Azole Resistance and Detection of the ERG11 gene in Clinical *Candida albicans* Isolated from Pregnant women with vulvovaginitis attending Federal Medical Centre, Yenagoa, Nigeria

Type of Article: Original Research Article.

Abstract

Introduction: *Candida albicans* is one of the most important aetiological agents causing vaginal candidiasis in pregnant women. Most women will experience at least one episode during their reproductive years. Antifungal resistance is a particular problem with Candida infections. Some types of Candida are increasingly resistant to the first-line and second-line antifungal medications.

Objective: To investigate the azole susceptibility of *Candida albicans* (*C. albicans*) from pregnant vulvovaginal candidiasis patients and to detect *ERG11* gene in these azole resistance isolates.

Methods: Forty-one clinical isolates of *C. albicans* were collected. Azole susceptibility was tested *in vitro* using microdilution techniques. The *ERG11* genes of 27 isolates of *C. albicans* (All resistant to azoles) were amplified using PCR method.

Results: Of the 67 isolates recovered, 41(61.19%) were *C. albicans*, of which 27 (65.85%) each, and 25(60.98%) were resistant to Fluconazole, Voriconazole, and Nystatin respectively. In total, *ERG11* genes were detected among 24(88.89%) of 27 *C. albicans* azole resistant isolates.

Conclusions: Twenty four *ERG11* genes were detected among 27 azole resistant *C. albicans* isolates, which indicates a possible relation with the increase in resistance to azole drugs and the recurrence of vulvovaginal candidiasis.

Key words: *Candida albicans*, *ERG11* gene, Azole resistance, Vulvovaginitis, Pregnant women.

Introduction

Of recent, there has been a marked increase in the frequency of azole treatment failures in patients with candidiasis and are being treated for long-term antimycotic therapy, this has posed a serious concern in its efficacious use in chemotherapy. Reasons had been that Candida can acquire multidrug resistance (MDR) during the course of the therapy [1, 2]. Various authors have documented that Candida species possessed different mechanisms of resistance to azole antifungal agents and these mechanisms are categorised mainly as (i) changes in the cell wall or plasma membrane, which can lead to impaired drug (azole) uptake [3, 4]; (ii) alterations in the affinity of the drug target *Erg11p* (lanosterol 14alpha-demethylase) especially to azoles or in the cellular content of *Erg11p* due to target site mutation or overexpression of the *ERG11* gene [4, 5, 6, 7] and (iii) the efflux of drugs mediated by membrane transport proteins belonging to the ATP-binding cassette (ABC) transporters, namely *CDR1* and *CDR2* or to the major facilitator superfamily (MFS) transporter, *CaMDR1* [8, 9]. Many such manifestations are associated with the formation of Candida biofilms, including those occurring on devices like indwelling intravascular catheters. According to Rodrigues and colleagues (2017) [3], and Sardi *et al*. [10], biofilm-associated Candida shows uniform resistance to a wide spectrum of antifungal drugs. Furthermore, studies conducted by Ksiezopolska and Gabaldón [1] revealed that a combination of different resistance mechanisms is responsible for drug resistance in clinical isolates of *Candida* species.

Flowers *et al*. [6] reported that in the modulation of the *ERG11* gene in the ergosterol biosynthetic pathway and the alteration of the *Erg11* protein targeted by azole antifungals contribute to azole resistance in *C. albicans*. The overexpression of *ERG11* transcripts, either by gain-of-function mutations (GOF) in the transcriptional regulator, *Upc2*, or increased chromosome 5 copy number (on which *ERG11* resides), can result in reduced azole susceptibility [11, 12, 13]. In addition, mutations

in the *Erg11* protein mediating lanosterol demethylation have been shown to alter the ability of azole antifungals to bind to and inhibit its activity and to result in enhanced resistance to this class of antifungal agents [14, 15, 16]. Previously, reports of mutations in *ERG11* have been demonstrated on three hot spot regions analogous to amino acids 105 to 165, 266 to 287, and 405 to 488, which are particularly tolerant to amino acid substitutions [17]. Investigators have also used several approaches, which includes: heterologous expression of mutant *ERG11* alleles in other microbial species (e.g. *Saccharomyces cerevisiae* and *Pichia pastoris*), enzyme inhibition with fluconazole (FLC) in cell extracts, and biochemical analysis [15, 16, 17, 18, 19] to demonstrate that *ERG11* mutations can contribute to azole resistance. While a number of different amino acid substitutions have also been associated with azole resistance [18]. This study was undertaken to investigate the azole susceptibility of the clinically isolated *Candida albicans* (*C. albicans*) from vulvovaginal candidiasis (VVC) patients to three (3) antifungal routinely used in gynaecological clinics and also to detect the presence of *ERG11* gene in these resistance isolates.

