"Screening, Identification and the Molecular Profiling of Sap1 gene from Candida albicans Isolated from the Clinical samples of Vulvovaginitis, at a Tertiary Care Centre, Uttar Pradesh"

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Abstract:

Introduction: Candida albicans, along with other closely related Candida species, are the primary causative agents of vulvovaginal candidiasis (VVC)—a multifactorial infectious disease of the lower female reproductive tract. Isoforms of aspartyl proteinase (Sap), which are encoded by at least nine related SAP genes, have been implicated to be a major virulence factor of the opportunistic yeast Candida albicans in experimental infections.

Aim and Objective: Screening, Identification and the Molecular Profiling of Sap gene from Candida albicans isolated from the Clinical samples of Vulvovaginitis, at a Tertiary Care Centre, Uttar Pradesh.

Material and Methods: A cross sectional study was conducted from August 2021 to August 2022 for a period of 1 year in the Department of Microbiology, at RMCH&RC. A total of 200 vaginal swabs was collected from patients in the reproductive age period presenting to the Obstetrics and Gynecology outpatients transported to the Microbiology lab.Vaginal swab specimens was subjected to direct Gram-stained smear examination as well as culture on Sabouraud dextrose agar (SDA) (Oxoid, UK) incubated at 37 °C for 24–48 h. The Candida isolates was tested by disk diffusion method using Muller-Hinton agar supplemented with 2% glucose and 0.5µg of methylene blue/mL. The DNA Extraction for the detection of Sap gene was done using the QiaAmp DNA Extraction kit and further confirmed by PCR.

Results: In our study a total of 200 clinically suspected cases of VVC were presented during the study period for 1 year in which 36 HVS (18%) yielded positive yeast on culture. The maximum number of positive cases of VVC was found in the age group of 21-30 years of age. The Direct microscopic examinations of the Gram's smear of HVS revealed budding yeast cell in only 11 (30.5%) cases. Majority of high vaginal swabs revealed few pus cells (< 5 pus cells/HPF). Candida glabrata was the most common isolate followed by Candida tropicalis, and Candida albicans. 83.3% isolate was susceptible to fluconazole. A total 16% isolates of Candida spp. was found to be fluconazole resistant of which 4 strains of C. krusei, 1 strains of C. glabrata and 1 strain of C. tropicalis. The DNA was isolated for the detection of Sap gene in Candida albicans using the QiaAmp DNA extraction kit followed by the PCR.

Conclusions: According to the point that excessive use of antifungal drugs without prescription for treatment of vaginal infection can lead to induction of Candida resistance, correct identification of Candida species could play an important role in treatment of VVC

Keywords: VVC, Sap gene, SDA

Introduction

Vulvovaginal candidiasis (VVC) is a frequent infection in females at the reproductive (FRT) age caused mostly by the polymorphic opportunistic fungus Candida albicans. [1], a member of the normal human microbiota. C. albicans commonly colonizes the vaginal lumen asymptomatically [2]. Candida is the second most common cause of vaginal infection after bacterial vaginitis [3]. In addition, the correct identification and differentiation of etiological agents is very important for early treatment and preventing of invasion [4].

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While C. albicans is the causative agent of over 90% of VVC cases, other non-albicans Candida (NAC) species have been identified as etiological agents [5]. For a long time, traditional microbiological procedures, such as morphology and biochemical methods, were used to identify Candida species, yet these methods are time-consuming, inaccurate to identify, and lacking the necessary validity [6]. Several DNA-based method, have been developed to improve the identification of fungi species [7]. The ITS regions of rDNA contains highly variable nucleotide sequences that have been used for identification of Candida species in the PCR assay. Secreted aspartyl proteases (Saps) are enzymes that are secreted by C. albicans and are coded for by the SAP gene family (SAP1-SAP10) [8]. The proteolytic activity of the Sap proteins is involved in the degradation of the host's barriers during infection [9], immune response evasion [10], and adhesion to the host's cells [11]. The present study is undertaken to

study the SAP1 gene as it appear to be essential for mucosal [12] and systemic infections (SAP4- SAP6) [13]. The expression and importance of SAP1 gene was detected by using PCR.

