Characterization of *Candida* species from Vulvovaginal Candidiasis - Emphasis on ALS3 Gene expression

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Abstract

For women, vaginitis is a severe health problem. Certain species of Candida can become resistant to certain antifungal therapies. The objective of the study is to scrutinize the rate of this condition, the differentiation of species from the clinical isolates and the development of resistance towards antifungal drugs and detection of ALS genes in isolates in a tertiary health-care facility. Patients who were suspected to vulvovaginal candidiasis, their vaginal swab were collected, subjected to microbiological investigation. AFST patterns were analysed and investigated for ALS gene expression. Pregnant women were found to have the highest prevalence among the 120 cases of Candida isolates in this investigation, with 43 (35.7%), while steroid users had the lowest frequency, with 9 (7.5%). The most common organism was 63 (52.5%) C. albicans and the most common NAC species were 32 (26.6%) C. tropicalis. The ALS3 gene has been found in Candida non-albicans (9.09%) and Candida albicans (36.6%).

As a result, NAC species was more common and showed that fluconazole was least efficient medication due to the high rate of resistance. VVC was more common in pregnant women. Candida isolates that express the ALS3 gene probably assisted to adhere to the vagina and create biofilms.

Keywords: Agglutinin-like sequence gene (ALS), *Candida albicans*, *non-albicans Candida* (NAC), vulvovaginal candidiasis, biofilm formation.

Introduction

Vaginal candidiasis are usual phrases used as a description of fungal infections in the area of the vagina. VVC is characterised as an infection by yeast like organism in the oestrogenised vaginal area and vestibulum that may extend to labia major on the outside[19].When these symptoms occur at least three times a year, the infection is considered as chronic in various regions of the world, millions of women have been affected by recurrent vulvovaginal candidiasis (RVVC). During their reproductive years, almost 75 percent of all women encounter at least a single case of VVC and around half of them do so at least once more[14,25]. A significant number of women, at least 20% of them, have vaginal colonization because of *Candida* organism and this percentage can reach 30% during pregnancy.

Over 90%, VVC affected women feature *Candida albicans* as the etiological culprit, however additional non-albicans *Candida* (NAC) organisms have also been detected. Some NAC species are abnormally common and make upto 50% of community[29]. The second most common NAC species to induce VVC is *C. tropicalis*, along with remaining NAC species *C. krusei, C. parapsilosis and C. glabrata*[2]**.** According to a prospective study of asymptomatic females between the age range of 18 and 30, 70% of the female participants had more than one species of *Candida*. Studies conducted in India found that among adult women within the reproduction-age range, there was a prevalence of 10% to 35% for laboratory-confirmed VVC**.** The diagnostic standards and the existence of risk factors may affect the prevalence differently.

The capability of *Candida* species, a yeast like fungus, to change morphologically between the yeast and the hyphal forms, a characteristic that is crucial to its pathogenicity and its ability to form biofilms, is primarily responsible for its transformation from a harmless commensal to an infectious pathogen. The majority of antifungal drugs are more resistant against *Candida* biofilms[23]. Phenotypic flipping, filamentation and secreted hydrolyses are only a few of the virulence factors that cause illness in the *Candida* species.

The agglutinin-like sequences (ALS), secretory aspartyl proteinase (SAP), as well as lipase groups are among the gene families[7,11]. Among these, the cell wall glycoprotein family encoded by the ALS gene family is connected to adhesion to host surfaces. A group of adhesions known as the ALS genes is implicated in early biofilm development and adherence[12,21,30]. These proteins are essential for adhering the *Candida*. It has the same three domain structure, which consists of the following components: 5' end domain of 1299–1308 bp in one end that is 55–90% identical across the group, a central domain with a varying amount of tandemly continual repeats of a 108 bp motif, plus a 3′ end domain that is typically variable in length and sequence. This is due to the fact that the growth of biofilm results in medication resistance[18].

