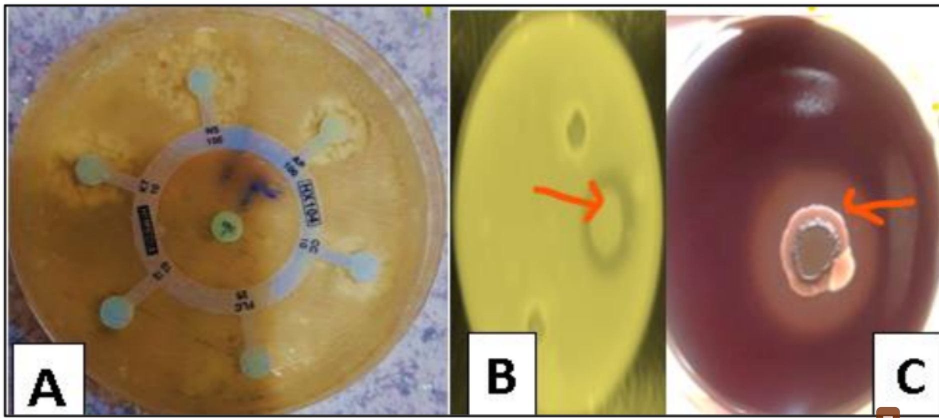

Figure 6: Antifungal susceptibility testing by disk diffusion method against 7 selected antibiotics of *Candida spp*. **A**, Ability of *Candida spp.* to produce proteinase **B**, and hemolysin enzyme **C**.

The frequency of virulence genes (*SAP9* and *SAP10*) among *Candida albicans*

The occurrence of the examined virulence genes in C. albicans isolates, specifically SAP9 (318bp) and SAP10 (102bp), was assessed using PCR product. The screening was conducted on an agarose gel, which was then stained with Safe Red stain (Biocat company) and visualized using a UV transilluminator. The gel electrophoresis results for the studied genes in Candida albicans isolates may be found in Figure 7 and Figure 8. In the subsequent stage, to verify the presence of the analyses genes, the purified PCR products were subjected to sequencing. The sequencing results corroborate the presence of the studied gene. Our study findings suggest that among *Candida albicans*, the frequency of SAP9 and SAP10 genes is 62.69% and 100%, respectively (Table 5).

Table 5: Frequency *SAP9* and *SAP10* genes in 27 *Candida albicans* isolates using PCR.

Genes	Frequency	%
SAP9	17	62.69%
SAP10	27	100%

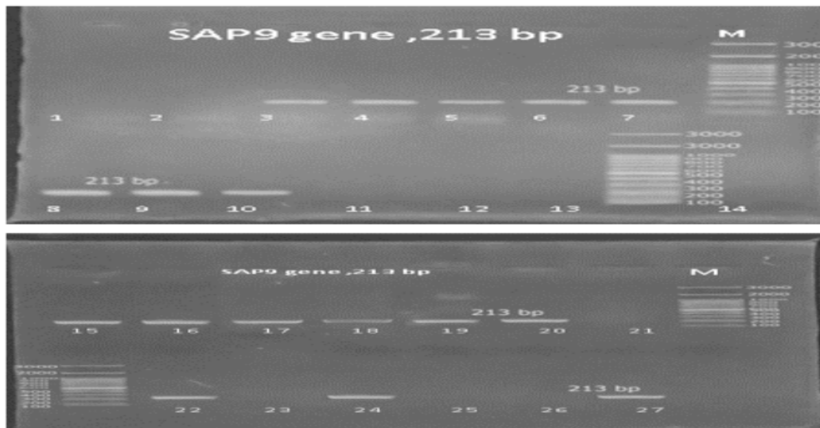

Figure 7: Gel electrophoresis for amplified PCR product of (*SAP9* gene). which show product size 213bp after electrophoresis in agarose 1.5%, TBE buffer (1X), stained with Safe Red stain (Biocat company). Agarose gel electrophoresis at 70-volt, 85 mA) for 40 min for PCR product visualized under U. V light after staining with safe red stain. Lane L: DNA ladder (3000-100) bp, Lanes (3-4-5 -6-7-M -8-9-10--M-14-15-16-17-18-19-20,22-24-27) represented positive results, while Lane (1 ,2, 11, 12,13, 21,23, 25, 26) represented Negative result.

