Biofilm Formation and Antifungal Susceptibility Profile of *Candida* Species Responsible for Vulvovaginal Candidiasis in Pregnant and Non-Pregnant Women visiting a Tertiary Care Hospital in Southern India

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**Abstract**

Most bacteria and fungi are capable of producing biofilms, enabling them to thrive in nature on distinct surfaces. Biofilm formation stands out as one of the most prominent virulence mechanisms that contribute to the infection’s chronicity by functioning as a defense against antimicrobials and host immune systems. Microbial isolates capable of generating biofilms have been discovered to possess higher resistance to frequently administered antifungal drugs. In this research study, 91 *Candida* isolates from Vulvovaginal Candidiasis (VVC) patients were tested for biofilm development. *Candida* species were identified, and clinical isolates were tested for antifungal susceptibility (AST). Three methods were used to screen the isolates: the Congo agar method (CRA), the visual tube method (VT), and the Microtitre plate method (MTP). Nearly 60% of the 91 clinical isolates tested were recognized as Non-Albicans Candida (NCAC) species. Itraconazole resistance was shown to be the highest in clinical isolates, followed by Amphotericin B resistance. There were 11(12.09%) isolates that formed strong biofilms, 35(38.46%) isolates that formed moderate biofilms, and 45(49.45%) isolates that formed no biofilm. Because there is a growing incidence of NCAC in the study, it is critical to speciate the *Candida* species as NCAC are more resistant to routinely used azole medicines. Furthermore, a spike in the prevalence of biofilm producers has been reported, implying greater pathogenicity and antifungal resistance.

**Keywords:** Microtitre Plate Method, Congo Red Agar Method, Itraconazole Resistance, Fluconazole Resistance, Antifungal Resistance

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INTRODUCTION

More than 70% of women will experience vulvovaginal candidiasis (VVC), a common gynecological illness, at some point in their life. VVC is usually not fatal; however, recurrence of VVC impairs the quality of life in afflicted women. VVC has been reported to be the second-leading cause of vaginitis. Candida colonizes roughly 10-15% of asymptomatic patients; almost fifty percent of women who were previously diagnosed with VVC will contract a second episode. Recurrent infections are also caused in about 5-10% of all women. Many variables, such as long-term usage of antifungal medications available over-the-counter and one-dose therapy of fungal infections, contribute to the increased incidence of VVC. Although it is thought that C. albicans, an opportunistic dimorphic fungal pathogen, is involved in majority of VVC infections, there is growing evidence of Non-Albicans Candida (NCAC) causing VVC. NCAC develops innate and acquired drug resistance to routinely given antifungal medications resulting in increasing antifungal resistance. Candida species produce virulence factors such as secreted aspartyl proteinases (SAP), hemolysin, lipases, coagulase, and biofilm formation, which contribute to pathogenicity, allow them to elude host immune responses, and establish themselves as a pathogen causing morbidity. Germ tube production, hyphal formation, phenotypic switching, and thigmotropism are other virulence factors that contribute to their pathogenicity. Candida forms biofilms by affixing to a wide range of surfaces, both abiotic and biotic. These have three-dimensional structural configuration composed of proteins, polysaccharides, and DNA embedded in the extracellular matrix (ECM) of fungal cell aggregates. The biofilm matrix forms a physical barrier that protects the Candida species that are immersed in it from environmental influences while also giving structural integrity to the biofilm. The biofilm matrix also prevents mechanical breakdown of the biofilm. Adherence, proliferation, maturity, and dispersion are the four critical steps in the creation of a biofilm. Fungal cells capable of generating biofilms are noted to be highly resistant to the antifungal drugs and to have a different metabolism than their planktonic counterparts. The biofilm works as a barrier, defending against attacks by the host immune system and antifungal medications. Candida species cause greater mortality in immunocompromised patients, necessitating the use of broad-spectrum antifungal medicines. However, continuous use of antifungal medicines, as well as abuse of over-the-counter drugs, leads to an increase in antifungal resistance in Candida species and cause greater mortality in immunocompromised patients, necessitating the use of broad-spectrum antifungal medicines. However, continuous use of antifungal medicines, as well as abuse of over-the-counter drugs, leads to an increase in antifungal resistance in Candida species. Furthermore, biofilm development is a major contributor to fungal resistance to antymycotic medications such as azoles, polyenes, and echinocandins. Several investigations on biofilm formation and antifungal resistance have revealed that they play a substantial part in pathogenicity of the infection. VVC, in particular, is believed to be a biofilm-mediated infection. This highlights the need for us to better our understanding of the infectious agent involved, in order to improve clinical outcomes and develop effective strategies for combating VVC. It is thus critical for researchers to contribute to the existing knowledge on Candida biofilm prevalence and antifungal resistance, as both of these Candidal attributes have been evolving considerably over the years. Furthermore, these are bound to alter based on the study population and its dynamics. Although several studies have demonstrated an increase in fluconazole resistance in VVC cases worldwide, there are only a limited number of studies that emphasize the evolving resistance of Candida species to other antifungals such as itraconazole, amphotericin B, and voriconazole. This study intends to shed light on the rising antifungal resistance and biofilm formation in both pregnant and non-pregnant women belonging to the suburban population. MTP (Microtitre plate method), CRA (Congo red agar), visual tube assay, bioluminescent assay, light or fluorescence microscopy, and other methods are available for biofilm screening. Although the MTP approach is considered the gold standard for identifying biofilm development, it is tedious and requires instruments such as a UV spectrophotometer, limiting its use in normal
laboratory processes. These drawbacks can be overcome by employing simpler alternative procedures such as CRA and VT.\textsuperscript{23} In this study, we examined 91 non-repetitive Candida vaginal isolates for biofilm development as well as their antifungal susceptibility profile. Biofilm formation was screened using three different techniques: MTP, CRA and VT to examine the efficiency of these methods.

