Original Research Article

Detection of mutated erg11 and fks1 genes among resistant *Candida* species isolated in pregnant women in Mbarara, Uganda

ABSTRACT

Background: Vulvo vaginal candidiasis is a universal health hazard that contributes to significant morbidity in pregnant women. The emergency of anti-fungal resistance in candida species against azoles and caspofungin is a rising concern because there is a limited range of choices of antifungals to be used in pregnant women with low toxicity. In Uganda, there is limited data regarding in vitro phenotypic and genotypic anti-fungal susceptibility patterns among candida species isolated from pregnant women. Thus, this study aimed to screen for the two mutated famous erg11 and fks1 genes that lead to anti-fungal resistance among clinical candida isolates.

Methods: A laboratory-based, cross-sectional study involving 90 candida species isolates previously collected from a larger study. Phenotypic susceptibility methods (Kirby-Bauer and minimum inhibitory concentration) while Polymerase chain reaction method and electrophoresis were used for detection of the amplified mutated ERG11 and FKS1 genes. Mean and chi-square tests were used to evaluate the associations of resistance patterns between resistant and susceptible isolates.

Results: Out of the 90 candida isolates recovered, 56% (50) were *Candida albicans* and 44% were non-*Candida albicans*. All the candida isolates were susceptible to Caspofungin while fluconazole resistance was 34.4%. The FKS1 mutated gene was not detected in randomly selected caspofungin susceptible isolates. The ERG11 mutated gene was detected in 80.6% of the fluconazole-resistant isolates and 87.5% of the isolates with intermediate activity towards fluconazole.

Conclusion: This study demonstrates that there is significant evidence that the mutated erg11 gene causes reduced in fluconazole drug susceptibility (*p-value*0.001). Susceptible dose dependence should not be ignored as it may be associated with ERG11 gene mutation leading to resistance to fluconazole.

Key words; erg11, fks1, Azoles, Echinocandins, Candida species

INTRODUCTION

Vulvo vaginal candidiasis (VVC) represents a universal clinical hazard that contributes to significant morbidity in pregnant women. Globally, vulvovaginal candidiasis is estimated to range between 35-60% among pregnant women (1, 2)while in Uganda, the burden of vulvovaginal candidiasis is estimated to be 60% (3). Indeed, VVC is a long-standing infection in this patient cohort. This is because, pregnancy increases the risk of vulvovaginal candidiasis and this may be attributable to the rise of progesterone and estrogen levels during pregnancy, especially in the final trimester (4). These two hormones are associated with a destabilization of the vaginal epithelial cells' integrity which affects *Candida albicans* colonization and thriving as a normal flora (Maruyama et al, 2017), on the other hand, progesterone suppresses neutrophil anti-*Candida* function (5). Undeniably, Candidiasis commonly occurs during a healthy pregnancy without seriously harming the fetus. However, untreated VVC during pregnancy can cause complications such as chorioamnionitis, which can result in abortion and preterm, neonatal congenital infection, and pelvic inflammatory disease (6). The etiology of VVC seems to be primarily dominated by *Candida albicans*, which is also considered normal flora in the vulva and vagina. However, certain health conditions lower the host's immunity, such as but not limited to pregnancy, increased antibiotic use, and corticosteroid therapy. *C. albicans* usually become pathogenic in the host and; are implicated in some of the most fetal opportunistic fungal diseases. Additionally, over the past 30 years, the etiology of VVC has been expanding and now includes non albicans candida (NACs) species such as *C. glabrata*, *C. tropicalis*, *C. dullness*, and *C. krusei*(7).

Unfortunately, VVC infections are associated with widespread asymptomatic colonization that can persist for years turning chronic in most cases (8). This has majorly been attributed to the ability of Candida species to symbiotically interact with the vaginal microbiota. The emergence of antifungal resistance in Candida species against azoles is due to the overexpression of the ERG11 gene which results in the production of a large amount of lanosterol 14α-demethylase which favors the continuous synthesis of ergosterol and maintenance of the integrity of the cell membrane (9). The resistance towards echinocandins is due to mutations in the FKS 1 gene resulting in amino acid changes in the proteins necessary and sufficient to confer reduced susceptibility to echinocandins (9).

