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Molecular identification, virulence gene profiling, and antifungal susceptibility of *Candida albicans* isolates from women with urinary tract infections

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Abstract. *Candida species are increasingly recognized as causative agents of urinary tract infections (UTIs), particularly in immunocompromised individuals. Among them, Candida albicans is the most prevalent and exhibits virulence factors that enhance adhesion, biofilm formation, and antifungal resistance. This study investigates the molecular identification, antifungal resistance profiles, and virulence gene prevalence (ALS1, ALS3, HWP1) in C. albicans isolates from women with UTIs in Thi-Qar Province, Iraq.*

Methods. A total of 150 urine samples were collected from women with UTIs and control groups. Candida species were isolated on Sabouraud Dextrose Agar and identified using phenotypic (Gram staining, germ tube test, CHROM agar) and molecular methods (PCR using ITS1 and ITS4 primers). Antifungal susceptibility testing was performed against seven antifungal agents using the disc diffusion method. Virulence genes (ALS1, ALS3, HWP1) were detected via PCR, and sequencing was conducted for ALS1 and ALS3 genes to assess genetic variation.

Results. C. albicans was the most frequently isolated species (54%), followed by C. krusei (24%), C. glabrata (16%), and C. tropicalis (6%). Antifungal resistance was highest against itraconazole (96.3%), fluconazole (88.9%), and voriconazole (85.2%), whereas amphotericin B (29.6%) and nystatin (18.5%) exhibited the lowest resistance rates. PCR analysis revealed high prevalence rates for virulence genes: HWP1 (96.3%), ALS1 (88.8%), and ALS3 (77.7%). DNA sequencing confirmed the presence of genetic diversity among isolates.

Conclusion. The study highlights the significant role of C. albicans in UTIs and its increasing resistance to azole antifungals. The high prevalence of virulence genes suggests a strong pathogenic potential, emphasizing the need for effective antifungal stewardship and molecular surveillance to manage Candida infections in clinical settings.

Key words: *Candida albicans, urinary tract infection, antifungal resistance, virulence genes, identification.*

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Молекулярна ідентифікація, профілювання генів вірулентності та чутливість до протигрибкових засобів ізолятів *Candida albicans*, виділених у жінок із інфекцією сечової системи

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Резюме. *Види Candida дедалі частіше визначаються як збудники інфекцій сечової системи (ІСС), особливо у імунокомпрометованих осіб. Серед них Candida albicans є найпоширенішим видом і проявляє фактори вірулентності, що сприяють адгезії, утворенню біоплівок та протигрибковій резистентності. Це дослідження присвячене молекулярній ідентифікації, профілю резистентності до протигрибкових засобів та поширеності генів вірулентності (ALS1, ALS3, HWP1) серед ізолятів C. albicans, виділених у жінок із ІСС у провінції Ді-Кар, Ірак.*

Методи. Було досліджено 150 зразків сечі жінок із ІСШ та контрольної групи. Види Candida ізольовано на агарі Сабуро та ідентифіковано за допомогою фенотипових методів (фарбування за Грамом, CHROM-агар) і молекулярних методів (ПЛР із використанням праймерів ITS1 та ITS4). Чутливість до протигрибкових засобів визначали методом дискового дифузійного тесту проти семи антимікотиків. Гени вірулентності (ALS1, ALS3, HWP1) виявляли за допомогою ПЛР, а секвенування генів ALS1 та ALS3 проводили для оцінки генетичних варіацій.

Результати. Найчастіше визначали C. albicans (54%), C. krusei (24%), C. glabrata (16%) та C. tropicalis (6%). Найвищий рівень резистентності спостерігався до ітраконазолу (96,3%), флуконазолу (88,9%) та вориконазолу (85,2%), тоді як амфотерицин В (29,6%) і ністатин (18,5%) мали найнижчі показники резистентності. Аналіз ПЛР показав високу поширеність генів вірулентності: HWP1 (96,3%), ALS1 (88,8%) і ALS3 (77,7%). Секвенування ДНК підтвердило наявність генетичного поліморфізму серед ізолятів.