Materials and Methods

Collection of Specimens, Isolation and Identification

Aseptically, specimens (Higher Vaginal swab "HVS"-66, and mid- stream urine catch-36) were collected from 102 pregnant women attending the Obstetrics and Gynaecology outpatient clinics in the Federal Medical Centre (a tertiary public health institution) in Yenagoa with genital infections (vulvovaginitis) in accordance to the protocols of McGowan [20] and Wang *et al*. [21].

Patients using any systemic or local antifungal therapy in the previous month were also included in this study.

Collected specimens were transported to the Laboratory unit of the Department of Medical Microbiology and Parasitology, Faculty of Basic Medical Sciences, College of Health Sciences, Niger Delta University, Wilberforce Island for further analysis in accordance to standard procedures [20].

In the Laboratory, standard procedure was used in the inoculation of specimens. In brief, loop-full of the aseptically diluted HVS and urine specimen were aerobically cultured at 37^0C for 24–48 hour on Cystine-Lactose-Electrolyte Deficient (CLED) agar, Mannitol Salt (MSA) agar, MacConkey agar, blood agar medium (Biotech Laboratories Ltd. UK) for bacteriological isolates, while, the Sabouraud Dextrose Agar (SDA, Oxoid, UK), and CHROMagar Candida (CMA; CHROMagar Company, Paris, France) were streaked for the fungi isolates. Isolates recovered from both the HVS and urine specimens were stored in 20% glycerol at -84°C.

Isolates (yeasts) on SDA were presumptively identified phenotypically as Candida by colony morphology, Gram staining, chromogenic medium (CHROMAgarCandida®), and were confirmed as at the species level biochemically by the API 20C AUX yeast identification kit (bioM´erieux SA, Marcy l'E toile, France), and genetically by PCR in accordance with procedures described by Santos *et al*. [22] as briefly described below. *C. albicans* standard strain (ATCC 6258) was employed as the control.

DNA Extraction:

The fungal DNA was extracted by boiling as described by Oliveira *et al*. [23]. Prior to extraction, pure isolates were subcultured in Luria-Bertani (Merck, USA) broth and incubated for 24 hours. Broth cultures were transferred to 2.00mL Eppendorf tubes. Then, tubes were centrifuged at 10,000 rpm for 1 min and the supernatant was discarded. To dislodge the sediments, 1.5mL sterile saline was added to the sediments and vortexed for a few seconds. This procedure was repeated twice. The tubes

were then transferred to a heating block at 95^0C and were heated for 20 minutes, after which they were fast freeze in a freezer (Thermocool, Nigeria) for 10 minutes.

The tubes were spun again for a minute and 300µgL of the sediment was picked and transferred to a new 1.5mL Eppendorf tube as the DNA extract. The extracted DNA concentration was quantified by spectrophotometry. Two µg/L of DNA extracted from each sample were placed directly on the spectrophotometer (NanoDrop, 2000, Thermo Cientific, USA) and measures in 260nm. The system software provides the DNA concentration in ng/µgL (software installed on a desktop computer).

PCR amplification for *Candida albicans* and of the *ERG11* gene

For genetic confirmation of the identified Candida isolates, the amplification reaction was performed following protocols reported by Vijayakumar *et al*. [24]. The ITS-1 and ITS-2 regions of *Candida spp*. were amplified using universal primers (Table 1). The amplification was performed in GeneAmp PCR Systems 9700 Thermal cycler (AB Applied Biosystem, USA) as previously published with modifications in the concentration of each primer (50 pmol/ reaction) and DNA template (5 lL extracted DNA/reaction), in addition to change the annealing temperature (53^0C).