From this standpoint, we hypothesized that susceptibility testing for antifungals is necessary for patients who raise suspicion index of VVC infection with specific etiology based on disease manifestation and clinical correlation. Understanding the anti-fungal susceptibility patterns of candida is crucial in guiding the successful treatment of vulvovaginal candidiasis (10). Yet, there is a limited range of choice of antifungals to be used in pregnant women with low toxicity; with a notably increasing resistance spectrum among the drugs of choice in our Ugandan local communities (11). In Uganda, there is limited data regarding in vitro phenotypic and genotypic anti-fungal susceptibility patterns among candida species isolated from

pregnant women. Thus, this study aimed at screening for the two mutational famous **erg11 and fks1 genes** that lead to anti-fungal resistance among clinical candida isolates.

MATERIALS AND METHODS

Study design and setting

This was a laboratory-based, cross-sectional study that included anti-fungal susceptibility analysis of candida isolates resistant to fluconazole and caspofungin and amplification of mutated ERG11 and FKS1 genes. The study was conducted at Mbarara University of Science and Technology in the Department of Medical Microbiology, mycology laboratory.

Retrieval of Archived *Candida* isolates

A total of 90 clinical isolates used were previously collected from pregnant women using high vaginal swabs, processed, and kept at -80°C from a prior investigation involving anti-fungal susceptibility patterns of vulvovaginal candida species among women attending antenatal clinic at Mbarara regional referral hospital, South Western Uganda (12) were retrieved following standard operating procedures.

Identification of *Candida* species

Preserved yeasts were sub-cultured on Sabouraud dextrose agar medium containing 50mg/ml of gentamycin, and the inoculated plates were incubated at 37°C for 24 to 48 hours. Further identification was performed by employing the Germ tube test and growth on Chromogenic Candida agar(HIMEDIA, India) growth (13).

Antifungal Susceptibility Testing

Antifungal susceptibility testing of the isolates was done by agar diffusion method for fluconazole (50g/ml) while caspofungin (6 to 0.045µg/ml) anti-fungal testing was determined by agar well diffusion according to the standards of the Clinical and Laboratory Standards Institute (CLSI) M27M44S-Ed3 (CLSI, 2022).Mueller-Hinton agar supplemented with 2% w/v glucose and 0.5 g/ml methylene blue dye was used for determining the anti-fungal susceptibility *of C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 4072 were used as controls.

Detection of FKS 1 and ERG11 mutated genes

DNA extraction

The chemical method (CTAB method) was used in candida DNA extraction. Polymerase chain reaction (PCR) amplification was used to confirm the existence of mutation of the ERGI1 gene was amplified using the following primers; 5'-CAAGAAGATCATAACTCAAT3' and 3′AGAACACTGAATCGAAAG-5′. The PCR

master mix consisting of 2.5 µL 10x standard PCR buffer,1.0 µL dNTPs, and 0.5 µL Taq polymerase; 1.0 µL forward, 1.0 µL reverse, 3 µL DNA template and 16µL PCR water making up to 25µL final reaction volume was prepared.

The PCR conditions were as follows; 94°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 70°C for 1 minute, and final extension at 72°C for 10 minutes.

During the PCR run, a positive control, the housekeeping actin gene primer was used as a positive control. DNA Amplicon was electrophoresed using 1.0% agarose gel, in 1x Tris-Borate EDTA buffer, 5µL Safe View ClassicTM DNA stain, 6x loading dye, and DNA ladder/marker 1000 bp for ERG11, and the gel electrophoresis was performed at 100V and 80mA for 1.3 hours. Bands were visualized using the Gene-Flash Trans-illuminator (14).

Primer detection of FKS1

The presence of FKS1 was established using PCR amplification using the following primer sets.

F641 FKS1 Mutation detection AATTGGTTGAATCTTATTTCTT Sense

S645 FKS1 Mutation detection CTAATAGGATCTCTTAAAGA Antisense

D648 FKS1 Mutation detection CGACAAGTTTCTAATAGGATC Antisense

P649 FKS1 Mutation detection GACATTGTCTTTAAGAGATCC Sense

R1361 FKS1 Mutation detection CGTTGATTGGATTAGACG Sense

The PCR master mix was prepared as follows: 5X of the multiplex PCR master mix; 1.0 µL of each primer, 5 µL DNA template, and 6µL RNAase-Free-H2O making up to 25µL final reaction volume. The following cycling temperature profiles were used for the PCR amplification in a conventional PCR Thermocycler (MultigeneOptimax): 94°C for 4 minutes of initial denaturation, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds of annealing, 70°C for 1 minute of elongation, and 72°C for 10 minutes of final extension. DNA amplicon was electrophoresed using 1.0% agarose gel, in 1x Tris-Borate EDTA buffer, 5µL Safe View ClassicTM DNA stain, 6x loading dye, and DNA ladder/marker 100bp for FKS. Electrophoresis ran at 100V and 80mA for 1 hour. Bands were visualized using the Gene-Flash Trans-illuminator (14) and the photos taken.

