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Original Article

Molecular Characterization of Biofilm-related Virulence and Resistance genes in *Candida albicans* Isolates from Women with Vulvovaginitis

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Abstract: One of the most prevalent reasons for gynecologic consultations is vulvovaginitis (VV), particularly vulvovaginal candidiasis (VVC). The etiology of VVC mostly associated with *Candida albicans* (*C. albicans*). The recurrence of VVs and the development of resistance to antimicrobials, along with efforts to find therapeutic alternatives are of paramount importance. Thus, this study aims to find the prevalence *C. albicans* virulence, resistance genes in addition to its susceptibility to antifungals. In this case control study, a total of 125 high vaginal cotton swabs attained in duplicate. from 100 wome clinically diagnosed with VVC and 25 controls (non-VVC). *C. albicans* was isolated with Hicrome differential agar and confirmed with species-specific primers using Polymerase chain reaction. Genes of the studied virulence determinants, *Aglutinin-Like-Sequence* (*ALS1*, *ALS3*), *Hyphal Wall Protein1* (*HWP1*) as well as resistance determinants associated such as multidrug-resistance (*MDR1*) and *Candida drug resistance* (*CDR1*, *CDR2*) were also tested. The prevalence of *Candida* species were 70% and 32% in case and control groups, respectively. Further, the frequency of *C. albicans* were 88.57% (case group) and 100% (control group). The most common virulence gene was *ALS3*, present in 96.7% of case group and 87.5% of control group. Additionally, the results indicated that 98.39% of case group and 100% of control group exhibited *MDR1* and *CDR2* from confirmed isolates of *C. albicans*. Lastly, the result showed the highest antifungal resistance rates in case group were against voriconazole (70.97%) and fluconazole (40.32%), whereas in control the antifungal resistance was 75% for both voriconazole and fluconazole. In conclusion, high rate of virulence and resistance genes amongst women with VVC and therefore, the study suggests the importance of these genes to be targeted in new antifungal drugs.

1. Introduction

The most prevalent infection of the female reproductive system during reproductive age is vulvovaginitis (VV), which is caused by a wide variety of pathogenic microorganisms. The majority of vaginal infections are bacterial vaginosis (BV), candidiasis and trichomoniasis. Vulvovaginal candidiasis (VVC) which is one of the most prevalent causes of vulvovaginal discharge and discomfort worldwide. VVC affects 75% of the women over the lifetime, particularly during their childbearing years [1–5]. Although the majority of VVCs have no symptoms, some women with vaginal *Candida* colonization suffer from itching as the most common defining symptom to VVC. Furthermore, some patients may complain of vaginal irritation, edema, dyspareunia, dysuria, or an increase in discharge [6].

Candida (C.) albicans, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* are the most identified *Candida* species associated with VVC women, of which *C. albicans* is the most prevalent species isolated and identified[2].

C. albicans is able to shift between a commensal and an opportunistic pathogen during favorable conditions within the host. This transition to pathogenicity is facilitated through ranges of virulence, including the Agglutinin-Like-Sequence (ALS) family and the *hyphal wall protein* (HWP), which are essential to adhere to the host epithelial surfaces [7–10]. The ALS gene family represents the largest gene family identified in *Candida albicans*, comprising eight members (ALS1–7 and ALS9) that encode extensive glycosylphosphatidylinositol-anchored cell surface glycoproteins, leading to increased adhesion to host cells. Among these essential proteins, ALS genes family contribute to generate fluconazole (FLC) resistance [11] . The HWP produced by the *HWP1* gene influences adhesion. HWP1 is encoded by one of a group of eight core genes that are upregulated during the *C. albicans* filamentation consequently, this protein is thought to be significantly associated with the virulence and pathogenicity of *C. albicans* [7].