Material and Methods

This was a cross sectional study carried out in the Department of Microbiology, RMCH&RC, Mandhana for a period of 1 year i.e, August 2021 to August 2022. The study was approved by the Ethical Committee and the informed consent was obtained from all individual participants included in the study.

Specimen collection and Processing

High vaginal swabs was collected from 200 married patients in the reproductive age period presenting to the Obstetrics and Gynecology outpatients Dept. Patients who was non-married, outside the reproductive age period or using any systemic or local antifungal therapy in the previous month was excluded from the study. Vaginal swab specimens was subjected to direct Gram-stained smear examination as well as culture on Sabouraud dextrose agar (SDA) (Oxoid, UK) incubated at 37 °C for 24–48 h. Isolates on SDA was identified as Candida by colony morphology and Gram staining.

Phenotypic identification of Candida species

Candida isolates was identified phenotypically by germ tube test (GTT), Rice Tween-80 agar performed as described in previous studies [14] in addition to Chrom ID Candida Agar (CAN2) (BioMérieux, France) and API 20C AUX (BioMérieux, France), which was performed according to the manufacturers' instructions. The Candida isolates was then stored in glycerol broth at −70 °C for further processing by PCR-restriction fragment length polymorphism (PCR-RFLP). Genotypic identification of Candida species: Genotypic identification by PCR was used as the gold standard method for Sap gene in Candida albicans species [15].

DNA extraction: The DNA extraction was performed using QIAamp DNA Mini kit (Qiagen) as per the manufacture's guidelines. Amplified PCR products was run on 2% agarose gel electrophoresis and visualized by UV transilluminator (BiometraTi 3) [16].

PCR

The Primers used to amplify SAP1 fragment was 5′ATCAAGCTTTAAAAAGAAGTGGGGATTGAAG AG-3′ and 5′-GATCCTCGAGAGTTTATTATTTGGTAGAGATTG-3′ [17]. The identified by PCR based on the method proposed by Bassyouni et al. (2015) [18], with some modifications was done. Reactions was performed at a final volume of 25 µl, containing approximately 20 µg of DNA, 12.5 µl of GoTaq Hot Start Green Master Mix (Promega, Madison, Wisconsin, USA) and 0.75 µl (20 pmol/µl) of each specific primer. Amplification conditions for SAP1 gene was: initial denaturation at 94°C for 3 minutes, 30 denaturation cycles at 94°C for 30 seconds, annealing at 46°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes. Amplification products was analyzed on 1% agarose gel electrophoresis containing DNA stain (Promega, Madison, Wisconsin, USA) and visualized under ultraviolet light [19].

Antifungal susceptibility testing

The Candida isolates was tested by disk diffusion method using Muller-Hinton agar supplemented with 2% glucose and 0.5 µg of methylene blue/mL . The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to 0.5 McFarland standards. The following antifungal disks were used: fluconazole (25 µg), voriconazole (1 µg), ketoconazole (50 µg), clotrimazole (50 µg), miconazole (50 µg) and amphotericin B (100 µg) (BioRad). Inhibition zones was interpreted using validated CLSI interpretive break points for fluconazole and voriconazole, while for other drugs, the interpretive break points was adopted from published studies [20].

Results

Vulvovaginal Candidiasis (VVC) is a common medical health problem of adult women. In our study a total of 200 clinically suspected cases of VVC were presented during the study period for 1 year. There was 36 HVS (18%) yielded positive yeast on culture. All positive yeasts were inoculated on both the culture medium – SDA and Candida CHROMagar. The maximum number of positive cases of VVC was found in the age group of 21-30 years of age followed by 31 to 40 years. Out of the 36 HVS positive yeast culture there was 19 (52.7%) patients lying in the age group of 31-40 years of age and 14 (38.8%) patients in the age group of 31-40 years.