The amplification of the *ERG11* gene was made using the following primers (Table 1). A 25µg/mL PCR mix was amplified with the following conditions: Initial denaturation at 94^0C for 4 minutes, denaturation at 94^0C for 30 seconds, annealing at 55^0C for 30 seconds, extension at 72^0C for 1 minute and final extension at 72^0C for 4 minutes. Amplified PCR products were run on 1.5% agarose gel electrophoresis and the DNA bands were visualized by UV transilluminator (BiometraTi 3) and photographed. The polymerase chain reaction (PCR) method was performed for amplification of genes with specific primers shown in table 1.

Table 1: Primers used in PCR

Gene	Orientation	Sequence 5′ to 3′	Reference
rDNA	ITS1 FW	5′-TCC GTA GGT GAA CCT GCG G-3′	White *et al.* [25]
	ITS4 RV	5′-TCC TCC GCT TAT TGA TAT GC-3	
ERG11	FW	5′-GTTGAAACTGTCATTGATGG-3′	Martínez *et al.*[26]
	RV	5′-TCAGAACACTGAATCGAAAG-3′	

Antimycotic susceptibility tests

The broth dilution susceptibility test was performed as recommended in the Clinical Laboratory Standards Institute (CLSI) M27-A3 reference document [27]. The antifungal agents used were Fluconazole (Sigma, UK), Nystatin (Sigma Aldrich, Steinheim, Germany) and Voriconazole (Sigma, UK).

The interpretive breakpoints for susceptibility assays were as follows. *C. albicans* strains showing minimum inhibitory concentrations (MICs) of ≤8µg/mL, ≤16µg/mL and ≤1 µg/mL with fluconazole, nystatin, and voriconazole, respectively were considered as susceptible (S). Strains with MIC values of ≥64 µg/mL, ≥16µg/mL and ≥4 µg/mL with fluconazole, nystatin and voriconazole, respectively were considered as resistant (R). *C. albicans* ATCC6258 is used as control strains.

Ethical Clearance

The study was approved by the Research and Ethical Committee of The Federal Medical Centre, Yenagoa (Ref. No. FMC/REC/19/013). Informed consent was also obtained from all individual participants included in this study.

Statistical Analysis

SPSS for Windows (version 20.0; SPSS) software was used for the analysis. Frequency distribution, mean, harmonic mean, standard deviation, analysis of variance (ANOVA) were determined. Categorical variables were compared by using Pearson's chi-squared test ($\chi 2$) or Fisher's exact probability tests. P-values were calculated and $P \leq 0.05$ was considered statistically significant

Results

Sixty-seven (65.69%) of the genitourinary specimens collected from the 102 pregnant outpatients' women attending the facility for suspicion of having vulvovaginitis during the period of study yielded significant microbial growth. As shown in Figure 1, of these 67 recovered isolates, 41 (61.19%) were identified and genetically confirmed as *Candida albicans* (Figure 2) and, the remaining ones (38.81%, n = 26) were identified to be bacteria such as *Escherichia coli* 10(14.93%), *Staphylococcus aureus* 8(11.94%), *Klebsiella spp*., 6(8.96%), and *Pseudomonas spp.* 2(2.99%). The mean age of these women was 32 ± 9.88years. As illustrated in Table 2, 19 (46.3%) of these isolates were recovered from HVS, while 22(53.7%) were from urine specimens. As shown in the table, the ratio of recovery of *C. albicans* from urine (21.52%) specimens was not significantly higher than that from the HVS (18.59%) ($P < 0.05$). Age-distribution wise, *C. albicans* were more frequent among age-group of 31-35years with 35(34.3%) isolates. This is followed by 26-30 years, 21-25years, and 15-20 years with recovery rate of 31(30.4%), 22(21.6%) and 6(5.9%) respectively, while the recovery rate for age 36-40, and >40 were with 4(3.9%) each.

Table 3, shows the *in vitro* antifungal susceptibility patterns of the isolated *Candida albicans*. As shown, 27(65.85%) of the 41 isolates were resistant to Fluconazole and Voriconazole each, while 25(60.98%) were resistant to Nystatin. Resistance to both azoles was found in 27(65.85%) of the

strains. There was no statistically significant difference in the susceptibility of the isolates to fluconazole, Voriconazole and Nystatin ($P > 0.05$).