Furthermore, these characteristics enable the organism to establish strong biofilms and to demonstrate antifungal resistance [2, 12–17]. *C. albicans* biofilms demonstrate increased resistance to azole-based antifungal drugs such as only FLC as well as newer triazoles including voriconazole (VRC) [18]. This resistance is believed to be related to the stage of biofilm development. The preferential activation of these efflux pumps is the result of the efflux proteome being primarily comprised of the *Candida Drug Resistance* (CDR) gene including *CDR1*, *CDR2* (ATP-binding cassette transporters from the *Candida Drug Resistance* gene family), as well as multidrug resistance (*MDR1)* (a member of the major facilitator superfamily), also referred as multidrug resistance protein [19, 20]. An *in vitro* study demonstrated that biofilm associated cells can have 1000 times more resistance to FLC than planktonic cells [18].These efflux mechanisms limit the intracellular accumulation of antifungals and thus result in reduced drug efficacy [20]. The increased expression of these genes is the most common pathways of *C. albicans* FLC resistance [21].

The recurrence of VVs, increasing antimicrobial resistance [16, 22–24], the relation between virulence and resistance genes is complex and requires more studies specific for each area. The lack of regional data highlights the need for local studies and the need for alternative treatments beyond the rationale of this study; therefore, this study is directed to explore the virulence and resistance genes prevalence and their association with sensitivity of *C. albicans* to antifungal drugs in Sulaimani city.

2. Materials and Methods

2.1. Ethics Approval

The ethics Committee of Sulaimani Polytechnic University (ethical approval number: 17/245 on 27/10/2024) approved the study's protocol. Consent was attained from all participants before enrollment. All collected data were analyzed anonymously.

2.2. Sample Collection

A total of 125 high vaginal swabs collected by a gynecologist using sterile swabs from the two groups of subjects involved in the study. The case group consisted of 100 samples from women clinically diagnosed by a gynecologist with vulvovaginitis symptoms such as itching, burning, odor and secretions [25]. The control group consisted of 25 samples from subjects without signs and symptoms. Collection took place from November 2024 to February 2025 from women aged between 18 and >50 years old referred to the Ali-Kamal Clinical Consultation Center in Sulaymaniyah, Iraq. Further laboratory examination was performed directly in the bacteriology and mycology laboratory.

2.3. Microscopic Examination, Colony Morphology, and Molecular Identification

Firstly, microscopic examination such as potassium hydroxide 10% (KOH 10%) and Gram staining were conducted for detecting yeast or hyphae, then the vaginal sample was grown on Sabouraud dextrose agar medium (SDA) (Liofilchem, Italy) with the addition of 0.05 g/L chloramphenicol (SolarBio, China) to prevent bacterial growth and subsequently incubated 35°C for 48 hours. Then, phenotypic-based identification performed using Hicrome Candida differential agar (HiCrome, India).

Primers for polymerase chain reaction (PCR) test designed using the basic local alignment search tool for nucleotides (BLASTn), based on sequences retrieved in FASTA format from the *Candida* genome available in National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nucleotide/CM084631.1.lnk). Forward and reverse primer sequences were selected from the *Candida* genome and their predicted amplicons were validated using BLASTn. To ensure specificity, the primers were further analyzed for sequence similarity against other fungal species using the NCBI BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast.lnk) [26] (Table 1).

Fungal DNA was extracted using colony PCR according to method described in a previous study [26] as follows: after one colony from the overnight culture and suspended in 40 µL of double-distilled water and incubating for 20 minutes at 95°C, the DNA was liberated. The supernatant was utilized as a PCR template after the DNA purified by centrifuging at 12,000 rpm for two minutes.