Висновки. Дослідження підкреслює значну роль C. albicans у розвитку ІСС та її зростаючу резистентність до азольних антимікотиків. Висока поширеність генів вірулентності свідчить про значний патогенний потенціал цього збудника, що підкреслює необхідність ефективного протигрибкового контролю та молекулярного моніторингу для оптимального лікування Candida-асоційованих ІСС в клінічній практиці.

Ключові слова: *Candida albicans, інфекції сечової системи, протигрибкова резистентність, гени вірулентності, ідентифікація.*

Introduction. Candida species are an uncommon cause of urinary tract infections (UTIs) in healthy individuals but are frequently found in hospital settings or among patients with predisposing illnesses and anatomical abnormalities of the urinary system [**1, 2**]. UTIs are inflammatory disorders caused by the abnormal presence and proliferation of microorganisms in the urinary system, and they represent the most common infection across all age groups [**3**]. Community-acquired UTIs are a frequent problem affecting both genders, but women are more susceptible due to differences in urogenital and reproductive anatomy, the proximity of the urethra to the gastrointestinal opening, physiology, and lifestyle [**4-6**]. The manifestation of a UTI varies depending on the specific pathogen involved, the extent of the illness, and the immune response of the affected individual [**7**]. Clinically, UTI symptoms include dysuria, hematuria, fever, chills, flank pain, and bacteremia, which can lead to serious complications such as hypertension, septicemia, and death [**8, 9**].

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The incidence of fungal UTIs caused by Candida species (candiduria) has increased significantly by two to three times in recent years, along with an increase in resistance to antifungal drugs used to treat them [**10-12**]. Candida species, particularly *Candida albicans*, are considered an important part of the normal vaginal microflora of most healthy women, colonizing the external genitalia in premenopausal and healthy females [**13, 14**]. In immune-deficient conditions, Candida may convert into an opportunistic pathogen, leading to Candida urinary tract infections (UTIs) in the host [**15**]. *Candida albicans* has become a significant health concern in immunocompromised patients and can cause a wide variety of mucosal and systemic infections [**16**].

Many virulence factors encoded by *C. albicans* genes play a central role in its pathogenicity, facilitating its invasion into host tissues and leading to infections [**17**]. Other factors include adherence to different tissues, biofilm formation, phenotypic switching, dimorphism, and hydrolytic enzyme production [**18**].

Secreted aspartyl proteinases, agglutinin-like sequence (ALS) genes, hyphal wall protein (HWP), and cell wall glycoproteins (adhesions) mediate adherence to various targets, including other microorganisms' cells, abiotic surfaces, and different host cells. This is a critical step in biofilm production and the development of infection [15, 19].

In *C. albicans*, the agglutinin-like sequence (ALS) gene family is the largest known gene family and is considered one of the significant traits in adhesion and biofilm formation [20]. The ALS gene family consists of eight genes, including ALS1-ALS7 and ALS9, which encode large glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins important for the production of cell surface glycoproteins, leading to increased adhesion to host cells [21, 22]. ALS1 and ALS3 genes have similar sequences and functions that mediate attachment to epithelial and endothelial cells and also play an important role in hyphal formation [23-26].

Another protein that affects adhesion and regulation during biofilm formation in *C. albicans* is hyphal wall protein (HWP1), produced by the HWP1 gene present in the hyphae [27, 28]. HWP1 is a mannoprotein-linked glycosyl-phosphatidylinositol that resembles the agglutinin-like sequence (ALS) proteins encoded by the ALS family of genes [29, 30]. Additionally, it is the substrate for transglutaminase activity derived from the host and plays a major role in contributing to the covalent attachment of *Candida albicans* to several tissue and host cell surfaces [29-33]. Several studies have shown that the HWP1-producing gene is highly expressed in the early stages of biofilm formation [20]. Therefore, hyphal wall protein 1 (HWP1) has shown a significant association with the pathogenicity and virulence of *C. albicans* [30].