Additionally, this gene plays a key role in the N-terminal domain's adhesion. The ALS proteins have a common three domain structure, however there might be significant sequence discrepancies amongst them that lead to the

possibility that the proteins have entirely separate purposes[13]. The ALS1, 2, 4, 5 and 9 genomes are found on the 6th chromosome, the ALS3 and ALS8 genomes are found on the R chromosomes and the ALS6 and ALS7 genomes are found on the third chromosome. The adherent isolation of *Candida* is more pathogenic[9]. The main aim of our research was to identify the frequency of vulvovaginal candidiasis, species differentiation by conventional methods, antifungal susceptibility pattern for isolates and ALS3 gene expression by RT PCR.

Material and Methods

Sample collection: This cross-sectional study was carried out at a Tertiary Healthcare centre between July 2022 - January 2023 and ethical approval (8417/IEC/2022) was obtained from the Institutional Ethical Committee. 120 *Candida* isolates from female patients between the age range of 18 to 40 had probable clinical signs of vulvovaginal candidiasis. The swab was collected in the aseptic setting with vaginal fluids and it was sent to a microbiology laboratory within 24 hours for further diagnosis

Speciation of *candida* species: The vaginal swabs were subjected for a microscopic examination, grown on SDA media (Hi-media) and then analysed for species using the Germ tube test (GTT), Dalmau plate method utilizing CMA media, sugar fermentation using 1% glucose, sucrose, maltose, lactose, galactose, trehalose and sugar assimilation tests using 1% glucose, maltose, sucrose, lactose, galactose, trehalose, dextrose.

Antifungal susceptibility testing: Antifungal susceptibility tests were performed by the Kirby Bauer disc diffusion method using fluconazole (25µg), voriconazole (1µg), itraconazole (10µg), amphotericin-B (100U) (Hi-media laboratories) on cation adjusted Muller Hinton Agar (MHA) supplemented with 2% glucose and methylene blue (0.5 µg/ml) according to CLSI-M44-A2 guidelines 2022. The zone size was interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2022.

Biofilm formation: To assess the level of biofilm formation, the isolates were tested using the "Congo red agar method" using Brain Heart Infusion (BHI) broth, 5% sucrose (50g/L) and Congo red dye.

Identification by genotypic methods: A 24-hour colony from the SDA agar medium was placed into an Eppendorf tube with a volume of 1.5 ml for RNA extraction. Glass beads, 200 µL of lysate buffer, the phenol- chloroform and isoamyl alcohol were added to the container. After centrifuging the tube for three minutes at 5000 rpm, the supernatant was transferred to a new tube and 300 µL of phenol-chloroform was then added. The liquid portion was transferred to a sterile Eppendorf tube after repeated centrifugation process. One volume of cool isopropyl alcohol (IPA) was added to the mixture and it was then kept at -20^{0}C for 15 minutes. The sample was then washed in 70 percent ethanol, 30 µL of distilled water was added and the sample was finally dried.

PCR protocol: Two-step RT-PCR was used to create the first strand of complementary DNA (cDNA). 1-2 µl of cDNA, phosphate Buffer A (10X), distilled water (18.5 µl), forward primer and reverse primer (0.35 µl) for specific gene in the microtube. Place it into the thermocycling device: A preliminary phase of 94°C for 5 min of denaturation was observed followed by 58°C for 1 min of annealing, 72°C for 1- 3mins of final extension. Finally, 25 µl of the amplicon has to be run across a gel made of 1% agarose. Primer for specific gene is shown in table 1.

Results and Discussion

A total of 120 *Candida* isolates of the vulvovaginal candidiasis individuals (women within the ages of 18 and 40) at a tertiary healthcare centre were used in the current study. The age population aged 24-29 years had the highest prevalence of vulvovaginal candidiasis, with 40 (33.2%) cases, followed by 18-23 years with 38 (31.6%) cases, 30-35 years with 36 (30.1%) cases and 36-40 years with 6 (5.1%) cases.

In this study, 120 women were included. Of these, 19 (15.8%) women complained of pruritus, 22 (18.3%) women reported discharge from the vagina, 13 (10.8%) women reported soreness, 15 (12.5%) women reported the genital area erythema, 16 (13.3%) women reported dysuria, 17 (14.1%) women reported burning sensations, 14 (11.6%) women reported dyspareunia, as well as 4 (3.6%) women reported odor.

120 research participants risk factors were distributed as follows: 35.7% of them were currently pregnant women, 7.5 percent were taking steroids, 17.5% had diabetic mellitus, 17.6% had used contraception and 21.7% had taken several antibiotics.