C. albicans was confirmed using NCBI-designed specific primer (GenBank Accession no (>CP115805.1)) and amplification by PCR. The primer sequences as follows: forward 5′- AGTGAAGGTGATGTGGCTGG -3′ and reverse 5′- GGGTGCTGCCTCCATATTGATT -3′. The final PCR mixture (25 µL) consisted of 12.5 µL of 2xGS Taq master Mix (Genesand, China), 1µL (10 pmol) of each primer, 4µL DNA template and 6.5 µL distilled water. The process of amplification involved 5 minutes of initial denaturation at 95°C, 35 cycles including denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C and extension for 30 seconds at 72°C and final extension at 72°C for 5 minutes. GoldView I nuclear staining dye (10000x) (Solarbio, China) was used to check the products of the PCR using 2% gel electrophoresis and a UV transilluminator (Biobase, China) was used to visualize the results with the expected PCR product of 519 base pairs (bp).

Table 1: List of the primer sequences and expected product base pairs used for virulence genes.

Primer names		Sequences (5'→3')	PCR Product sizes (bp)	annealing	Sources
*ALS1**	F	AACAGCACAACCTCCGATTTG	864	60°C	This study: GenBank Accession no >XM_712984.2
	R	TGGAGCTTCTGTAGGACTGG			
*HWP1**	F	ATGACTCCAGCTGGTTC	503	60°C	[12]
	R	TAGATCAAGAATGCAGC			
*ALS3**	F	CCAAGTGTTCCAACAACTGAA	185	60°C	[12]
	R	GAACCGGTTGTTGCTATGGT			
*MDR1**	*F*	GGTGTTGGCCCATTGGTTTT	909	60°C	This study: GenBank Accession no >Y16405.1
	R	AGTAGTGGCAGCACCAAACA			
*CDR1**	*F*	AATGGTCACGCTTTGGATTCT	615	60°C	This study: GenBank Accession no >DQ462360.1
	R	TTTGGCATGTGAACCTGGTG			
*CDR2**	*F*	CTGCCATGTCACTCTCCACA	418	60°C	This study: GenBank Accession no >XM_718076.2
	R	TCCCCCTTTTGCATAGCACC			

*Agglutinin like sequence (ALS1, ALS3), Hyphal wall protein (HWP1), resistance genes Multi Drug Resistance (MDR1) And Candida Drug Resistance (CDR1, CDR2); F: Forward; R: Reverse.

2.4. Virulence and Resistance Genes Detection

Extracted and purified DNA used for the amplification of the *ALS1, ALS3, and HWP1* virulence genes, *MDR1*, *CDR1*, and *CDR2* resistance genes from confirmed *C. albicans* isolates. The PCR

amplification conducted using a thermal cycler (Biobase, China) with the amplification condition detailed previously in *C. albicans* isolation with annealing temperatures (60°C) [12].

2.5. Antifungal Susceptibility Testing

Disk diffusion antifungal susceptibility testing was carried out following the CLSI document M44-A [27]. Briefly, preparing Glucose Muller Hinton Methylene blue agar by preparing Mueller Hinton agar (MHA) (Himedia-India) supplemented with (2% glucose, 0.5 µg/ml methylene blue) was used. The agar surface was inoculated by a swab full of cell suspension standardized to a 0.5 McFarland turbidity standard, which yields a yeast suspension of 1×10^6 to 5×10^6 cells per mL. (VRC) 1 µg, (FLC) 10 µg, Nystatin (NS) 50 µg, Amphotericin B (AP) 100 Units (Himedia- India) discs added to the surface of the inoculated plates and subsequently incubated at 35°C and read after 24 hrs. The standard *C. albicans* strain (ATCC 10231) was applied as a control to monitor the reliability of antifungal testing.

2.6. Statistical Analysis

Data analysis was performed using GraphPad Prism version 10.5.0. Categorical variables were expressed as frequencies and percentages. Comparisons between variables were assessed using Fisher's exact test with 2 × 2 contingency tables. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Microscopic Examination, Colony Morphology, and Molecular Identification