Twenty-four (88.89%) of the 27 isolates that were determined to be azole resistant were positive for *ERGII genes* (Figure 3).

Discussion

The study was able to isolate and identified 41(61.19%) *Candida albicans* from the pregnant women with vulvovaginitis attending FMC, Yenagoa during the period of study. However, the presence of *E. coli*, *Klebsiella* spp., *Pseudomonas* and *S. aureus* in some vaginal samples (n = 26) agrees with prior reports presenting bacterial vaginitis as also a cause of vaginal infections [28, 29].

The outcome of this present study is in consistency with earlier reports from different parts of the world were the rates of isolation of *C. albicans* in cases of VVC has been reported to range between 47 and 89%. For illustration, studies conducted in Egypt [30] recorded higher rate of *C. albicans* in VVC (86.6%), while rates of 59%, 65.95% and 73.9% were reported from Saudi Arabia [31], Yemen [32] and Kuwait [33] respectively. Furthermore, studies from Nicaragua [34], Australia [35, 36], Turkey [37], Iran [38], China [39], Nigeria [40] and India [41] collaborates this isolation range.

Among the isolates studied, there was no significant isolation rate of *C. albicans* from the HVS when compared with the urine specimen among the patients with vulvovaginitis, thus, supporting species distribution isolation rates of *C. albicans* previously reported in India [42].

The highest frequency of vaginal candidiasis was observed among age group of 31-35yrs, with the mean age of 32 ± 9.88years. However, the frequency of vaginal candidiasis in women aged ≥ 40 years was low. This finding is similar to the previous findings reported [43, 44]. Furthermore, supporting earlier observed reports that women of child bearing age groups are more susceptible to

vaginal candidiasis. Similarly, Achkar and Fries [45], reported that vaginal candidiasis is an extremely common infection in 60-70% women during their reproductive age, and that every women will have candidiasis at least once in their life-time. Reasons have it that the high level of reproductive hormones and increase glycogen content of vagina favours candidiasis in pregnancy [46]. Hence this might be the common predisposing factor associated with vaginal candidiasis in the present study. Furthermore, the level of social activities, such as drug abuse and sexual promiscuity, may also be important in the distribution frequency of *Candida* species in different age groups and locations.

Due to the increased antifungal resistance of *C. albicans* species, their emergence to antimycotic agents remains a concern and this is terrifying because the indiscriminate use of azoles for the treatment of VVC over time has resulted in the selection of strains resistant to these compounds [47]. The resistance rate of our isolates to Fluconazole and Voriconazole were 27(65.85%) each. This recorded high rate is comparable to that earlier observed in various parts of the globe [28, 48, 49, 50, 51, 52, 53]. The level of fluconazole resistance found in this study was significantly higher, possibly because fluconazole is more frequently used in our environment. Notwithstanding, the high frequencies of strains resistant to fluconazole and Voriconazole in this study could further be explained by the high use of these fluconazole in combination with clotrimazole as prophylaxis and as the gold-standard treatment of fungal infections. Additionally, fluconazole is almost ineffective against most moulds.in our environment, given that this is the most commonly used therapy against VVC. Our results are consistent with the observation that Candida species isolated in different geographical regions differ in their sensitivity to fluconazole [54]. With this outcome, our findings negates earlier reports by Hazirolan *et al*. [55] that pronounces the activity of fluconazole weaker than

196 itraconazole and that itraconazole is weaker than Voriconazole. Because, there is no significant
197 difference in the frequency of resistance against fluconazole as observed to Voriconazole.

198 The *C. albicans* strains described in this study were resistant to nystatin (n = 25(60.98%). This is in
199 sharp contrast to reports in other studies [21, 28, 56, 57] that found nystatin to be highly efficacious.
200 This result outcome suggests that nystatin can neither be used as empirical therapy nor as an
201 alternative for the treatment of vaginal infections caused by strains of *C. albicans* which are resistant
202 to azoles as earlier suggested by Achkar & Fries [45]. There is need to draw the attention of clinicians
203 in our environment to this situation so that they can sought improve treatment via different
204 approaches, which may include the combination (synergistic) of antifungals as evidence has shown
205 that combinatory therapy contributes to reducing toxicity and could be an alternative for treatment of
206 candidiasis due to *C. albicans* [58, 59]. However, the possibility of some system bias cannot be
207 excluded due to the potential reasons of the different specimen, test method, and regional disparity
208 [60, 61].

