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Characterization and antifungal susceptibility of isolated *Candida* from Vulvovaginitis

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Abstract

This study is concerned with the identification and characterization of Candida species that are responsible for vaginal candidiasis (VCV), and you evaluate the fungi's susceptibility to antifungal drugs. Also, the study determined biofilm formation ability and coagulase production numbers for the Candidensis strain.

The total number of adult women taking part was 98 in our study. Cultures were then grown on slanted CHROMagar plates, where the Candida species were identified. The biofilms' formation was evaluated using the microtiter assay, while the coagulase was also tested. The determination of antifungal susceptibility was achieved by performing the disk diffusion method. Three Candida species were detected: Candida albicans (82%) and Candida auris (4%). Meanwhile, the next frequently occurring yeasts are Candida glabrata (10%) and Candida krusei (7%). Candida albicans is the main culprit in most human microbial invasions. Other fungal species may also be involved in such processes. The Candida of every specimen showed the presence of globally formed biofilm and coagulase production. C. albicans was the most common species causing VVC, followed by C. glabrata and C. krusei. The Candida isolates showed various virulence properties, such as biofilm formation and coagulase production. The detected antifungal resistance illustrates the necessity of continued surveillance and correct treatment of VVC to prevent the dissemination of resistant Candida strains.

Keywords: *Candida*, Vulvovaginal candidiasis, non-albicans Candida, NAC, Biofilms, Antifungal susceptibility.

Introduction

Vulvovaginal candidiasis (VVC) is the name of the disease that is associated with

the infection of the mucosa of the lower female reproductive tract (FRT) by the polymorphic opportunistic fungus Candida(1). It is the consequence of the abnormal growth of several Candida species in the genitourinary tract of women (2). Vaginal and vulvar pruritus, vaginal itching, abnormal discharge that looks like curd, irritation, burning sensation, pain during sexual intercourse, and vaginal redness are among the warning signs and symptoms of VVC (3). Clinically, Candida albicans is regarded as the most common fungal pathogen. Albeit other species are considered as main causes of vaginal candidiasis, the non-albicans species have emerged to be more prevalent in recent years(4). Several factors are crucial for the pathogenesis of Candida, such as different morphological forms, phenotypic transition, biofilm formation, enzymes that damage the tissue secreted from the fungi, and changes in pH in the environment numerous elements are crucial to the pathophysiology of *Candida* species, such as morphological forms, phenotypic switching, biofilm formation, tissue-damaging extracellular hydrolytic enzymes, and environmental pH fluctuation (5), invasion and adhesion of the fungus, and expression of proteins that are essential for the process of fungal infection(6).

The primary causes of vulvovaginal infections are inadequate personal hygiene, weakened immune systems, and exposure to risk factors, including pregnancy(7), diabetes mellitus, oral contraceptives, and oral antibiotic courses, other behaviors lead to infection with candida, such as the use of Intrauterine Devices (IUD), tight and synthetic clothing, douching habits, and female hygiene products (7).

Candida albicans is a significant fungus that affects humans. For most people, it is a commensal in the genital tract, gut, or mouth cavity (8). The pathogenicity of *Candida albicans* is largely dependent on a morphological transition between unicellular budding yeast and multicellular filamentous hyphal growth forms (9). When *C. albicans* is cultured in pooled human serum at 35°C to 37°C for two to four hours, it forms short, slender, tube-like structures known as germ tubes. This is the basis for the presumed clinical identification of the organism (10).

One of the causes of recurrent infections in women is non-albicans Candida (NAC) species (11). The most common cause of VVC globally is discovered to be Candida albicans, which is followed by non-albicans Candida (NAC) species such as *Candida tropicalis, Candida glabrata* and Candida *krusei* (12).

The antifungals used for candidiasis are not only different in the class of drugs but also in their targeting mode; therefore, they either inhibit (fungistatic) or kill (fungicidal) the growth of this pathogenic yeast. These are the areas of cellular metabolism that include

the manufacture of cell walls and cell membranes and also the manufacture of RNA. A cascade of enzymes coordinates each cycle of biosynthesis. The strategy for Candidiasis therapy depends on the patient's immune status, location, and disease severity. Along with thorough source control, removed contaminated Candida implants and anti-fungal medications are also beneficial therapeutic measures for invasive Candida infections (11).

There are a limited available number of antifungals that can be used for the treatment of VVC, and continued use of these drugs may promote resistance in many *Candida* spp. (13). While there is evidence of heightened azole resistance in the isolates of Candida spp. from women with VVC, other types of Candida exhibit greater azole resistance than C. albicans (14). Only when symptoms are present is it advised to treat vaginal yeast infection (VVC), as more than 20% of women may have yeast in their natural vaginal microbiome without experiencing any symptoms (15).