Out the 100 samples, 18 (18%) were positive for KOH 10% and 21 (21%) were positive for Gram staining in microscopic examination, while all controls yielded negative results. Results from cultures on SDA showed 70% (70/100) positive results for *Candida* spp. and 32% (8/25) for the control group. A total of 81 *Candida* of different species were recovered from 70 patients (Table 2), with 11 (15.6%) cases having mixed infections with two species, while the remaining 59 (84.29%) had only one *Candida* species identified on HiCrome agar. The most prevalent species among patients, 62 (88.5%) and controls, 8 (100%), was *C. albicans* (Table 2, Figure 1). Non-Albicans *Candida* spp. accounted for the remaining 11.4% of the case group. Isolated *C. albicans* were identified and confirmed via PCR, with 62 (88.5%) isolates from case group and eight (100%) from controls confirmed as *C. albicans* (Table 2). This was then verified by gel electrophoresis, showing an expected size of 519 base pairs (Figure 2).

Table 2: Culture and polymerase chain reaction results of *C. albicans* identification samples of patients and controls.

***Candida* Species**	**Culture on HiCrome agar**		**Molecular identification**	
	Patients	**Controls**	**Patients**	**Controls**
C. albicans	62 (88.6%)	8 (100%)	62 (88.6%)	8 (100%)
Non-Albicans *Candida* spp.	8 (11.4%)	0 (0%)	8 (11.4%)	0 (0%)
Total	70 (100%)	8 (100%)	70 (100%)	8 (100%)

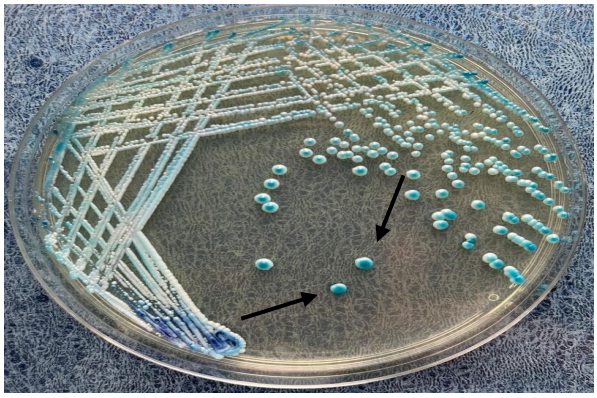

Figure 1: Culturing result on Hicrome Candida differential agar: Light green color is *C. albicans*.