209 In this study, the association of azole resistance phenotypes (fluconazole/ Voriconazole) was
210 identified in 27(65.85%) of the strains (Table 3), whereas *ERG11* was found in 24(88.89%) (Figure
211 3). The detection of *ERG11*genes conforms with several studies that have implicated this gene to
212 azole resistances [11, 18, 28, 48, 62, 63, 64, 65, 66].

213 However, the gap difference of 3(11.11%) in the detection of this gene in the present study can be
214 explained by the idea that azole resistance is not only conferred by *ERG11* gene alone, but also
215 caused by *CDR1*, an ATP-binding cassette (ABC) transporter [63, 64] or by MFS-transporter,
216 *CaMDR1* [8, 9]. A better understanding of this mechanism of resistance to these agents as well as
217 detection of *ERG11* genes are essential for the patient management, as the *ERG11*gene has been

linked to clinically-relevant increases to azoles and which is also correlated with the increase in recurrence of VVC [21].

Conclusion

This study found that *C. albicans* was associated with VVC among the pregnant women and that the strains that infects Yenagoa patients suffering from VVC are highly resistant to azoles, nystatin and that those resistant to the azoles are habouring *ERG11* genes. It is therefore vital that regimens should be adjusted according to local surveillance and *in vitro* susceptibility results, as high-level azole resistance is a problem of critical importance in our setting.

Acknowledgment

We are grateful to members of staff of the Departments of Obstetrics & Gynecology and Medical laboratory Science (Microbiology unit) of the Federal Medical Centre, Yenagoa for allowing us access to their patients and assisting in specimen collections. We are also grateful to the participating patients for partaking in this study.

Conflict of interest statement

We declare that we have no conflict of interest

All the authors read and approved the final manuscript.

References

1. Ksiezopolska E, Gabaldón T. (2018). Evolutionary Emergence of Drug Resistance in Candida Opportunistic Pathogens. Genes. 2018; 9(9):461. doi:10.3390/genes9090461
2. Jyoti T, Shrayanee D, Zeeshan F, Saif H. Multidrug Resistance: An Emerging Crisis. Interdisciplinary Perspectives on Infectious Diseases, 2014, Article ID 541340, 7 pages, 2014. https://doi.org/10.1155/2014/541340
3. Rodrigues C, Rodrigues M, Silva S, Henriques M. Candida glabrata biofilms: How far have we come? J. Fungi, 2017; 3: 11
4. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol. Mol. Biol. Rev. 2011;75: 213–267. doi: 10.1128/MMBR.00045-10.
5. Berkow E, Lockhart S. Fluconazole resistance in Candida species: A current perspective. Infect Drug Resist. 2017; 10:237–245. doi: 10.2147/IDR.S118892.
6. Flowers SA, Colón B, Whaley SG, Schuler, MA, & David R-P. Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans. Antimicrob Agents Chemother. 2015; 59: 450–460. doi: 10.1128/AAC.03470-14.
7. Xiang M J, Liu JY, Ni PH, Wang S, Shi C, Wei B, et al. Erg11 mutations associated with azole resistance in clinical isolates of Candida albicans. FEMS Yeast Res. 2013; 13: 386–393. 10.1111/1567-1364.12042 [PubMed] [CrossRef]
8. Morschhäuser J., Barker K.S., Liu T.T., Blaß-Warmuth J., Homayouni R., Rogers P.D. The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in Candida albicans. PLoS Pathog. 2007;3:1603–1616. doi: 10.1371/journal.ppat.0030164.
9. Lupetti A, Danesi R, Campa M, Del Tacca M, Kelly S. Molecular basis of resistance to azole antifungals. Trends Mol Med 2002; 8: 76–81. [PubMed]
10. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini M.J.S. Candida species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J. Med. Microbiol. 2013;62:10–24. doi: 10.1099/jmm.0.045054-0
11. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Morschhauser J, Rogers PD. Gain-of-function mutations in UPC2 are a frequent cause of ERG11

upregulation in azole-resistant clinical isolates of Candida albicans. Eukaryot Cell. 2012; 11:1289–1299. doi:10.1128/EC.00215-12.