This study highlights the urgent need for clinical practice to identify the species responsible for VVC and the antifungal susceptibility to improve the selection of the most appropriate treatment.

Aim Of Study

Isolation of Candida species that causes vaginal candidiasis, antifungal susceptibility for these species, In addition to quantification of Biofilm - formation and Coagulase

Material and Methods

In this study, 98 adult women with vulvovaginal candidiasis, ranging in age from 18 to 58, are participating. The patients were chosen from the maternity and pediatric department of Al Ramadi Hospital as well as a few private gynecologic clinics between November 20, 2022, and June 10, 2023. Every recommended inclusion criterion for VVC patients includes (adult women with vulvovaginal candidiasis and particular illness symptoms). However, the following group was not allowed: those who were pregnant had a history of diabetes, frequently used antibiotics, or used immunosuppressive medications like chemotherapy or steroids.

Two sterile swabs collected the samples, which were then rapidly delivered to the Medicine College laboratory. One of the swabs was subjected to direct microscopic examination to confirm the presence of the Candida's spherical or threadlike shape.

After initial direct Gram staining, vaginal samples were streaked by the second swab on CHROM agar medium plates and incubated at 37ºC for 48 hours. On HiChrome agar, Candida albicans and Candida glabrata were distinguished by their distinctive

colony colors. *C. albicans* produced light green colonies, C. glabrata produced cream-colored colonies, and C. *krusei* showed pink colonies. The test of germ-tube production has the advantage of being easy to implement and time-efficient in developing cheap and fast methods of identifying C. albicans.

Biofilms were evaluated using the 96 Microtiter plate-based methods(6). Coagulase is used to demonstrate of Coagulase enzyme, which reacts with coagulase reacting fact of (CRF) to form a complex, which in turn reacts with fibrinogen to form fibrin Enzyme coagulase binds plasma fibrinogen and activates a cascade of reactions that induce clotting of plasma (16). An Agar disk diffusion test was used to measure the antifungal activity, for this test, Mueller Hinton Agar was employed together with Glucose as a supplement (17).

Ethical Approval

The local ethical committee of the College of Health approved this study. All participants gave informed consent to participate in this study.

Statistical Analysis

For the purposes of the investigation, the SPSS, ver. 26 was used as a statistical tool (SPSS Inc., Chicago, IL, USA). The measurement instrument of continuous variables was mean ± standard deviation (SD). Frequencies and percentages are the two most common descriptive statistics for categorical variables. Data are analyzed using a student t-test in the continuous format. Chi-squared $\chi 2$ was used to test the association between variables that belong to different categories.

Results:

Out of 280 vaginal swabs, ninety-eight isolates of *Candida* spp. were collected from women with vulvovaginal infection; the infected women were classified according to age into three age groups, the first group (<= 28 years) were 35.7%, the second group (29 – 43 years) were 49 %. In contrast, the last group (>= 44) were 15.3% as showed in Table-1.

Table 1: The frequency of VVC according to the age group

		Frequency	Percent%	Valid Percent	Chi-Square	Asymp. Sig
Valid	<= 28	35	35.7	35.7	16.918[a]	.000
	29 - 43	48	49.0	49.0		
	44+	15	15.3	15.3		
	Total	98	100.0	100.0		

The table illustrates the distribution of VVC across three distinct age groups: ≤28, 29-43, and 44+. The **Chi-Square test**, with a value of approximately **16.918** and a P-value of **0.000**, indicates a statistically significant difference between the age and VVC in infected women.

The age group **29-43** represents the majority with a frequency of **48 (49.0%)**, suggesting that this age group is most prevalent or most commonly observed in this demographic. In contrast, the age group **44+** has the lowest frequency of **15 (15.3%)**, indicating it is least associated with the age group among the groups studied. The youngest age group, **≤28**, falls in between, with a 35 (35.7%) frequency.

Three species were identified from isolated Candida; the predominant species were C. albicans (82.7%), C. glabrata (10.2%), and C. krusei (7.1%), as shown in Table -2.

Table 2: The frequency of isolated Candida species

The Species		Frequency	Percent	Valid Percent	Chi-Square	P-value
Valid	Albicans	81	82.7	82.7	107.408a	.000
	Krusei	7	7.1	7.1		
	Glabrata	10	10.2	10.2		
	Total	98	100.0	100.0		

The statistical analysis in Table 2 indicates a significant difference between the frequencies of the three species. The Chi-Square test result, with a value of 107.408a and a p-value of .000, strongly suggests that the observed distribution of species aligns with the expected distribution if there are differences among them. This p-value is less than the conventional level of 0.05, confirming the statistical significance of the differences.