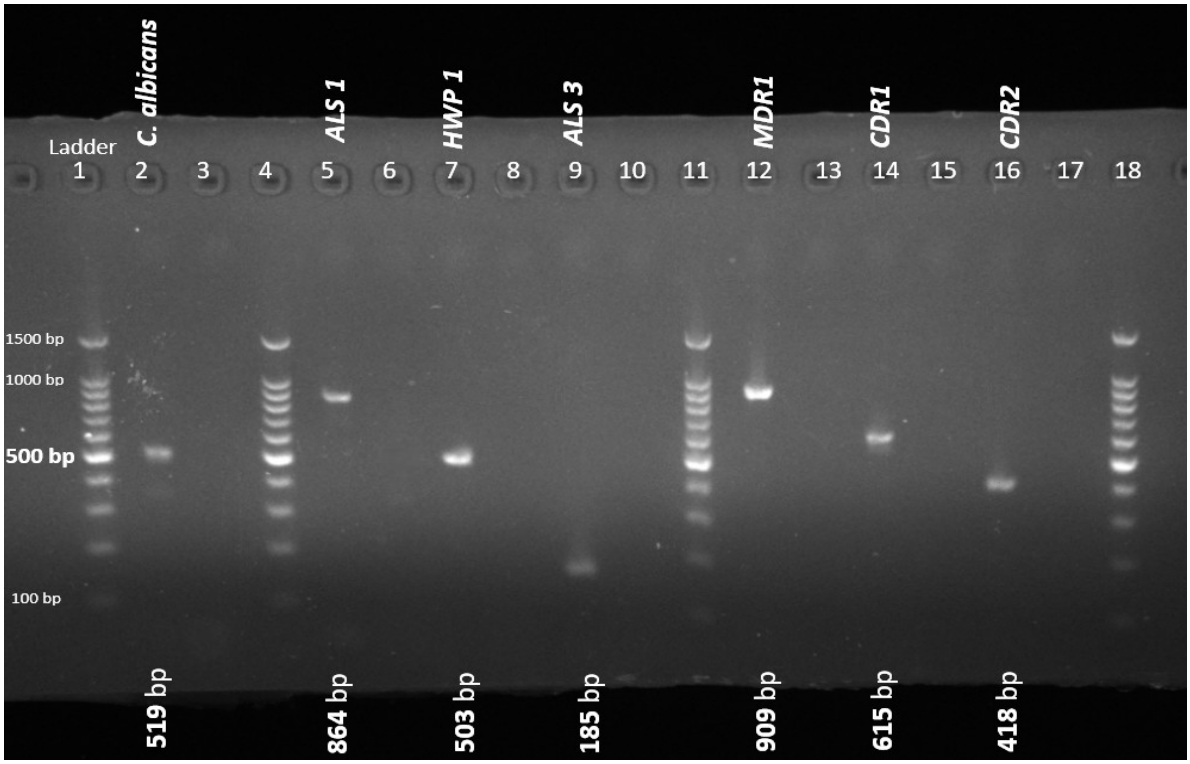

Figure 2: PCR amplification and Agarose gel electrophoresis results for detecting *C. albicans* confirmation, virulence (*ALS1, HWP1* and *ALS3*) and resistance genes (*MDR1, CDR1* and *CDR2*) of *C. albicans*. Lane 2: *C. albicans* (519 bp); Lane 5: *ALS1* gene (864 bp); Lane 7: *HWP1* gene (503 bp); Lane 9: *ALS3* (185 bp); Lane 12: *MDR1* (909 bp); Lane 14: *CDR1* (615 bp); Lane 16: *CDR2* (418 bp); Lane 1, 4, 11, 18: 100 bp Ladder and Lanes 3, 6, 8, 10, 13, 15, 17 are negative controls (PCR mix without DNA).

3.2. Virulence and Resistance Genes Detection

Virulence genes detected for confirmed *C. albicans* isolates, with predicted amplification products for *ALS1*, *HWP1* and *ALS3* genes observed as 864, 503 and 185 bps on 2% agarose gel, respectively (Figure 2). PCR analysis identified *ALS3* in 60 (96.7%), *ALS1* in 51 (82.2%) and *HWP1* in 48 (77.4%) of *C. albicans* from patients, while the corresponding values for control group were 7 (87.5%), 5 (62.5%) and 2 (25%) for *ALS3*, *ALS1* and *HWP1*, respectively (Table 3).

Further, *MDR1*, *CDR1*, and *CDR2* resistance genes were detected with predicted amplification products of 909, 615 and 418 bp on 2% agarose gel, respectively (Figure 2). Prevalence of detected genes of confirmed isolates in patients showed 61 (98.39%) for *MDR1* and *CDR2*, 38 (61.29%) for *CDR1*m, while it was 8 (100%) for *MDR1* and *CDR2* and 6 (75%) for *CDR1* in control group (Table 3).

Table 3: Prevalence of molecular detected virulence genes *Agglutinin like sequence* (*ALS1*, *ALS3*) and *Hyphal wall protein* (*HWP1*) and resistance genes *Multi Drug Resistance* (*MDR1*) And *Candida Drug Resistance* (*CDR1*, *CDR2*) of isolated *C. albicans* from 62 patients and 8 controls using PCR.

	Genes	Patients	Controls	P-value*
Virulence genes	*ALS1*	51 (82.2%)	5 (62.5%)	0.1926
	HWP1	48 (77.4%)	2 (25%)	0.0145
	ALS3	60 (96.7%)	7 (87.5%)	0.3091
Resistance genes	*MDR1*	61 (98.39%)	8 (100%)	NA
	CDR1	38 (61.29%)	6 (75%)	0.7009
	CDR2	61 (98.39%)	8 (100%)	NA
Total		62 (100%)	8 (100%)	

*p value calculated using fisher's exact test.
NA: Not applicable.