The presence of virulence genes and the increasing prevalence of resistance to antifungal agents contribute to the pathogenicity of *Candida albicans* [34]. Antifungal resistance is rising due to empirical management, the overuse of selective therapies, and the frequent use of antifungal agents for fungal infection prophylaxis in humans [35]. The relationship between virulence genes and the resistance profiles of antifungal therapies in *Candida albicans* has not been adequately investigated; new studies are still needed on these issues [32, 36].

Many studies in Iraq focus on the role of pathogenic bacteria in the occurrence of UTIs, with only a few addressing the role of *C. albicans*. Therefore, our study aims to investigate the molecular identification, antifungal resistance profiles, and the prevalence of virulence genes (ALS1, ALS3, HWP1) in *C. albicans* isolates from women with UTIs in Thi-Qar Province, Iraq.

Materials and methods. Ethical approval. Ethical approval for this study was obtained from the Institutional Review Board (IRB) and the Ethics Committee of the Department of Biology, College of Science, University of Thi-Qar (approval number 3/11/29, dated 08/01/2023). Permission for patient participation was granted by the respective hospitals, with patient selection facilitated by gynecologists at Al Nasiriyah Teaching Hospital, Bint Al Huda Hospital, and private clinics in Thi-Qar Governorate, Iraq.

Sample collection. A total of 150 urine samples were collected from women (both patients and healthy controls) aged 15-60 years attending Al Nasiriyah Teaching Hospital, Bint Al Huda Hospital, and private clinics in Thi-Qar Province, southern Iraq, between December 2022 and March 2023. The samples were transported to the Microbiology Laboratory in sterile containers and cultured within 3 hours of collection.

Isolation and identification of Candida species. Urine samples were inoculated onto Sabouraud Dextrose Agar (SDA) and Malt Extract Agar (MEA) supplemented with chloramphenicol to inhibit bacterial growth. Candida species colonies were purified by subculturing on SDA and incubated at 30°C for 48-72 hours. Identification of Candida isolates was performed based on colony morphology, Gram staining, and the germ tube test in serum. Further confirmation was achieved by subculturing on CHROM Agar Candida (CONDA, Spain), where colonies were identified based on their characteristic color, as per the manufacturer's guidelines, after 72 hours of incubation [37].

Antifungal susceptibility testing by disk diffusion method. Antifungal susceptibility testing was performed on all *Candida albicans* isolates using the disk diffusion method on Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/mL of methylene blue. The following antifungal agents were tested: Amphotericin B, Clotrimazole, Itraconazole, Voriconazole, Fluconazole, Ketoconazole, and Nystatin. The methodology followed the Clinical and Laboratory Standards Institute (CLSI) guidelines (2019) for antifungal resistance testing [38].

Control strains. The standard *C. albicans* strain (ATCC 10231) was used as a quality control for antifungal susceptibility testing.

Molecular study of isolated C. albicans isolates DNA extraction. Genomic DNA was extracted from the *C. albicans* isolates using the Geneaid Genomic DNA Extraction Kit (Geneaid, Taiwan) according to the manufacturer's instructions. DNA quality was assessed by running the samples on a 1.5% agarose gel.

Molecular identification. Conventional PCR was employed for molecular identification of *C. albicans* using specific primers targeting the Internal Transcribed Spacer (ITS1, ITS4) regions of the rDNA. PCR conditions were as described by previous studies [36], and the primers used are listed in Table 1.

Table 1

Primer sequences and PCR product sizes for molecular study of *C. albicans* isolates

Gene	Primer Sequence (5′–3′)	Product Size (bp)	Reference
ITS1	TCCGTAGGTGAACCTGCGG	500	[40]
ITS4	TCCTCCGCTTATTGATATGC		
HWP1-F	ATGACTCCAGCTGGTT	572	[32]
HWP1-R	TAGATCAAGAATGCGC		
ALS3-F	CCAAGTGTTCCAACAACTGAA	185	[20]
ALS3-R	GAACCGGTTGTTGCTATGGT		
ALS1-F	GACTAGTGAACCAACAAATACCAGA	318	[36]
ALS1-R	CCAGAAGAAACAGCAGGTGA		

PCR Amplification Procedure. The PCR amplification was conducted using a thermal cycler with the program outlined in Table 2.