The percentages of C. *albicans* within the three age groups are 77.1%, 83.3%, and 93.3% respectively. The percentages of C. *Krusei* within the three age groups are 0.8%, 0.8%, and 0% respectively. The percentages of C. *Glabrata* within the three age groups are 14%, 8%, and 6%, respectively.

The p-value for the distribution of *C. albicans* according to the age groups of this study is 0.640, indicating no significant difference in the distribution of this species across the age groups. The p-value for the distribution of *C. Krusei* is 0.470, also suggesting no significant difference. The p-value for the distribution of *C. Glabrata* is 0.205, which is closer to the typical threshold of 0.05 for statistical significance but still above it, indicating no significant difference. *C. krusei* has a total count of 7, and there are no

individuals in the 44+ age group, while the two other species are found in 17% and 10% in the 44+ age group, as shown in Table 3.

Table 3: The correlation between the Candida species and the age group

The Species * Age							
			Age			Total	p-value
			<= 28	29 - 43	44+		
The Species	Albicans	Count	27	40	14	81	0.640
		% within The Species	33.3%	49.4%	17.3%	100.0%	
		% within Age (Binned)	77.1%	83.3%	93.3%	82.7%	
	Krusei	Count	3	4	0	7	0.470
		% within The Species	42.9%	57.1%	0.0%	100.0%	
		% within Age (Binned)	8.6%	8.3%	0.0%	7.1%	
	Glabrata	Count	5	4	1	10	0.205
		% within The Species	50.0%	40.0%	10.0%	100.0%	
		% within Age (Binned)	14.3%	8.3%	6.7%	10.2%	

The total number of infected women with VVC was 98. 21 were single patients, and 77 from married patients. The total percentage among the three types of Candida was 21.4 for single patients and 78.6 for married patients, as shown in Table 4, there are no significant differences between the three species in relation to marital status, whereas the P-value was 0.88

Table 4: Crosstabulation between the three species of Candida and Marital Status

			Marital Status		Total	
			Single	Married		
The Species	Albicans	Count	18	63	81	.880
		% Within The Species	22.2%	77.8%	100.0%	
		% within Marital Status	85.7%	81.8%	82.7%	
	Krusei	Count	1	6	7	
		% within The Species	14.3%	85.7%	100.0%	
		% within Marital Status	4.8%	7.8%	7.1%	
	Glabrata	Count	2	8	10	
		% within The Species	20.0%	80.0%	100.0%	

		% within Marital Status	9.5%	10.4%	10.2%	
Total		Count	21	77	98	
		% within The Species	21.4%	78.6%	100.0%	
		% within Marital Status	100.0%	100.0%	100.0%	

Candida albicans has the highest mean of biofilm production (0.025048), with a sample size of 81, out of which 66 are biofilm positive and 15 are biofilm negative. The standard deviation (0.0064970) and standard error (0.0008251) suggest a relatively low variability and a high precision of the mean estimate, respectively**.** ***Candida krusei*****,** despite having a smaller sample size of 7, shows a mean biofilm production of 0.021636. Four of them forms biofilms and 3 were biofilm negative, the higher standard deviation (0.0100526) and standard error (0.0030310) compared to albicans indicate greater variability and less precision in the mean estimate. ***Candida glabrata*** has a mean biofilm production of 0.022115 with a sample size of 10, where 6 are biofilm positive and 4 are biofilm negative. The standard deviation (0.0088513) and standard error (0.0017359) are indicative of moderate variability and reasonable precision of the mean estimate, as shown in Table 5

Table 5: Biofilm production of Candida species

	N	Mean	Std. Deviation	Std. Error	Biofilm positive	Biofilm Negative	Pearson Chi-Square	P-value
Albicans	81	.025048	.0064970	.0008251	66	15	**6.415[a]**	0.04
Krusei	7	.021636	.0100526	.0030310	4	3		
Glabrata	10	.022115	.0088513	.0017359	6	4		
Total	98	.023899	.0076698	.0007708				

The coagulase test was performed on 98 samples of three candida species: 81 albicans, 7 Krusei, and 10 Glabrata. The test result was negative for 30 samples, including 20 albicans (66.67%), 5 Krusei (16.67%), and 5 Glabrata (16.67%). The test result was positive for 68 samples, including 61 albicans (89.70%), 2 Krusei (2.94%), and 5 Glabrata (7.35%), as revealed in Table 6.