**MATERIALS AND METHODS**

This cross-sectional study involved 91 non-repetitive species of Candida identified from both pregnant and non-pregnant women visiting SRM Medical College Hospital and Research Centre with complaints of curdy white vaginal discharge from May 2022-October 2022. Standard microbiological procedures were used to identify the clinical isolates, such as identification of yeast cells by KOH met mount, germ tube formation, chlamydospore formation, sugar fermentation and sugar assimilation assays. Furthermore, speciation of C. albicans and NCAC was done using HiCrome Candida differential agar (Himedia Mumbai, India). On Candida differential agar (CDA), Green colonies are formed by C. albicans, C. krusei formed pink to purple colonies, C. tropicalis was identified by formation of dark blue colonies, and cream to white colonies were formed by C. glabrata. (Figure 1). The culture was kept viable by subculturing it on Sabouraud’s dextrose broth (SDB) and storing it in the deep freezer until needed.

**Phenotypic detection of Biofilm production Congo red agar (CRA)**

CRA is a qualitative evaluation method for spotting microorganisms that produce biofilm. CRA is prepared by adding 0.8g of Congo red along with 36g of sucrose to 37 g/L of Brain heart infusion (BHI) agar. The clinical isolates to be evaluated were streaked over CRA petri dishes and were cultured at 37°C for 24 hours. Biofilm producers were distinguished by the emergence of dark black colonies with a brittle crystalline texture, while non-biofilm formers did not change colour and maintained pink colony morphology. Positive results of the assay is indicated by formation of black coloured colonies with dry crystalline consistency. Weak biofilm formers are normally pink, with intermittent darkening of the colonies’ centres.\textsuperscript{24,25}

**Visual Tube method**

In 5ml of SDB, a loopful of overnight Candida culture is introduced and cultured at 37°C for 24 hours. After the incubation period, the tubes were decanted and further washed with Phosphate buffered saline (PBS). The tubes were kept in upside down position for drying. The dried tubes were then stained with 0.1% Crystal violet. The excess stains in the tubes were removed and sterile water was used to clean the stained tubes. The tubes were then dried inverted. Formation of visible film lining the tube’s wall and its bottom indicates biofilm formation. Biofilm production in tubes was evaluated and classified as weak/non (+), moderate (++), or strong (+++).\textsuperscript{24,26}