Gram staining method of the swabs revealed Gram positive budding yeast cells with few pus cells and epithelial cells and no clue cells seen. Out of 120 symptomatic *Candida* isolates, 63 (52.5%) produced germ tubes, all of the *Candida* positive samples that produced germ tube formation, were recognized as *Candida albicans.*

Table 1
ALS3 specific primers

Gene	Primer name	Sequence (5'⟶ 3')	PCR product size
ALS 3	ALS3-F	CCAAGTGTTCCAACAACTGAA	183 bp
	ALS3-R	GAACCGGTTGTTGCTATGGT	

Out of the 120 *Candida* species that were isolated, *Candida albicans* had the highest frequency, with 63 (52.5%), followed by *Candida tropicalis* with 32 (26.6%), *Candida parapsilosis* with 15.5%, *Candida glabrata* with 6% and *Candida krusei* with 3.3%.

By using the Kirby Bauer disk diffusion method, an analysis of the antifungal susceptibility pattern revealed that amphotericin B (96.6%) had the highest reported sensitivity, followed by voriconazole (93.3%), itraconazole (91.6%) and fluconazole (82.5%). Additionally, it shows that Fluconazole (17.5%) had the highest resistance pattern, next to itraconazole (8.4%), voriconazole (6.7%) and amphotericin B (3.4%) (Table 2). *C. albicans* had the highest rate of antifungal drug resistance, 20 (46.5%), next to *C. tropicalis* 13 (30.2%) and *C. parapsilosis* 6 (13.9%), while *C. glabrata* 2 (4.6%) and *C. krusei* 2 (4.65%) exhibited the lowest rates of resistance.

The Congo red agar method was then used to test any resistant *Candida* isolates for biofilm generation and it was discovered that roughly 11 (25.5%) of the isolated were biofilm producers. Among these, *C. albicans* 5 (45.5%) produced the most biofilm, which was followed by 2 (18.1%) strains of *C. tropicalis*, 2 (18.1%) isolates of *C. parapsilosis*, 1 (9.1%) strain of *C. glabrata* and 1 (9.1%) isolate of *C. krusei*. However, 32 (74.4%) of the isolates did not generate biofilms (Table 3).

All of the *Candida* isolates that formed biofilm were subjected to RT-PCR analysis for the ALS 3 gene identification (figure 1).

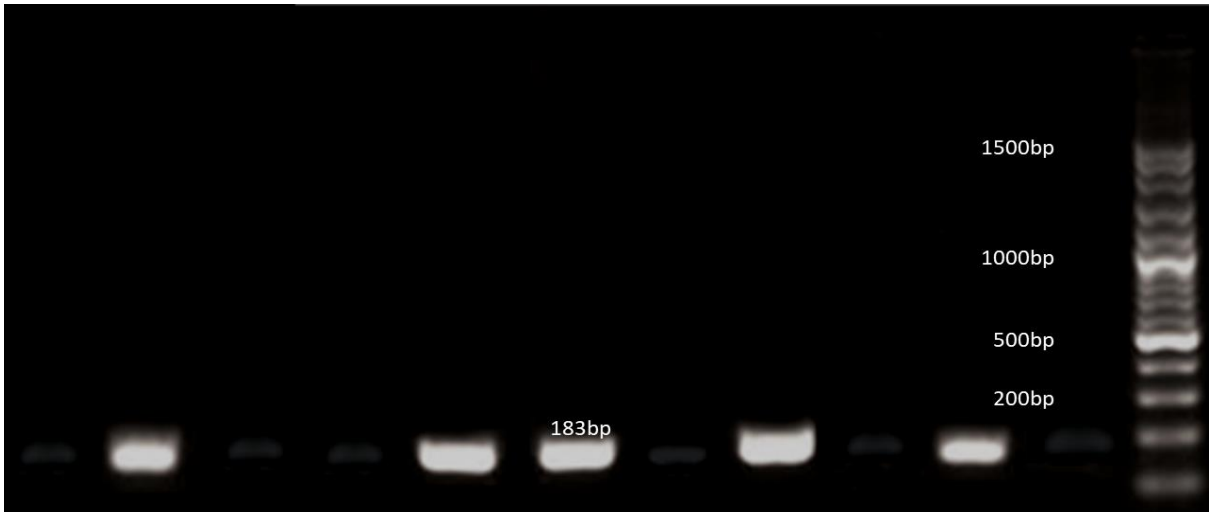

Figure 1: Gel Image showing ALS 3 gene

Table 2
Antifungal susceptibility pattern of *Candida* isolates by Kirby Bauer disc diffusion method (n=120)