Statistical Analysis

The acquired quantitative data was double-checked for accuracy. The data for resistant and susceptible isolates were compared using chi-square, and the results were shown as mean ± SD. To evaluate statistical significance, a P-value of 0.05 was utilized. STATA 17.0 (StataCorp Texas USA) was used to perform all statistical computations on the susceptibility pattern of the vulvovaginal candida isolates.

RESULTS

Among the 90 Candida isolates analyzed, 50 isolates (55.56%) were identified as *C. albicans*. Furthermore, out of the 40 non-*Candida albicans* isolates that were sub-cultured on Chromogenic agar, *C. parapsilosis*accounted for 4.44% (4/90). *C. glabrata* accounted for 31.11% (28/90). *C. tropicalis* accounted for 1.11% (1/90), *C. krusei* accounted for 3.33% (3/90) and *C. famata* accounted for 4.4% (4/90) (see Figure 1).

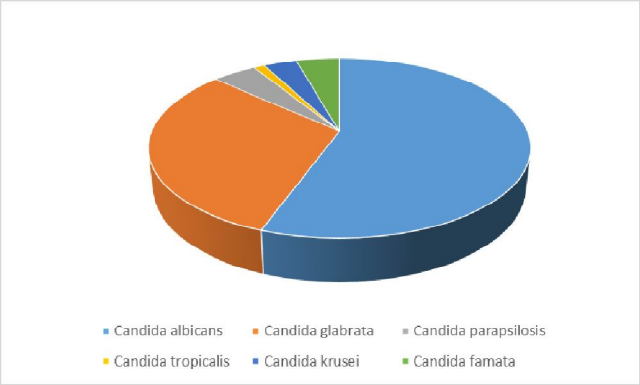


Figure 1:Candida species distribution. Majority of the isolates were *Candida albicans* (56%) while *C. tropicalis* being the least isolated.

In vitro Susceptibility Profile of the Candida Species to Fluconazole

In this study, we observed that 51 (56.7%) of the isolates of candida were susceptible to fluconazole, 8 (8.9%) had intermediate susceptibility and 31 (34.4%) candida isolates were resistant. *Candida krusei* and *Candida tropicalis* were the most susceptible (100%). *Candida parapsilosis* and *Candida famata*showed fluconazole resistance of 50% each while *Candida albicans and Candida glabrata* demonstrated 34% and 35.7% fluconazole resistance respectively, as indicated in Table 1 below.

Table 1: In vitro susceptibility profile of the Candida Species to Fluconazole (50 µg)

Species	**Total number**	**S (%)**	**I (%)**	**R (%)**
Candida albicans	50	29 (58%)	4 (8%)	17 (34%)
Candida krusei	3	3 (100%)	0 (0%)	0 (0%)
Candida famata	4	2 (50%)	0 (0%)	2 (50%)
Candida glabrata	28	15 (53.57%)	3 (10.71%)	10 (35.71%)
Candida parapsilosis	4	1 (25%)	1 (25%)	2 (50%)

Candida tropicalis	1	1 (100%)	0 (0%)	0 (0%)

Key: S= Susceptible, I = Intermediate, R= Resistant

In vitro Susceptibility Profile of the *Candida* Species to Caspofungin.

All the Candida isolates were susceptible to caspofungin at a minimum inhibition concentration of 0.009µg/ml. However, the concentration of 6.125 micro-grams was the most effective and the concentration of 0.0045 micro-grams was the least effective as shown in figure 2.