Table 1: Age wise distribution of positive cases of VVC

S.N.	Age group (Years)	Total number (N=36)	Percentage %
1.	20-30	2	5.5%
2.	31-40	19	52.7%
3.	41-50	14	38.8%
4.	51-60	1	2.7%

The Direct microscopic examinations of the Gram's smear of HVS revealed budding yeast cell in only 11 (30.5%) cases. All of them yielded *Candida* on culture. Majority of high vaginal swabs – 40 (80%) revealed few pus cells (< 5 pus cells/HPF). In our study SDA and Candida CHROMagar were able to isolate yeast in all positive cases. In our study we also observed 5 to 10 pus cells/HPF. In the present study the most common isolate was *Candida glabrata* observed in 12 cases, followed by 11 cases of *Candida tropicalis*, 11 cases of *Candida albicans*, 2 cases of

Candida krusei infections. All 11 isolates of *C. albicans* was germ tube test positive.

Table-2 Distribution according to the type of Isolates

S.N.	Type of Isolates	Number of Isolates	Percentage %
1.	*Candida glabrata*	12	33.3%
2.	*Candida tropicalis*	11	30.5%
3.	*Candida albicans*	11	30.5%
4.	*Candida krusei*	2	5.5%

From our study we found that the antifungal susceptibility testing revealed that, 30 isolates (83.3%) was susceptible to fluconazole. A total 16% isolates of Candida spp. was found to be fluconazole resistant of which 4 strains of *C. krusei*, 1 strains of *C. glabrata* and 1 strain of *C. tropicalis*.

All the isolates of *C. krusei* were considered resistant to fluconazole as it is intrinsically resistant to fluconazole. There were 4 isolates (11%) of S-DD to fluconazole which include 3 of *C. glabrata* and one of *C. tropicalis*. All isolates of *C. albicans* was susceptible to fluconazole. All *Candida spp.* was susceptible to voriconazole. In case of *C. glabrata*, 4 out of 12 isolates was found resistant (24%) to fluconazole. Among the *C. tropicalis* isolates 9 isolate was susceptible (81%) and 2 isolate (18%) each of S-DD and resistant to fluconazole.

The DNA Extraction for the detection of Sap1 gene in *Candida albicans* was done using the QiaAmp DNA extraction kit followed by the PCR.