Table 2

PCR amplification program

Cycle	Time	Temperature	Step
1	5 min	95°C	Initial Denaturation
35	30 sec	95°C	Denaturation
	30 sec	50-58°C	Annealing (specific temperatures for each primer: 58°C for ITS, 52°C for HWP1, 55°C for ALS3, 50°C for ALS1)
	1 min	72°C	Extension
1	10 min	72°C	Final Extension

Validation of molecular assays. Twenty-five Candida isolates were confirmed by sequencing and recorded in the NCBI database (Accession Numbers LC791623 to LC791648). Results were verified using the NCBI BLAST tool to detect any sequence alterations.

Detection of virulence genes (HWP1, ALS1, ALS3). PCR amplification was conducted to detect the virulence genes HWP1, ALS1, and ALS3 using the primers listed in Table 1. PCR reactions were performed in a 25 µL mixture containing 2 µL genomic DNA, 2 µL each forward and reverse primers, 5 µL Master Mix, and nuclease-free water. The amplification protocol involved an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 30 seconds at 95°C for denaturation, 30 seconds of annealing at temperatures ranging from 50°C to 55°C, and a 10-second extension at 72°C. A final extension step was performed at 72°C for 10 minutes. The PCR products were visualized using 1.5% agarose gel electrophoresis at 70V for 45 minutes, with sizes compared against a 100-3000 bp DNA ladder (Bioneer, South Korea).

DNA sequencing of ALS1 and ALS3 virulence genes. The PCR products for ALS1 and ALS3 were sent to Macrogen Inc. (South Korea) for sequencing. Sequencing results were analyzed using NCBI BLAST for alignment and comparison to reference sequences.

Statistical analysis. Statistical analyses were performed using SPSS software (version 26). Data were presented as frequencies and percentages. Chi-square tests and independent sample t-tests were used to assess associations, with a significance level set at $p < 0.05$.

Results. Identification of *Candida albicans* isolates. A total of 50 Candida species isolates were obtained from 150 urine samples, representing four species within the Candida genus. The most prevalent species was *Candida albicans* (54%), followed by Candida krusei (24%), Candida glabrata (16%), and Candida tropicalis (6%). Of the *Candida albicans* isolates, 27 clinical isolates were identified using the germ tube test, Gram staining, and chromogenic agar, and confirmed by PCR amplification of the ITS1 and ITS4 regions. A significant difference in the distribution of Candida species was observed ($p < 0.05$) (Fig. 1).

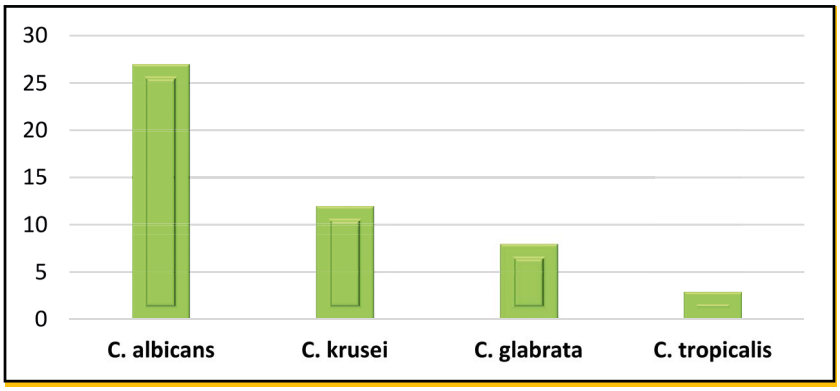

Fig. 1. Frequency of *Candida albicans* isolated from total urine samples.

Antifungal resistance of *Candida albicans*. Resistance patterns of *Candida albicans* isolates to seven selected antifungal agents were assessed. The highest resistance was observed to Itraconazole (96.3%), Fluconazole (88.9%), Voriconazole (85.2%), Clotrimazole (85.2%), Ketoconazole (77.8%), Amphotericin B (29.6%), and Nystatin (18.5%). Statistical analysis revealed significant differences in antifungal resistance ($p < 0.05$) (Table 3, Fig. 2).