12. Selmecki A, Gerami-Nejad M, Paulson C, Forche A, Berman J. An isochromosome confers drug resistance in vivo by amplification of two genes, ERG11 and TAC1. Mol Microbiol. 2008; 68:624–641. doi:10.1111/j.1365-2958.2008.06176.
13. Selmecki A, Forche A, Berman J. Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science. 2006; 313:367–370. doi:10.1126/science.1128242.
14. Warrilow AG, Mullins JG, Hull CM, Parker JE, Lamb DC, Kelly DE, Kelly SL. S279 point mutations in Candida albicans sterol 14-alpha demethylase (CYP51) reduce in vitro inhibition by fluconazole. Antimicrob Agents Chemother. 2012; 56, 2099–2107. doi:10.1128/AAC.05389-11.
15. Kelly SL, Lamb DC, Kelly DE. Y132H substitution in Candida albicans sterol 14alpha-demethylase confers fluconazole resistance by preventing binding to haem. FEMS Microbiol Lett 1999a; 180:171–175. doi:10.1111/j.1574 6968.1999.tb08792.x.CrossRefPubMedWeb of ScienceGoogle Scholar
16. Kelly SL, Lamb DC, Loeffler J, Einsele H, Kelly DE. The G464S amino acid substitution in Candida albicans sterol 14alpha-demethylase causes fluconazole resistance in the clinic through reduced affinity. Biochem Biophys Res Commun 1999b; 262:174–179. doi:10.1006/bbrc.1999.1136.CrossRefPubMedWeb of ScienceGoogle Scholar
17. Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, et al. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida albicans. Microbiology. 1999; 145:2701–2713.CrossRefPubMedWeb of ScienceGoogle Scholar
18. Morio F, Loge C, Besse B, Hennequin C, Le Pape P. Screening for amino acid substitutions in the Candida albicans Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. Diagn Microbiol Infect Dis. 2010; 66(4), 373–384.
19. Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant Candida albicans clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother 1998; 42:241–253. doi:10.1093/jac/42.2.241.

20. McGowan K. Specimen Collection, Transport, and Processing: Mycology. In Jorgensen J, Pfaller M, Carroll K, Funke G, Landry M, Richter S, Warnock D (ed), Manual of Clinical Microbiology, Eleventh Edition. ASM Press, Washington, DC. 2015.p 1944-1954. doi: 10.1128/9781555817381.ch114
21. Wang B, Huang Li-Hua, Zhao Ji-Xue, Wei Man, Fang Hua, et al. ERG11 mutations associated with azole resistance in Candida albicans isolates from vulvovaginal candidosis patients. Asian Pac J Trop Biomed. 2015; 5(11): 909–914.
22. Santos MS, Souza ES, Junior RM, Talhari S, Souza JV. Identification of fungemia agents using the polymerase chain reaction and restriction fragment length restriction fragment length polymorphism analysis. Braz J Med Biol Res 2010;43(8):712–6.
23. Oliveira C F, Paim T G, Reiter K C, Rieger A, D'Azevedo PA. Evaluation of four different DNA extraction methods in coagulase-negative staphylococci clinical isolates. Rev Inst Med Trop Sao Paulo, 2014;56(1), 29–33. doi:10.1590/S0036-46652014000100004
24. Vijayakumar R, Giri S, Kindo AJ. Molecular species identification of Candida from blood samples of intensive care unit patients by polymerase chain reaction – restricted fragment length polymorphism. J Lab Phys 2012;4(1):1–4.
25. White T J, Bruns T D, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ eds. PCR protocols, a guide to methods and applications. San Diego, California: Academic Press. 1990; p315-322.
26. Martínez M, López-Ribot J L, Kirkpatrick W R, Bachmann S P, Perea S, Ruesga M T., et al. Heterogeneous mechanisms of azole resistance in Candida albicans clinical isolates from an HIV-infected patient on continuous fluconazole therapy for oropharyngeal candidosis, J. Antimicrob. Chemother. 2002;49(3): 515–524.
27. Clinical Laboratory Standard Institute (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition—Document M27-A3. Wayne, Pa, USA: CLSI; 2008.
28. Monroy-Pérez E, Paniagua-Contreras G L, Rodríguez-Purata P, Vaca-Paniagua F, Vázquez-Villaseñor M, Díaz-Velásquez C., et al. High Virulence and Antifungal Resistance in Clinical Strains of Candida albicans. Can J Infect Dis Med Microbiol. 2016; 2016, 5930489. doi:10.1155/2016/5930489