**Microtitre plate method (MTP)**

Millsap et al., MTP test was used to screen 91 Vulvovaginal Candidiasis isolates for biofilm development. In brief, 100μl of the overnight grown cell suspension of Candida was added in triplicate to the 96 welled MTP plates. The sterility and the non-specific binding of the media was tested by adding merely the broth in the wells of the MTP plate in triplicates followed by incubation at 37°C for 72 hours. Following the incubation period, the contents of the plate were removed by gently by tapping off the plates. To eliminate planktonic (free floating) organisms, the plates were rinsed with PBS. To detect sessile organisms, 0.1% Crystal violet was used to stain the plates. Distilled water was utilized to remove excess stains. Solubilization of the stain was done by adding acetic acid to the wells and a wavelength of 450 nanometers was applied to assess the absorbance using an ELISA reader (Merilzyzer, Elisa reader, and washer).\textsuperscript{26} The mean OD values are used to estimate the Candida species’ adhering capacity (Table 1).

**Antifungal Susceptibility Testing**

The Antifungal susceptibility profile of the clinical isolates were identified for the most commonly suggested antifungal drugs by the Disc diffusion method according to the CLSI guidelines.\textsuperscript{6,7} Mueller-Hinton agar (MHA) was used
to carry out the susceptibility testing with some minute modifications. Briefly, MHA for *Candida* was prepared by adding 0.5mg/ml of Methylene blue to the MHA medium, followed by autoclaving. Candidal cell suspensions were prepared to a turbidity to match 0.5 McFarland standards ($10^6$ CFU/ml), similar to the standard procedure for testing antibacterial substances. These *Candida* cell suspensions were then spread onto agar plates. Antifungal discs (fluconazole (25g), amphotericin B (100g), itraconazole (10g), and voriconazole (1 g)) were positioned on the inoculated plates and were subsequently incubated at 37°C for a duration of 24-48 hours. The diameter of the inhibition zone was measured after the incubation period. The results were evaluated in accordance with CLSI.27,28

In this investigation, ATCC 10231 of *C. albicans* was utilised as the reference strain.

**RESULTS**

*C. albicans* was detected in 38(41.76%) of the 91 clinical isolates investigated, *C. glabrata* in 22(24.17%) isolates, *C. krusei* in 19(20.88%) isolates, *C. tropicalis* in 8(8.79%) isolates and *C. parapsilosis* in 4(4.39%) isolates. In the MTP assay, 11(12.09%) of the isolates were found to be strong formers, while 35(38.46%) isolates were moderate biofilm formers and 45(49.45%) of the clinical strains were either weak or Non-biofilm formers (Figure 2) (Table 2). According to the VT method, 19(20.88%) isolates were strong biofilm producers, 35(38.46%) isolates were moderate biofilm producers, and the remaining 37(40.66%) isolates were weak/non biofilm producers (Figure 3). By the CRA method, the majority of *Candida* isolates, 82% (90.11%), displayed a red colony shape with no dry crystalline consistency, 4 (4.39%) of the clinical isolates had black colonies with dry crystalline consistency, while 5 (5.49%) had red to pink colonies with dry crystalline consistency (Figure 4) (Table 3). Among the 91 clinical isolates, 33(36.36%) vaginal isolates were from pregnant women with complaints of vaginal discharge and 58(63.73%) isolates were from non-pregnant
women. *C. albicans* was detected in 15(45.45%) of 33 clinical isolates from pregnant women, while NCAC was found in 18(54.54%). *C. albicans* was found in 23(39.65%) vaginal isolates from non-pregnant women, while NCAC was isolated from 35(60.34%) clinical strains. Antifungal susceptibility profiles of clinical strains were evaluated for fluconazole, amphotericin B, itraconazole, and voriconazole. Fluconazole sensitivity was detected in 86(94.50%) clinical isolates, intermediate sensitivity in 02(2.20%) isolates, and resistance in 03(3.30%) isolates. Amphotericin B sensitivity was found in 49(53.85%) clinical isolates, with 23(25.27%) showing moderate sensitivity and