3.3. Antifungal Susceptibility Testing

Disk diffusion antifungal susceptibility testing for *C. albicans* isolated from patients revealed statistically significant differences compared to the control group for VRC. Specifically, for VRC (1 μg), a higher proportion of isolates from patients showed resistance (44, 70.97%) (p=0.0024) and were sensitive (11, 17.7%) (p=0.0019), while no significant difference was found for Susceptible-Dose Dependent (SDD) isolates (7, 11.2%) (p=1.0). For FLC, no statistically significant differences were observed between patients and controls for resistance (25, 40.32%, p=0.242), SDD (5, 8.1%, p=0.531), and sensitive isolates (32, 51.6%, p=0.275). All isolates in both groups were sensitive to NS and AP (62,100% and 8,100%, respectively). Among the controls, only one (12.5%), 1 (12.5%), and six (75%) isolates were resistant, SDD, and sensitive to VRC and FLC, respectively, while 0, 0 and eight (100%) isolates were resistant, SDD, and sensitive to NS and AP, respectively (Table 4)."

Table 4: Comparison of antifungal susceptibility testing for *C. albicans* isolated from patients (62) and controls (8) groups using disk diffusion method.

Antifungal discs	Resistance (%)		*P	SDD (%)		*P	Sensitive (%)		*P
	Patients	Controls	value	Patients	Controls	value	Patients	Controls	value
Voriconazole (1 μg)	44 (70.9%)	1 (12.5%)	0.0024	7 (11.2%)	1 (12.5%)	1.0	11 (17.7%)	6 (75%)	0.0019
Fluconazole (10 μg)	25 (40.3%)	1 (12.5%)	0.242	5 (8.1%)	1 (12.5%)	0.53	32 (51.6%)	6 (75%)	0.275
Nystatin (50 μg)	0 (0%)	0 (0%)	NA	0 (0%)	0 (0%)	NA	62 (100%)	8 (100%)	NA
Amphotericin B (100 Units)	0 (0%)	0 (0%)	NA	0 (0%)	0 (0%)	NA	62 (100%)	8 (100%)	NA

SDD: Single Dose Dependent.
*P-value using fisher's exact test.
NA: Not applicable.

4. Discussion

VVC ranks among the most prevalent infections in women of childbearing age worldwide [5, 14, 28–31]. In the present study, the frequency, virulence, resistance genes and the antifungal susceptibility of *C. albicans* from women with VV were compared to the control group. Results in this study showed that fungal infection was the most prevalent cause of VVC (70%) among women suffering from VV, which is similar to findings in other studies in Yemen and Saudi Arabia [29]. This may be attributed to similarity in geographic region, socioeconomic status of the population and cultural behaviors of the area. Of the *Candida* species isolated, *C. albicans* was the most frequent. among both patients and control groups. This domination of *C. albicans* is observed in several countries including the United States, China, India, Australia, and Turkey [32, 33]. The predominance of *C. albicans* species is attributed to ability of this species to switch from the commensal to the opportunistic pathogens when the conditions allow (e.g., systemic antibiotics, a length of presence of disease, or modulated host immune response) [34]. Its good adherence properties and ability to produce biofilms also render it efficient as a pathogen [35]. Notably, 15.6% of cases had mixed infection involving two *Candida* species, which implies a possibly more complicated microbial picture of VV in this area than other past studies with emphasis on single-organism infections.