***Candida* species**	**FLC**		**ITR**		**VRC**		**AMP B**	
	S	**R**	**S**	**R**	**S**	**R**	**S**	**R**
C. albicans (n=63)	52	11	58	5	60	3	62	1
C. tropicalis (n=32)	26	6	29	3	30	2	30	2
C. parapsilosis (n=15)	13	2	14	1	13	2	14	1
C. glabrata (n=6)	5	1	6	-	5	1	6	-
C. krusei (n=4)	3	1	3	1	4	-	4	-
Total (n=120)	**99**	**21**	**110**	**10**	**112**	**8**	**116**	**4**
Percentage (%)	**82.5%**	**17.5%**	**91.6%**	**8.4%**	**93.3%**	**6.7%**	**96.6%**	**3.4%**

Table 3
Biofilm production among *Candida* species by Congo red agar method (n=43)

***Candida* species**	**Total no. of isolates**	**Biofilm production among *Candida* species (%)**	**Non – biofilm producers**
C. albicans (n=63)	20 (46.5%)	5 (45.5%)	15 (46.8%)
C. tropicalis (n=32)	13 (30.2%)	2 (18.1%)	11 (34.3%)
C. parapsilosis (n=15)	6 (13.9%)	2 (18.1%)	4 (12.5%)
C. glabrata (n=6)	2 (4.6%)	1 (9.09%)	1 (3.1%)
C. krusei (n=4)	2 (4.65%)	1 (9.09%)	1 (3.1%)
Total	**43**	**11**	**32**

Table 4
Distribution of Molecular detection of *ALS 3* gene (n=11)

***Candida* species**	**Total no. of isolates**	**Expression of *ALS 3* gene in Candida species**
C. albicans (n=63)	5	4 (80%)
C. tropicalis (n=32)	2	1 (20%)
C. parapsilosis (n=15)	2	-
C. glabrata (n=6)	1	-
C. krusei (n=4)	1	-
Total	**11**	**5**

Out of 11 biofilm-producing *Candida* isolates which were tested for ALS 3 gene expression, the highest levels were detected in 4 (36.3%) *C. albicans* isolates and 1 (9.09%) *C. tropicalis* isolates. The ALS gene was not expressed by other *Candida non-albicans* (Table 4).

The most typical problem impacting women nowadays is vulvovaginal candidiasis, which has increased significantly during the past few years[24]. It may result in a number of genital signs and symptoms, such as a thick discharge like cottage cheese coupled with vulvar and vaginal itching, pain, burning, redness and edema. Also possible are external dysuria and dyspareunia. After bacterial vaginitis, candidiasis of the vulvovaginal tract is the second most prevalent fungal infection[17]. In their lifespan, 50% to 80% of people will have many bouts of vaginal candidiasis[3]. The age range of 24-29 years had the highest prevalence of vulvovaginal candidiasis at 33.2%. These findings were consistent with those of Zaman et al[28] who found that 33% of the study's participants were between the ages of 25 and 30.

In this investigation, a maximum of 57 (47.5%) NAC spp. were isolated from the vulvovaginitis patients, compared to 63 (52.5%) *Candida albicans*. Ahamad et al[1] conducted a similar investigation on vulvovaginitis cases, which revealed that *Candida albicans* made up 46.9% of the organisms and NAC spp. accounted for roughly 50.2%. The results of the current investigation contradicted those of Trama et al's[26] study were *C. tropicalis* 32 (26.6%) was the most prevalent species isolated from NAC spp. it was discovered that *C. glabrata* had the second-highest recovery rate, at 14.3%, after *C. parapsilosis,* which came in at 5.9% and *C. tropicalis,* which came in at 8.0%.