Table 3

Antifungal resistance patterns of *Candida albicans* in the present study

Antifungal agent	Susceptible	Intermediate	Resistant	P-value
Voriconazole	4 (14.8%)	0	23 (85.2%)	<0.001
Amphotericin B	12 (44.4%)	7 (25.9%)	8 (29.6%)	<0.001
Clotrimazole	3 (11.1%)	1 (3.7%)	23 (85.2%)	<0.001
Fluconazole	3 (11.1%)	0	24 (88.9%)	<0.001
Itraconazole	1 (3.7%)	0	26 (96.3%)	<0.001
Ketoconazole	4 (14.8%)	2 (7.4%)	21 (77.8%)	<0.001
Nystatin	19 (70.4%)	3 (11.1%)	5 (18.5%)	<0.001

Fig. 2. A. *Candida albicans* on Candida chromogenic agar. B. Antifungal susceptibility pattern of *Candida albicans*.

Frequency of virulence genes in *Candida albicans*. The frequency of virulence genes, including HWP1, ALS1, and ALS3, was assessed in 27 *Candida albicans* isolates using PCR. Gel electrophoresis showed the presence of the following genes: HWP1 (96.29%), ALS1 (88.88%), and ALS3 (77.77%). The most frequently detected gene was HWP1, followed by ALS1, while ALS3 was the least frequent. The prevalence of these genes in *Candida albicans* isolates is shown in Table 4, and representative gel images are presented in Figures 3-5.

Table 4

Prevalence of virulence genes in *Candida albicans* isolates by PCR

Gene Type	Positive	Negative	t-test P-Value
ALS1	24 (88.88%)	3 (11.11%)	0.273
ALS3	21 (77.77%)	6 (22.22%)	0.852
HWP1	26 (96.29%)	1 (3.70%)	0.059

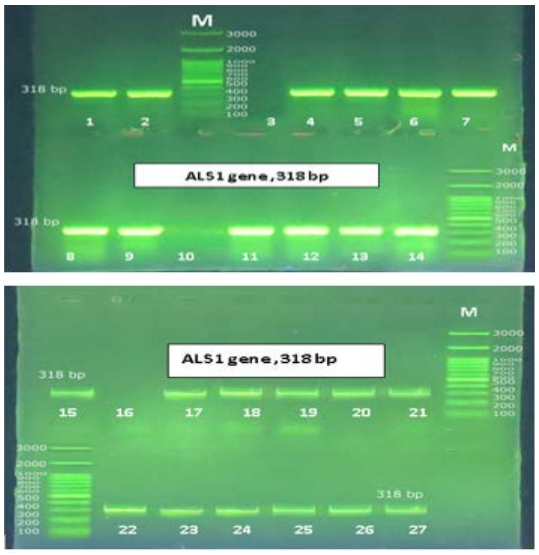

Fig. 3. Agarose gel electrophoresis of PCR products for ALS1 gene amplification (318 bp). Lane L: DNA ladder (3000-100 bp); lanes 1-15, 17-21, and 22-27 show positive results, while lanes 3, 10, and 16 show negative results.

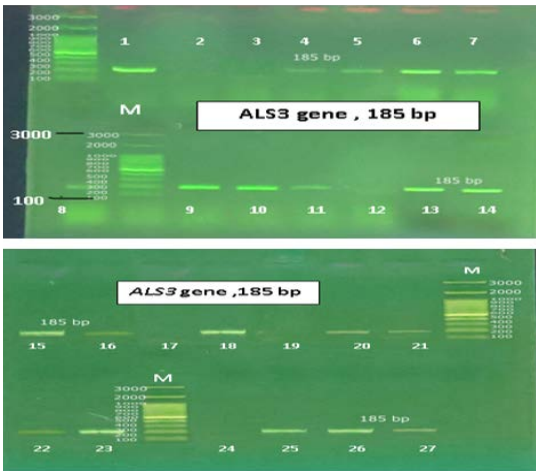

Fig. 4. Agarose gel electrophoresis showing PCR product analysis of the ALS3 gene (185 bp) in *Candida albicans* isolates. Lane L: DNA ladder (3000-100 bp); lanes 1-27 show positive results for the ALS3 gene.