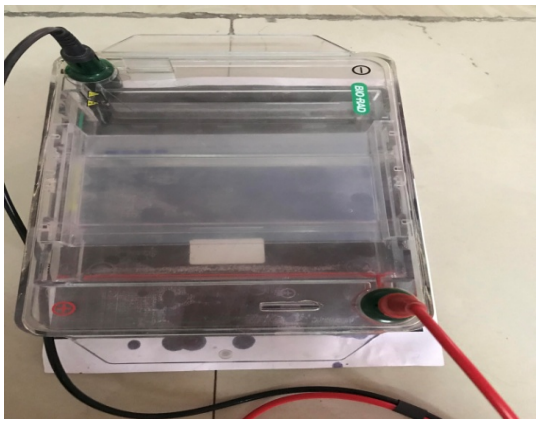

Figure No.1: Gel Electrophoresis for the DNA Extraction

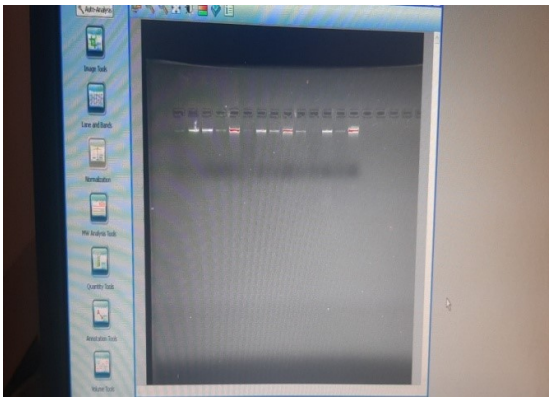

Figure No 2: DNA Extraction

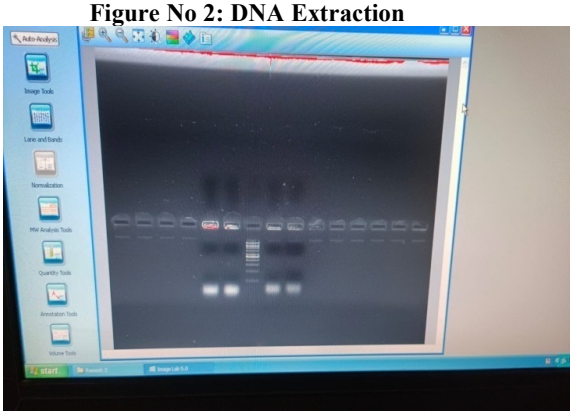

Figure No 3: Photograph of Amplified Sap1 gene in *Candida albicans*, the amplified DNA band size was obtained 161 bp, L corresponding to 100bp ladder used; Lane 1 is the Positive Control; Lane 2,3 and4 is the sample positive for sap Gene ; Lane 5 is the Negative control and Lane 6 is the sample negative for Sap gene.

Discussion

Vulvovaginal Candidiasis (VVC) is a common medical health problem of adult women. It is caused by overgrowth of Candida species in the vagina [20] is most commonly caused by Candida albicans. The manifestations of VVC may range from asymptomatic colonization to severe acute symptomatic infection. Although it has a wide clinical presentation but there is no sign or symptoms which are pathognomonic of VVC [21]. Therefore, laboratory support may be required for accurate diagnosis. In our study a total of 200 clinically suspected cases of VVC was studied in which 36 cases of HVS (18%) yielded positive yeast on culture. This study was similar to the study performed by the other author where the positive VVC cases was found similar to our finfing[22-23]. In our study maximum number of positive cases of VVC was found in the age group of 21-30 years of age followed by 31 to 40 years. Our finding was parallel with the other study where the maximum number of cases recorded was in the age group of 21 to 40 years[22] [24].

The Direct microscopic examinations of the Gram's smear of HVS revealed budding yeast cell in only 11 (30.5%) cases. All of them yielded Candida on culture. Majority of high vaginal swabs – 40 (80%) revealed few pus cells (< 5 pus cells/HPF) in our study, which was in support with the study performed by the other author Sulaiman SP et al [25].

In the present study the most common isolate was Candida glabrata observed in 12 cases, followed by 11 cases of Candida tropicalis, 11 cases of Candida albicans, 2 cases of Candida krusei infections. All 11 isolates of C. albicans was germ tube test positive. There was many studies parallel and contract to our present findings. A study from Goswami R et al. observed C. glabrata as the most common species isolated [26]. The current study is among the few study from India which depicted isolation of C. glabrata as more than 50% of VVC. Mohanty S et al. and Ray D et al., from AIIMS, New Delhi, India, observed 50.4% and 61.3% VVC by C. glabrata respectively [27,28]. The high isolation of C. glabrata in our hospital may be due to the fact that complicated cases, chronic cases or unresponsive cases of VVC may be presented at this tertiary care hospital. There were many other studies performed by the authors where the Candida albicans was the most predominant isolate [29-31]. Raghunathan L et al., [24] recently studied AFST of 40 vaginal Candida isolates at Puducherry by E-test method and observed that 87.5% were susceptible to fluconazole, 7.5% were S-DD and only 5% were resistant. The increased resistant against fluconazole in the current study was mainly because of high isolation of C. glabrata and C. krusei strains which have increased resistance against fluconazole.In our study a total 16% isolates of Candida spp. was found to be fluconazole resistant of which 4 strains of C. krusei, 1 strains of C. glabrata and 1 strain of C. tropicalis. There are several studies reported on fluconazole resistance in C. glabrata [32].

Conclusion

It is important to know at least the causative agent as C. albicans or NAC so that accordingly fluconazole based regimen or non-fluconazole based azole regimen respectively can be provided. There should be continuous monitoring of antifungal susceptibility testing to guide empirical treatment and in vitro susceptibility testing should be required for NAC isolates to guide appropriate treatment of VVC because recurrent vulvovaginal candidiasis has become a challenge which is difficult to be treated due to drug resistance.

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