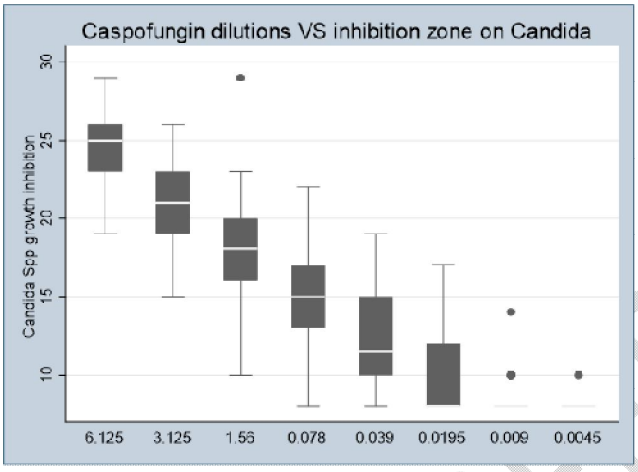


Figure 2: MIC Values of Caspofugin

Screening fungal isolates for mutated ERGII gene

After the PCR run, 25 resistant isolates showed clear bands while 7 intermediate isolates had clear bands on the DNA stain and trans-illuminated agarose gel. PCR amplified fluconazole-resistant and intermediate candida isolates (figure 3 and 4). Presence of the ERG11 gene was 80.6% and 87.5% respectively.

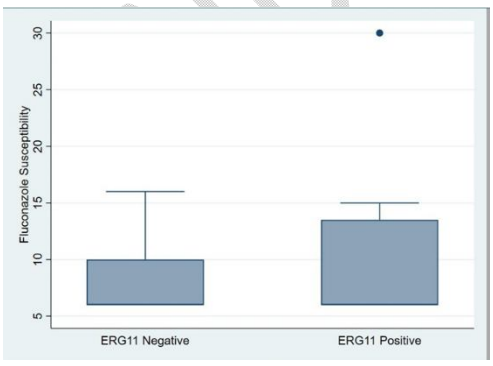


Figure 3: Prevalence of mutated ERG11 gene. The fluconazole resistant and intermediate isolates which were tested for the presence of mutated ERG11 gene.Presence of the mutated ERG11 gene was 80.6% and 87.5% respectively.

PC NTC 1 2 3 4 5 6 Ladder

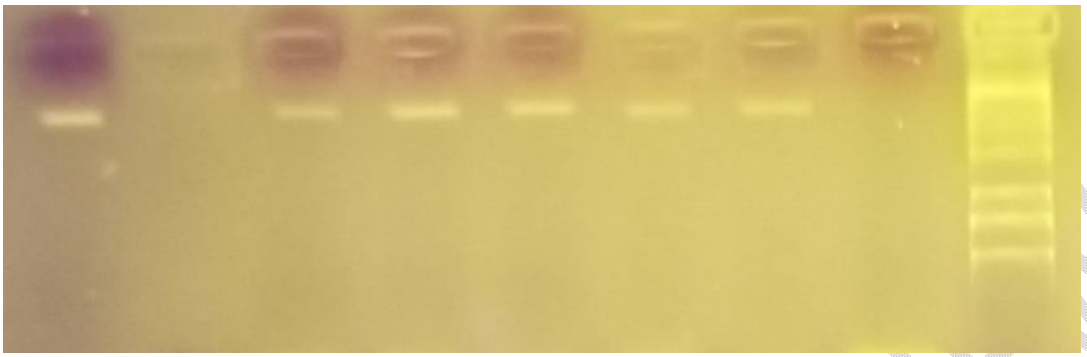
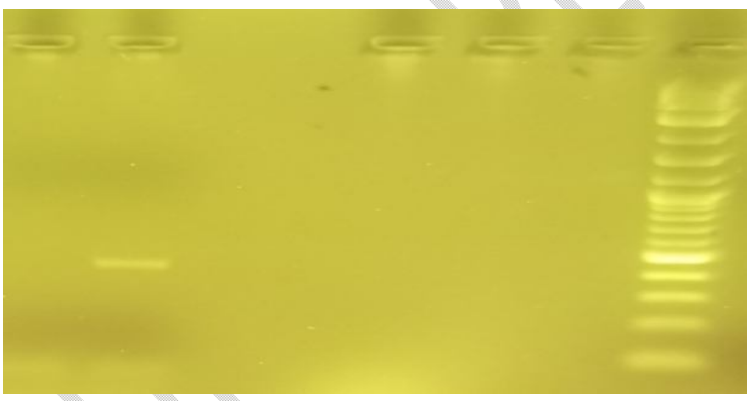


Figure 4: Amplification of ERG11 Gene: PC (Positive Control), NTC – (Non-Template control); wells labeled 1-5 have bands for *ERG11* gene positive isolates, 1Kb Ladder (ERG11-1587bp)

Screening fungal isolates for FKS1 gene

Candida species may have mutations in the FKS1 gene and this leads to echinocandin resistance. Amplification of the FKS1 450base pair of randomly selected 10 susceptible isolates was done. However, none of the isolates showed the presence of the gene (figure 5).