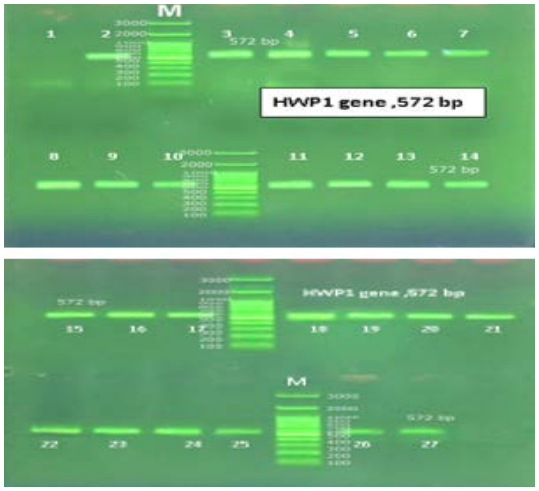

Fig. 5. Agarose gel electrophoresis showing PCR product analysis of the HWP1 gene (572 bp) in *Candida albicans* isolates. Lane L: DNA ladder (3000-100 bp); lanes 1-27 show positive results for the HWP1 gene.

Sequencing and alignment of *Candida albicans* genes. The nucleotide sequences of the ALS1, ALS3, and ITS regions were aligned using BioEdit software (v. 7.2.5) and compared to available sequences in the NCBI GeneBank using the BLAST tool. Phylogenetic trees were constructed for the ALS1 and ALS3 genes using Mega X software. Sequencing of the ITS region confirmed the identification of *C. albicans* isolates, while the sequencing results for ALS1 and ALS3 genes revealed genetic diversity within the isolates.

Discussion. *Candida albicans* has emerged as a significant nosocomial uropathogen, with morbidity and mortality often linked to the presence of virulence genes and antifungal resistance [36]. In the present study, *Candida albicans* was the most prevalent species, accounting for 54% of the isolates, followed by Candida krusei at 24%, Candida glabrata at 16%, and Candida tropicalis at 6%. These findings align with a study in Iraq, which also found *Candida albicans* to be dominant over other species [37]. However, they contrast with other studies that reported non-albicans Candida species as the more frequently isolated from urine samples [41]. The recovery rate of Candida species from urinary tract infection (UTI) patients can vary across studies, likely due to regional and population-specific factors, as multiple variables contribute to the development of UTIs [4].

Antifungal susceptibility testing is a crucial tool for determining antifungal resistance and guiding appropriate therapy for fungal infections [42]. The emergence of multidrug-resistant (MDR) strains of Candida presents a significant challenge in the treatment of invasive infections, as it limits available antifungal treatment options [43]. Prolonged use of antifungals, especially in the treatment of *Candida albicans* infections, has led to the development of resistance [44]. In our study, in vitro antifungal susceptibility testing using the disc diffusion method revealed resistance of *Candida albicans* isolates to seven selected antifungal agents: Itraconazole, Clotrimazole, Fluconazole, Voriconazole, Ketoconazole, Amphotericin B, and Nystatin. Our results demonstrated that Nystatin and Amphotericin B were the most effective agents, while resistance was most commonly observed to Itraconazole, Clotrimazole, and Fluconazole. These findings are consistent with studies by [45, 46], but contradict others [47-49]. The resistance of *Candida albicans* to antifungal drugs, including azoles and polyenes (e.g., Nystatin and Amphotericin B), varies in different studies. The underlying cellular and molecular mechanisms of antifungal resistance may differ depending on the drug's mode of action [50]. For example, resistance may be linked to a decrease in ergosterol content in the fungal cell membrane. Additionally, resistance to azole antifungals may result from mitochondrial dysfunction and the presence of specific Candida drug resistance genes [18].