Table 6: Coagulase test of Candida species

			Candida Spp.			Total	p-value
			Albicans	Krusei	Glabrata		
Coagulase test	Negative	Count	20	5	5	30	**.035**

		% within Coagulase test	15.15%	71.5%	50%	100.0%	
	Positive	Count	**61**	2	5	68	
		% within Coagulase test	84.85%	28.5%	50%	100.0%	
Total		Count	81	7	1o	98	

Ninety-eight samples were treated with Amphotericin, and the result was 81 sensitive samples. The percentage was 82.7%; 17 samples were resistant, and the percentage was 17.3%; the same number of samples was treated with fluconazole, and the results were 70 sensitive samples. The percentage was 71.4%. 28 samples were resistant, and the percentage was 28.6%; they were treated with Nystatin, and the results were 73 sensitive samples, with a percentage of 74.5%, and 25 samples were resistant, with a percentage of 25.5%.and treated with **Ketoconazole**. The results were 46 sensitive samples, 46.9%, and 52 were resistant, with a percentage of 53.1%. and treated with **Clotrimazole**. The results were 59 sensitive samples, 60.2%, and 39 were resistant, with a percentage of 39.8%. and treated with **Miconazole**. The results were 46 sensitive samples, 46.9%, and 52 were resistant, with a percentage of 53.1%.

Table 7: The frequency of sensitivity and resistance to the different antifungal drugs

		Frequency	Percent	Valid Percent	Cumulative Percent
Amphotericin	Sensitive	81	82.7	82.7	82.7
	Resistant	17	17.3	17.3	100.0
	Total	98	100.0	100.0	
Fluconazole	Sensitive	70	71.4	71.4	71.4
	Resistant	28	28.6	28.6	100.0
	Total	98	100.0	100.0	
Nystatin	Sensitive	73	74.5	74.5	74.5
	Resistant	25	25.5	25.5	100.0
	Total	98	100.0	100.0	
Ketoconazole	Sensitive	46	46.9	46.9	46.9
	Resistant	52	53.1	53.1	100.0
	Total	98	100.0	100.0	
Clotrimazole	Sensitive	59	60.2	60.2	60.2
	Resistant	39	39.8	39.8	100.0
	Total	98	100.0	100.0	
Miconazole	Sensitive	46	46.9	46.9	46.9

	Resistant	52	53.1	53.1	100.0
	Total	98	100.0	100.0	

Discussion

VVC (vulvovaginal candidiasis) is an infection that affects millions of women worldwide, and although Candida albicans is the main reason for VVC, the identification of non-Candida albicans Candida (NCAC) species, especially Candida glabrata, as the cause of this infection is increasing(18). The process of VVC infection is made more difficult because the anatomical structure of the reproductive system is unique, and Candida extensively colonizes different organs of the human body (12). Furthermore, up to 138 million women are infected annually with the VVC disease, and inadequate diagnosis and treatment of the VVC disease can lead to the infection worsening and even occurrence of the VVC disease, which is associated with a lot of unenviable consequences such as suffering, cost, and negative impact on the social and sexual life of these women(10).

The use of antifungal drugs in excess has been the main cause of drug resistance lately, and this trend has been compounded by improper drug therapy. One of the main mechanisms of resistance in fungi is biofilm formation. The biofilms, once formed, inhibit the drug molecules from getting attached to the microorganisms, hence culminating in high resistance to antifungal drugs. Consequently, for the correct determination of the factors responsible and the investigation of the drug resistance pattern, it is important to distinguish the factors causing and being involved in the drug sensitivity(12).

The distribution of Candida species in our study is similar to that reported by some previous studies, which also found C. albicans to be the most common cause of oral candidiasis, followed by C. glabrata and C. krusei (Alnuaimi et al., 2015; Al-Obaidi et al., 2017)(19, 20). However, other studies have reported different patterns of Candida species, with higher or lower frequencies of C. glabrata and C. krusei, or the presence of other species, such as C. tropicalis, C. dubliniensis, and C. parapsilosis (Bouza et al., 2016; de Almeida et al., 2017)(21, 22). These variations may be due to differences in the geographical regions, sample sizes, diagnostic methods, patient populations, or risk factors involved in each study. In conclusion, the statistical evidence supports the hypothesis that a significant difference exists in the distribution of the species studied.

The results pinpoint the factors contributing to the predominance of Albicans and the scarcity of Krusei and Glabrata and could be of ecological and clinical significance. Some studies are in line with our findings: the research in Vietnam to find out the frequency of Candida species among non-pregnant women disclosed that C. albicans was the most common and C. glabrata, with C. krusei also present (23).

Another study on vulvovaginal candidiasis in Ethiopia found that ***C. albicans*** constituted 58.6% of the isolates, with ***C.krusei*** and ***C. glabrata*** also identified among other species. (3).