29. Sobel J D. Vaginitis. N Engl J Med. 1997; 337(26):1896–1903. doi:10.1056/NEJM199712253372607.
30. El-sayed H, Hamouda A. Candida albicans causing vulvovaginitis and their clinical response to antifungal therapy. Egypt J Med Microbiol, 2007;16 (1):53-62.
31. Al-Hedaithy S. Spectrum and proteinase production of yeasts causing vaginitis in Saudi Arabian women. Med Sci Monit, 2002;8(7): 498-501.
32. Al-Mamari A, Al-Buryhi M, Al-Heggami MA, Al-Hag S. Identify and sensitivity to antifungal drugs of Candida species causing vaginitis isolated from vulvovaginal infected patients in Sana'a city. Der Pharma Chemica, 2014;6(1), 336-342.
33. Alfouzan W, Dhar R, Ashkanani H, Gupta M, Rachel C, Khan ZU. Species spectrum and antifungal susceptibility profile of vaginal isolates of Candida in Kuwait. J Mycol Med. 2015; 25(1): 23-28.
34. Bello MD, Gonzalez A, Barnabé C, Larrouy G. First characterization of Candida albicans by Random amplified polymorphic DNA method in Nicaragua and comparison of the diagnosis methods for vaginal candidiasis in Nicaraguan women. Mem Inst Oswaldo Cruz. 2002;97(7): 985-989.
35. Holland J, Young M, Lee O, Lee S. Vulvovaginal carriage of yeasts other than Candida albicans species. Sex Transm Infect. 2003;79 (3):249-250.
36. Pirotta M, Garland S. Genital Candida species detected in samples from women in Melbourne, Australia, before and after treatment with antibiotics. J Clin Microbiol. 2006;44 (9):3213-3217.
37. Gültekin B, Yazici V, Aydin N. Distribution of Candida species in vaginal specimens and evaluation of CHROMagar Candida medium. Mikrobiyol Bul, 2005;39 (3): 319-324.
38. Pakshir K, Yazdani M, Kimiaghalam R. Etiology of vaginal candidiasis in Shiraz, Southern Iran. Res J Microbiol. 2007;2: 696-700.
39. Xu Y, Chen L, Li C. Susceptibility of clinical isolates of Candida species to fluconazole and detection of C. albicans ERG11 mutations. J. Antimicrob. Chemother. 2008;61 (4): 798-804.
40. Emmanuel N, Romeo O, Mebi A, Mark O, Scordino F, Bessy E I. et al.. Genotyping and fluconazole susceptibility of Candida albicans strains from patients with vulvovaginal candidiasis in Jos, Nigeria. Asian Pac. J. Trop. Dis. 2012; 2012:48-50.