The pathogenicity of *Candida albicans* is enhanced by its ability to produce hyphae, adhere to host cells, produce extracellular enzymes, and form biofilms, which allow the fungus to evade host immunity and fa-

cilitate tissue invasion [51, 52]. In this study, the ALS1, ALS3, and HWP1 genes, which are critical for adhesion to epithelial cells, mucosal surfaces, and biofilm formation, were examined. These genes also play a role in the fungus's resistance to antifungal agents such as Fluconazole [53]. Our study revealed that the most frequently detected virulence gene was HWP1 (96.29%), followed by ALS1 (88.88%) and ALS3 (77.77%). A similar study in Iran reported a higher prevalence of HWP1 (96%), ALS3 (94%), and ALS1 (92%) [19], while another study found different prevalence rates: HWP1 (89.4%), ALS3 (91.5%), and ALS1 (78.7%) [20]. In Iraq, a previous study reported a higher prevalence of HWP1 (100%) but a lower prevalence of ALS1 (45.71%) [48]. The variations in the prevalence of these virulence genes can be attributed to differences in the number of isolates studied and the isolation sites of *C. albicans* [54].

The presence of ALS1, ALS3, and HWP1 genes in *Candida albicans* not only facilitates the transition of Candida from a commensal to a pathogenic organism but also plays a critical role in the formation of biofilms, which may hinder treatment efficacy. These genes also enhance the production of fungal hyphae, which enable tissue penetration and infection. Thus, the detection of these genes is valuable for accurate diagnosis and the selection of appropriate treatments.

In terms of patient management and public health, increased attention to the presence of *Candida albicans* in urine is necessary. If left untreated, infections may progress from acute to chronic forms, lead to ureteral obstruction due to fungal ball formation, or progress to candidemia, which increases the economic burden on healthcare systems.

Our study has several limitations. First, the sample size of 150 urine samples, although providing valuable insights, may not fully represent the diversity of Candida species and their antifungal resistance patterns in the broader population. Second, the study was conducted in a single geographic region, Thi-Qar Province, which limits the generalizability of our findings to other regions with different demographic and environmental factors. Third, while phenotypic and molecular methods were used for identification and resistance testing, some species of Candida may not have been detected due to limitations in the diagnostic methods, particularly for less common or atypical Candida species. Additionally, the study focused primarily on the prevalence of three virulence genes (ALS1, ALS3, and HWP1), but other important virulence factors were not included in the analysis, potentially overlooking other pathogenic mechanisms. Finally, the antimicrobial resistance testing was performed using the disc diffusion method, which may not be as precise as other techniques, such as broth microdilution, for determining minimal inhibitory concentrations (MICs). Future studies should address these limitations by including larger and more diverse populations, exploring additional virulence factors, and utilizing more advanced diagnostic techniques.

Conclusions. The study highlights the prevalence of antifungal resistance in *Candida albicans*, as well as the high expression rates of virulence genes, reflecting the high pathogenicity of this species. The study found *Candida albicans* to be the most frequent Candida species, with clinical isolates identified by germ tube testing, Gram staining, chromogenic agar, and PCR. The isolates exhibited significant resistance to all selected antifungal agents. The frequency of ALS1, ALS3, and HWP1 genes among *Candida albicans* isolates was found to be high, with HWP1 being the most commonly detected gene and ALS3 the least prevalent.

Further molecular sequencing studies of ALS1 and HWP1 genes should be conducted to explore their potential for identifying *C. albicans* isolates. Additionally, gene expression studies of these virulence genes using real-time PCR should be performed. Greater attention should be paid to *Candida* in urine samples, and antifungal treatment should not be overlooked, as infections can progress from acute to chronic forms, cause ureteral obstruction, or lead to candidemia.

Conflict of interest. The authors declare no conflict of interest.

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Contributions.

Muna F. Jihad: Conceptualization, data curation, investigation, methodology, project administration, resources, software, validation, visualization, writing – original draft, writing – review & editing.

Manal B. Salih: Conceptualization, data curation, investigation, methodology, project administration, resources, supervision, writing – review & editing.

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