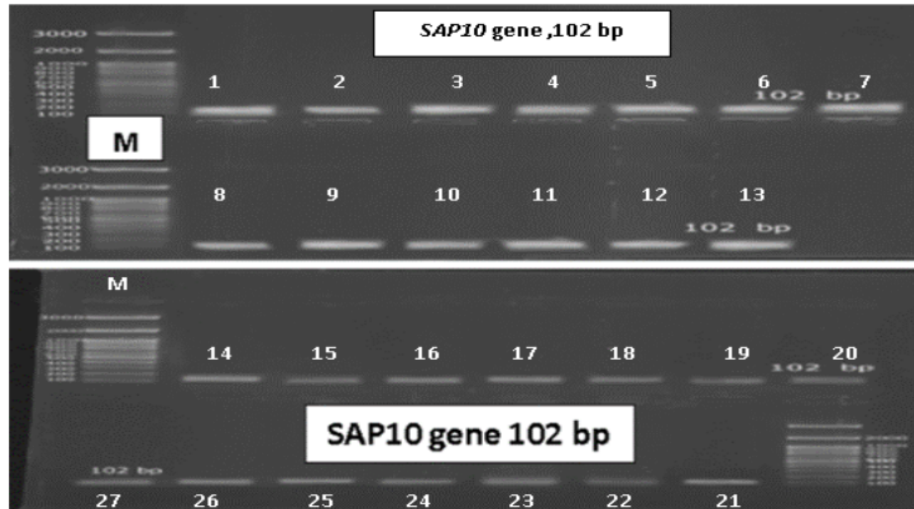

Figure 8: Agarose gel electrophoresis image that show the PCR product analysis of ***SAP10*** gene in Candida albicans isolates. Where Marker ladder (3000-100bp), lane (1-27) represented positive Candida albicans isolate *SAP10* gene at **102 bp**.

Discussion

Candida species are fungi that often do not cause disease but can become harmful in people with weakened immune systems or an imbalance of competing bacteria [30]. *Candida albicans* is the predominant causative agent of fungal infections, however, there has been a rise in the prevalence of non-*Candida albicans* species that exhibit varying levels of virulence and resistance to antifungal treatments [27]. The current study aimed to identify the prevalence of various Candida species isolates and their distribution among women with urinary tract infections (UTIs) using PCR for identification. Additionally, the study examined the expression of virulence factors by different Candida species. This study collected 50 isolates of Candida species from urine specimens. The overall proportion of Candida isolates was 33.33%, which were obtained from both patients and the control group. The findings revealed that the prevalence of Candida species in patients was 45.37%, whereas in the control group it was 2.38%. Our findings align with the results reported by Zafar et al in Pakistan [27] and are also consistent with those of [31]. The current findings contradict the results of prior research conducted by [32,33]. The variation in the recovery rate of Candida species from patients with urinary tract infections in different studies can be attributed to population and regional differences, as there are several factors that contribute to the formation of urinary tract infections [34]. In this study, it was found that *C. albicans* is

usually the most common species isolated from urinary tract infections, compared to non-albicans species. Specifically, the prevalence of *C. albicans* was 54%, while non-albicans species accounted for 46% of the cases. These results are like the findings reported in reference [35]. In contrast, several investigations have indicated a higher frequency of non-albicans [36,37]. This investigation successfully isolated many species of non-*C. albicans*, including Cain isolating (24%), Candida glabrata (16%), and Candida tropicalis (6%). This conclusion is in line with recent research [35,38] that has demonstrated the occurrence of candiduric resulting from several Candida species [39].