### Table 4. Antifungal susceptibility profile of *Candida* species

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<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
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<tbody>
<tr>
<td>Fluconazole</td>
<td>86(94.50%)</td>
<td>02(02.20%)</td>
<td>03(03.30%)</td>
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<tr>
<td>Amphotericin B</td>
<td>49(53.85%)</td>
<td>23(25.27%)</td>
<td>19(20.88%)</td>
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<tr>
<td>Itraconazole</td>
<td>16(17.58%)</td>
<td>24(26.37%)</td>
<td>51(56.04%)</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>89(97.80%)</td>
<td>00(00.00%)</td>
<td>02(02.20%)</td>
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More than half of the clinical strains were identified to be responsive as compared to MTP technique, our study found 11 (12.08%) high biofilm producers and 35 (38.46%) intermediate biofilm producers while 45 (49.45%) isolates didn’t form any biofilm. These results are similar to the prior studies made on biofilm production in VVC. More than half of the C. albicans were observed to develop biofilm. The VT technique found 19 (20.88%) strong biofilm formers and 35 (38.46%) intermediate biofilm formers. Furthermore, the VT method was more sensitive than the CRA method. In biofilms produced by certain clinical strains, it proved challenging to visually differentiate among moderate and weak/non-Biofilm formers applying the VT method. This aligns with the results of a previous investigation that compared various approaches for screening biofilm. More than half of the clinical strains were itraconazole resistant, and more than 20% of the isolates were amphotericin B resistant. Similar to the previous investigation, the vaginal Candida strains were identified to be responsive to Fluconazole and Voriconazole although many research have found increased fluconazole resistance in VVC, which contradicts the findings of the current study.

CONCLUSION

NCAC predominance was found in vaginal candidiasis. It is being consistently identified as the most predominant species followed by C. glabrata, C. tropicalis, and C. krusei. As a result, screening for biofilm formers, particularly NCAC, is considered imperative as it helps them to survive in adverse conditions, making the biofilm the most predominant species followed by C. glabrata, C. tropicalis, and C. krusei. As a result, screening for biofilm formers, particularly NCAC, is considered imperative as it helps them to survive in adverse conditions, making the biofilm resistant to various antifungals and helps them to survive in adverse conditions. It is also found that in vivo conditions are comparatively more favourable for rapid biofilm production.13 As a result, screening for biofilm formation in instances of VVC is critical, especially in recurring cases of vulvovaginal Candidiasis. A prior study found 44% biofilm formation in VVC, as well as an increased incidence of NCAC.15 By MTP technique, our study found 11 (12.08%) high biofilm producers and 35 (38.46%) intermediate biofilm producers while 45 (49.45%) isolates didn’t form any biofilm. These results are similar to the prior studies made on biofilm production in VVC. More than half of the C. albicans were observed to develop biofilm. The VT technique found 19 (20.88%) strong biofilm formers and 35 (38.46%) intermediate biofilm formers. Furthermore, the VT method was more sensitive than the CRA method. In biofilms produced by certain clinical strains, it proved challenging to visually differentiate among moderate and weak/non-Biofilm formers applying the VT method. This aligns with the results of a previous investigation that compared various approaches for screening biofilm. More than half of the clinical strains were itraconazole resistant, and more than 20% of the isolates were amphotericin B resistant. Similar to the previous investigation, the vaginal Candida strains were identified to be responsive to Fluconazole and Voriconazole although many research have found increased fluconazole resistance in VVC, which contradicts the findings of the current study.

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patients with VVC. Itraconazole resistance was found in more than half of the clinical isolates tested while the clinical strains were found to be sensitive to fluconazole. Information on antifungal susceptibility becomes crucial as prompt treatment is required as VVC causes inflammation and vulvovaginal pruritus. Fluconazole should continue to be the first-line of drugs prescribed by the clinicians because VVC isolates are very responsive to it, followed by Voriconazole, even though some studies show an increase in fluconazole resistance in VVC. Almost half of the clinical isolates tested positive for Strong and Moderate biofilm formers. Furthermore, the prevalence of biofilm producers continues to rise, indicating increased pathogenicity and antifungal resistance, emphasizing the significance of screening for biofilm formation in VVC.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS’ CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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