Women who are pregnant, showed the highest frequency in this study (43) followed by those who take broad-spectrum antibiotics (21%) and patients taking steroids (7.5%) exhibited the lowest frequency. Similar findings were found in a study by Nwadioha et al[22] who showed that broad-spectrum antibiotic users (67%) and steroid users (63%) had the lowest frequency of vaginal candidiasis respectively and that pregnant women (about 40%) had the highest frequency. Additionally, the findings of the study by Babin[4] revealed that steroid users (19.83%) and pregnant women (29.75%) had the highest frequency of use. Once the yeast cell clings to the surface and begins to grow, a mature, highly organised biofilm forms. The growth circumstances and co-infection of other microbes can have an impact on the production of biofilms, which adhere to solid surfaces. Biofilms can act as a reservoir for microorganisms and can promote the development of antimicrobial agent resistance[15]. In our investigation, 11 (25.5%) isolates had biofilm creation shown by Congo agar plate and of those, 11.6% were *C. albicans*, which formed biofilm more frequently than NAC species.

Similar to this, Bansal et al[5] revealed that 57% of the 100 clinical *Candida* samples they examined produced biofilm. In comparison with *non-albicans Candida* spp. (58.2%), *C. albicans* showed a slightly lower percentage of positive biofilms (44.4%)[5].

Contrarily, a study by Bansal et al[6] revealed that *Candida tropicalis* (52.86%) is the most typical *Candida* species to be separated as biofilm producers and that biofilm producers were also more likely to be antifungal resistant.

The *Candida* species in this investigation showed the highest level of amphotericin B sensitivity (96.6%). As a result, Noake et al[20] found that certain kinds of *Candida* are more amphotericin-B sensitive (92%), while 60% of all isolates tested positive for azole resistance. The antifungal agent with the largest spectrum of activity is thought to be amphotericin B. The current study reveals that when compared to other evaluated antifungal drugs, fluconazole showed the highest reported resistance (17.5%). Fluconazole resistance was shown to be 11.7% in a study by Xess et al[27] which was determined to be consistent with the results of the current investigation. Giri et al[10], on the other hand, found fluconazole resistance rates of 30.8%, which were greater than the findings of our investigation.

Structured microbial communities called biofilms are affixed to solid surfaces. Als3 is important for the development of biofilms. These suggest that despite the involvement of numerous adhesins on the creation of biofilm *in vivo*, ALS3 plays a crucial function in this process. It has been demonstrated that ALS3 proteins play a part in how fungus adhere to the vaginal mucosa. HWP1 and ALS1 are two additional *Candida* species that produce adhesins, which help to create biofilms[16]. In this investigation, four *C. albicans* strains (80%) and one *C. tropicalis* strain (20%) both showed the expression of the ALS3 gene.

Our findings are in agreement with those of Cheng et al's[8] study. It revealed that the *Candida* species with the highest degree of ALS3 expression of genes was *C. albicans*, which accounted for around 86.7% of the total. It was determined that ALS3 is one of the main overproduced genes in biofilm development and adherence, which is crucial for medication access limitation to the fungus.

Conclusion

Our statistics show that vulvovaginal candidiasis constitutes a common infection in our area. Both albicans- and NAC species causing VVC are prevalent in women at reproductive age. The presence for *Candida* species in women's cultures indicates that candidiasis of the vulvovaginal area cannot be detected solely based on clinical criteria but also calls for the use of vulvovaginal complaints and diagnostic procedures. *C. albicans* was among the most prevalent isolated species, accounting for 63 (52.5%) whereas *C. tropicalis* made up 32 (26.6%) of all species. An efficient strategy for assuming the presence of *Candida* species was to conduct fermentation of sugar and sugar assimilation tests. The ALS 3 genes were often detected in *Candida albicans* and *Candida tropicalis*.

Clinical strains of *Candida* species discovered in the vagina are affected by ALS gene expression in terms of antifungal treatment resistance and adhesion due to biofilm formation. Since the prevalence of vulvovaginal candidiasis varies geographically and over time, it is important to continuously monitor changes in prevalence and susceptibility rates and features of Agglutinin-like sequence genetic family in species of *Candida*. This will help with future research on the ALS gene family within other *Candida* species and the uniqueness of ALS family gene function.

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