1 2 3 4 5 6

Figure 5: FKS1 gel electrophoresis. Wells 1 & 2 are No template control and positive control respectively. Wells 3, 4 and 5 are clinical samples representatives. Well 6 is the 100Bp ladder (FKS 641Bp). No FKS1 genes were detected among the clinical isolates.

DISCUSSION

The primary opportunistic yeast infection, vulvovaginal candidiasis (VVC), is caused by many Candida species, primarily *Candida albicans*(15, 16). Due to the sugar content and the altered pH of vaginal secretions, vaginal candidiasis is linked to pregnancy. If not managed, vaginal infections can lead to infertility, abortion, premature birth, fallopian tube scarring, postpartum infections, and systemic inflammation (15).

Candida albicans had an overall resistance profile of 34.4% towards fluconazole and *C. glabrata* showed a resistance of 35.7% among the most prevalent resistant strains. Our findings here are in agreement with several other studies including but not limited to 48.1% in Ghana (17), 100% in Iran (18),11.9% in Nigeria (19), and 23% in Sudan (20). However, a prevalence of 0% resistance against fluconazole in 2018 in Arua regional referral hospital, West Nile Region of Uganda (21). In most of these studies, *Candida albicans* stands out as the most resistant strain at an average resistance rate of 54.84% whilst non-albicans candida species are averagely reported at 45.16%. Thus, the susceptibility differences seen here could be associated with the differences in the accessibility of fluconazole in the various locations. *C. glabrata* also appears to be the most resistant non-albicans candida species with an average resistance level of 7.4%. *C.glabrata* azole resistance is known to be associated with selection pressure.

The azole resistance molecular pathways in Candida are heavily reliant on the mutation of the ERG11 gene. ERG11 mutated gene detection of the resistant and intermediate strains was 80.6% and 87.5% respectively. Such a relatively high rate of gene mutation indicates the current overuse of azoles in the absence of a thorough laboratory diagnosis and an authorized medical prescription in the management of vaginal infections in Uganda. Studies conducted by Ikenyi(19) in Nigeria and by Hnaya in South Africa (22), showed a higher prevalence (100%) in ERG11 gene mutation of resistant isolates. This may be due to repeated treatment with the same drug that has resulted in azole-resistant clinical isolates with increased mutations. In contrast, a study conducted in Burkina Faso showed a lower prevalence of 9.79% (23) and 11.1% in Ghana (24). These findings may be due to the difficulty to access fluconazole hence minimal misuse of the drug. Fluconazole is fungistatic therefore constant exposure provides the opportunity for acquired resistance.

However, the inability to fully sequence the ERG11 gene to identify the actual mutations that predominate in our clinical environment limited the scope of our analysis. Additional research should attempt to accomplish this. Additionally, other than the ERG11 and FKS1 genes, the study was unable to examine additional causes of specific antifungal resistance in the drugs used.

CONCLUSION

This study shows the increasing prevalence of fluconazole resistance among vaginal Candida species. Caspofungin was the most effective drug with 100% susceptibility. This study demonstrates that there is significant evidence that the erg11 mutated gene causes a reduction in fluconazole drug susceptibility (p-value = 0.001). Susceptible dose dependence should not be ignored as it may be associated with ERG11 genes leading to resistance to fluconazole resistance.

Testing for *Candida spp*. Antifungal susceptibility should be a requirement during VVC management to guide prescription. We recommend continued surveillance and comprehensive epidemiological investigation to assess the extent of the distribution of ERG11 genes.

ETHICAL STATEMENT

The research proposal was approved by the Mbarara University of Science and Technology Ethical Review Committee, reference number; MUST-2021-147. Permission to use the stored isolates was granted by Mr. Kiguli James, the PI of the study from which these isolates had been archived with storage number; (IRB NO.02/09/12).

AVAILABILITY OF DATA AND MATERIALS

The analyzed datasets are available from the corresponding author upon request.

CONSENT FOR PUBLICATION

Not applicable

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