The development of Candida species is strongly linked to the generation of virulence factors such as proteinase, hemolysin, lipase, phospholipase, and esterase [40]. The virulence characteristics of Candida species can differ based on the type of infection, specific species, geographic location, site of infection, and the reaction of the host [41]. Identifying Candida virulence factors can aid in the development of new drugs to enhance innovative therapeutic strategies [42]. The current investigation found that most Candida species isolates generate hemolysin with varying scores, while proteinase synthesis was seen in 84% of Candida isolates. Our findings corroborated various research documented by [43]. All clinical isolates of Candida species exhibited a hemolysin activity of 100%. These findings closely align with those of [42], who reported a hemolytic activity of 96%. The current analysis revealed proteinase activity in 84% of Candida spp. isolates, which is consistent with the findings of previous studies [44,45].

The occurrence of multidrug resistance (MDR) in fungal pathogens is a significant issue in the management of invasive Candida infections and presents a significant danger by restricting the availability of antifungal medications [46]. The present study conducted in-vitro susceptibility testing of Candida strains using the disc diffusion method. The results revealed that all isolated Candida species exhibited resistance to the seven selected antifungal drugs. These findings indicate that Nystatin and Amphotericin B were the most efficacious antifungal agents against Candida species. The prevalence of resistance was highest for Itraconazole, Clotrimazole, and Fluconazole. These findings are consistent with the investigations conducted by researchers [37,47,48]. However, the results demonstrated that Clotrimazole was not efficient in suppressing most isolated Candida species, which contradicts the findings of [49]. These investigations reveal high levels of resistance to itraconazole (86%), fluconazole (72%), and voriconazole (58%). The findings are consistent with the results reported in references [33, 50], but contradict the findings in references [51, 52]. The study found that fluconazole represented 16.21% of the instances, whereas voriconazole accounted for 13.15%. The susceptibility of Candida species to several antifungal medications, including azoles and polyenes such as Nystatin and Amphotericin-B, exhibited variability among different investigations. The cellular and molecular mechanisms that contribute to resistance against antifungal chemicals vary based on the specific mode of action of the antifungal medications [53]. Furthermore, the resistance was likely caused by a reduction or complete lack of detectable ergosterol in the cell membranes [54]. The resistance of Candida spp. to azole antifungal drugs may be caused by mitochondrial malfunction and the presence of Candida drug resistance genes [33].

Candida albicans utilizes various pathogenic mechanisms to avoid the host's immune response and facilitate tissue invasion, ultimately causing infection. One such mechanism involves the production of secretory aspartyl proteinases [55]. Secreted aspartic proteases (Sap) are enzymes that are produced by a group of genes known as the SAP gene family. In the current study, the prevalence virulence genes in *Candida albicans*, *Sap9* gene was detected (62.69%) and *Sap10* gene was detected (100%) isolates. This result agreed with previous study in Egypt [14]. Another study in India established by Das *et al* revealed higher frequency for SAP9 (100%), [8,56]. Ahmed et al. [57] discovered varying prevalence rates for the SAP9 and SAP10 genes, with a lower frequency of 27.7% and 12.9% respectively [25]. The variation in the prevalence rates of virulence genes can be attributable to other factors, such as the number of isolates examined and the variation in the places where C. albicans was isolated [52]. Sap9 is mostly found in the cell membrane, while Sap10 is found in both the cell wall and membrane [8]. Unlike, other members of the Saps family, the Saps 9-10 proteases, when seen in both laboratory and living circumstances, are not influenced by pH or morphotype [58]. Sap 9 and Sap10 exert their effects on specific cell wall activities by the enzymatic cleavage of covalently attached cell wall proteins. These proteins play a crucial role in biofilm formation, as well as in facilitating the attachment to host cells and invasion into epithelial cell layers [14,59,60].

Conclusions

The current study emphasizes the frequency of Candida species in women with urinary tract infections (UTIs). Our findings indicate that Candida albicans was the most commonly occurring fungal species. Furthermore, the elevated levels of expression of both virulence factors containing SAP genes indicate a heightened level of pathogenicity in Candida. The significance of these findings lies in their implications for managing invasive fungal infections, which can provide a significant challenge for women.

Acknowledgment

We would like to thank all of the investigators and supervisors who participated in the reflection's writing our manuscript.

Ethical Consideration

To ensure ethical research practices, explicit consent was acquired from the hospital and all parties involved in this study. The patient selection process was carried out in collaboration with gynecologists from both the hospital and private clinics in Thi-Qar Governorate.

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