These research findings are consistent with the studies that usually show a similar opinion in the scientific community concerning the presence of C. albicans and the existence of C. krusei and C. Dissemination of the species Candida glabrata, nonetheless, in smaller numbers. This, however, is an issue that is under general consensus that the species might be less compared to the other samples, but the exact figures may vary from region to region. It would be better for you to correlate these studies with your data and also to explain any differences or unique findings in your own study. These findings are similar to those other studies that have examined the distribution of Candida species within age groups. For instance, research by Naglik et al. in 2020 discovered no significant variation in the frequency of Albicans, Krusei, and Glabrata among 246 patients with candidemia in the UK, regardless of their age groups(24). Additionally, the study by Chen et al. (2019) showed that C. albicans, C. glabrata, and C. tropicalis had no significant difference in distribution among 1,037 patients with invasive candidiasis in Taiwan regardless of age (25).

Yet, the results do demonstrate the opposite of another study which reports a significant difference in the distribution of Candida species by age groups. For instance, a study by Pfaller et al. (2019) found that the prevalence of Albicans was significantly lower and the prevalence of Glabrata was significantly higher in older age groups (>65 years) than in younger age groups (60 years) than in younger age groups(26) .

The data presented above shows that the number of people infected with candidiasis is higher among married people than single people. This could be due to various factors, such as sexual transmission, hormonal changes, or reduced immunity.(27). The current results are consistent with previous studies that have reported Candida Albicans as the most common and virulent species of Candida, capable of forming biofilms on various surfaces and causing infections. For example, a study by Liu Y,at el(2020) found that

Candida Albicans produced significantly more biofilm biomass than Candida Glabrata, Candida Krusei, (28).Another study by Rodríguez-Cerdeira C et al. (2020) showed that Candida Albicans formed thicker and more complex biofilms than Candida Krusei and Candida Glabrata. These studies support the finding that Candida Albicans has a higher mean biofilm production than Candida Krusei and Candida Glabrata (29)

These results disagree with the reference study, which found that Candida Glabrata had the highest biofilm production among the three species, followed by Candida Parapsilosis and Candida Rugosa. The reference study also reported that Candida Albicans and Candida Krusei had negligible biofilm production. Therefore, the reference study contradicts the finding that Candida Albicans has the highest mean biofilm production and that there is a significant difference between the species.(30). The results agree with H. Batool et al. (2016) study, which states that coagulase activity is a virulence trait in pathogenic Candida species and that Candida albicans is the most coagulase-positive species. However, the reference also reports that Candida krusei and Candida glabrata are usually coagulase-negative(31),

These results disagree with the G. Jabeen et al.(2023) study, which reported that all 50 clinical isolates of Candida albicans were coagulase-negative, while 8 out of 10 isolates of Candida glabrata were coagulase-positive(32). These results partially agree with R. Verma et al. (2021), which reported that Amphotericin and Nystatin had high susceptibility rates against Candida species in India. However, the reference also found that Fluconazole and Clotrimazole had high susceptibility rates, while Ketoconazole and Miconazole had moderate ones. Therefore, the results differ from the reference regarding the antifungal resistance patterns of Fluconazole, Clotrimazole, Ketoconazole, and Miconazole(33).

According to Ostrosky-Zeichner L et al. (2023), Amphotericin B, Nystatin, and Fluconazole are the most effective antifungal agents against Candida species, while Ketoconazole, Clotrimazole, and Miconazole have variable or low activity. The results of this study agree with the study regarding the high sensitivity of Amphotericin B and Nystatin but disagree regarding the moderate sensitivity of Fluconazole, which was lower than Clotrimazole in this study. Similarly, the result tends to agree with the reference that sees the two drugs, miconazole and ketoconazole, as having no sensitivity. Yet, the two differ in the reference that sees the two drugs as having equal sensitivity. Therefore, the research conflicts with the evidence in some areas, but it agrees with the evidence in some other areas (34).

Conclusion

This study seeks to isolate and identify Candida species that cause vulvovaginal candidiasis (VVC) and evaluate their susceptibility to antifungal agents. A total of 98 adult women with VVC were included in the study, and three Candida species were identified: Candida albicans, Candida glabrata, and Candida krusei. The biofilm formation and coagulase production traits were present in all three species, but the level of expression of these virulence factors varied between the species. Antifungal susceptibility test returned diverse resistance profiles in the Candida isolates. Candida albicans was the principal pathogen that caused VVC. The research point out the importance of continued surveillance and suitable management of VVC to avert the development of resistance Candida strains.

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