An array of virulence genes such as *ALS1*, *ALS3* and *HWP1* was observed and which play a role in the adhesion of the microorganism to mucosal tissues and have also been shown to be essential in *C. albicans* biofilms formation in both *in vivo* and *in vitro* studies [36, 37]. Genes that associated with resistance, including *MDR1*, *CDR1* and *CDR2* were also identified. High-detection rates of *ALS1*, *ALS3* and *HWP1* in the *C. albicans* isolates as observed in this study underline their importance in the pathogenesis of VVC. Among ALS genes, *ALS3* was the most prevalent, followed by *ALS1* and *HWP1*, in *C. albicans* strains of both patients and control groups. This particularly high abundance of *ALS3* is of interest as this gene is known to be one of the main *Candida*te genes for adhesion and biofilm formation, as well as colonization and establishment of *C. albicans* in and on the host [7]. We observed a higher *ALS1* frequency in the present study which is in contrast with some other studies [38, 39]. The

frequency of *HWP1* in the patient isolates was significantly greater than that in the control isolates, lending further support for its pathogenicity. The significantly higher frequency of *ALS3*, *ALS1* and *HWP1* in *C. albicans* isolated from patients may suggest a relationship between virulence and persistence. Moreover, the high frequencies of these genes may reflect the adaptive responses of *C. albicans* to host defenses and antifungal therapies.

Molecular characterization of the resistant genes showed high frequency of *MDR1* and *CDR2* among both cases and control groups isolates, in association with a moderate frequency of *CDR1*. Overexpression of *CDR1*, *CDR2* or *MDR1* genes has been well-established as a major mechanism of azole resistance in *C. albicans*, as a result of reducing intracellular drug concentrations [20, 21]. The high prevalence of *MDR1, CDR1* and *CDR2* genes in samples isolated from patients indicates strongly that efflux pump mechanisms are upregulated and play a crucial role in the resistance pattern of the yeast investigated in the current study [9, 40] and this supports our result of antifungal resistance.

The rise in *C. albicans* resistance to antifungal agents, particularly azoles, represents a major public health issue that complicates the treatment of VVC [14]. This study revealed significant differences in antifungal susceptibility profiles between patient and control isolates, with notable findings for azole and polyene antifungals. Our most significant finding was the higher proportion of VRC resistance observed in the patient group (44, 70.97%) compared to the control group (1, 12.5%), which was statistically significant (p=0.0024). This concerning level of resistance to a front-line azole antifungal is of high medical importance. It is possible that the resistance mechanisms involved are complex, potentially including the overexpression of efflux pump genes[9] or prior exposure to related antifungal agents in the patient population. In contrast, no statistically significant difference in resistance to FLC was observed between the patient and control groups (p=0.242). This suggests that while resistance to VRC may be more prevalent, resistance to FLC has not yet reached statistically discernible levels in this specific cohort. Further studies are needed to understand the factors influencing the differential susceptibility to these azole antifungals. A key clinical finding of this study was the complete absence of resistance to Nystatin and Amphotericin B in both patient and control isolates. This finding is highly relevant as it demonstrates that these older antifungal agents can still serve as reliable and effective alternatives for treating infections where azole resistance is a concern. However, there is possibility that the resistance genes in both patients and control groups are present but not expressed; therefore, further studies need to check expression of those genes using real time PCR.

The primary limitation of this study is the small size of the control group. Future studies should focus on evaluating the biofilm-forming ability of *Candida* isolates and assessing the expression levels of the examined virulence genes to better understand their role in pathogenicity and antifungal resistance.

5. Conclusions

The study reveals the high rates of virulence genes, particularly *ALS3* and the prevalence of resistance genes of *MDR1*, *CDR1* and *CDR2*. These mediate concerning levels of antifungal resistance, especially to azoles (VRC and FLC), However, polyene antifungals such as Amphotericin B and Nystatin retained good activity against most isolates, suggesting that these genes might have a major role in pathogenicity of the disease, multi-drug resistance cases and enhanced adaptive capabilities of *C. albicans* and thus treating VVC. Potentially, these genes might be a good target for new antifungal drugs.

Author contributions: Solhan Mustafa Ahmadessa: Conceptualization, Investigation, Methodology, Project administration. **Solaf Jawhar Ali:** Supervision, Validation, Writing – original draft, Writing – review & editing.

Data availability: The data has been used is confidential.

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