41. Babin D, Kotigadde S, Rao P, Rao T. V. Clinico-mycological profile of vaginal candidiasis in a tertiary care hospital in Kerala. Int J Res Biol Sci, 2013;3(1):55-59.
42. Agwan V, Butola R, Madan M. Comparison of biofilm formation in clinical isolates of Candida species in a tertiary care center, North India. Indian J Pathol Microbiol. 2015;58:475-478
43. Deepa B, Subbannayya K, Sunil Rao P, Rao TV. Clinico-mycological profile of vaginal candidiasis in a tertiary care hospital in Kerala. Int. J. Biol. Sci. 2013; 3(1): 55-59.
44. Reddy A, Mustafa M. Phenotypic Identification of Candida Species and their Susceptibility Profile in Patients with Genitourinary Candidiasis. International J. Adv. Res. 2014; 2(12):76-84.
45. Achkar J M, Fries BC. Candida infections of genitourinary tract. Clin. Microbiol. Rev. 2010;23(2):253-273. DOI: 10.1128/CMR.00076-09
46. Okungbowa FI, Isikhuemhen OS, Dede AP. The distribution frequency of Candida species in the genitourinary tract among symptomatic individuals in Nigerian cities. Rev. iberoam. Micol. 2003;20(2), 60-63.
47. Richter SS, Galask R. P, Messer SA, Hollis RJ, Diekema DJ, Pfaller M.A. Antifungal susceptibilities of Candida species causing vulvovaginitis and epidemiology of recurrent cases. J. Clin. Microbiol. 2005;43(5):2155–2162. doi: 10.1128/JCM.43.5.2155-2162.2005.
48. Chowdhary A, Prakash A, Sharma C, Kordalewska M, Kumar A, Sarma S, et al. A multicentre study of antifungal susceptibility patterns among 350 Candida auris isolates (2009–17) in India: role of the ERG11 and FKS1 genes in azole and echinocandin resistance. J. Antimicrob. Chemother. 2018; 73(4):891–899. https://doi.org/10.1093/jac/dkx480
49. Lockhart S.R, Etienne K.A, Vallabhaneni S, Farooqi J, Chowdhary A, Govender N.P, et al. Simultaneous emergence of multidrug-resistant Candida auris on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. Clin. Infect. Dis. 2017; 64(15):134–140. doi:10.1093/cid/ciw691
50. Morales-López S. E, Parra-Giraldo C. M, Ceballos-Garzón A, Martínez H P, Rodríguez G J, Álvarez-Moreno C A, et al. Invasive Infections with Multidrug-Resistant Yeast Candida auris, Colombia. Emerg Infect Dis. 2017; 23(1): 162–164. doi:10.3201/eid2301.161497

51. Schelenz S, Hagen F, Rhodes J.L, Abdolrasouli A, Chowdhary A, Hall A., et al. First hospital outbreak of the globally emerging Candida auris in a European hospital. Antimicrob Resist Infect Control. 2016;5:35. doi:10.1186/s13756-016-0132-5

52. Magobo R E, Corcoran C, Seetharam S, Govender N P. Candida auris-associated candidemia, South Africa. Emerg Infect Dis 2014;20(7):1250–1251. doi:10.3201/eid2007.131765

53. Yang C W, Barkham T M, Chan F Y, Wang Y. Prevalence of Candida species, including Candida dubliniensis, in Singapore. J. Clin. Microbiol. 2003;41(1):472–474. doi:10.1128/jcm.41.1.472-474.2003

54. Yang Y L, Cheng H H, Ho YA, Hsiao C.F, Lo H.JFluconazole resistance rate of Candida species from different regions and hospital types in Taiwan. J Microbiol Immunol Infect. 2003;36(3):187–191.

55. Hazirolan G, Canton E, Sahin S, Arikan-Akdagli S. Head-to-head comparison of inhibitory and fungicidal activities of fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole against clinical isolates of Trichosporon asahii. Antimicrob. Agents Chemother. 2013;57(10):4841–4847. doi:10.1128/AAC.00850-13

56. Choukri F., Benderdouche M., Sednaoui P. In vitro susceptibility profile of 200 recent clinical isolates of Candida spp. to topical antifungal treatments of vulvovaginal candidiasis, the imidazoles and nystatin agents. J Mycol Med. 2014;24(4):303–307. doi: 10.1016/j.mycmed.2014.05.001. [PubMed] [CrossRef] [Google Scholar]

57. Fan S, Liu X, Wu C, Xu L, Li J. Vaginal nystatin versus oral fluconazole for the treatment for recurrent vulvovaginal candidiasis. Mycopathologia, 2014;179:95–101. doi: 10.1007/s11046-014-9827-4. [PubMed] [Google Scholar]

58. Liu X, Li T, Wang D, Yang Y, Sun W, Liu J, et al. Synergistic Antifungal Effect of Fluconazole Combined with Licofelone against Resistant Candida albicans. Front Microbiol. 2017;8:2101. doi:10.3389/fmicb.2017.02101

59. Cui J, Ren B, Tong Y, Dai H, Zhang L. Synergistic combinations of antifungals and anti-virulence agents to fight against Candida albicans. Virulence. 2015;.6(4): 362-371. doi: 10.1080/21505594.2015.103988.

60. Pfaller MA, Jones RN, Castanheira M. Regional data analysis of Candida non-albicans strains collected in United States medical sites over a 6-year period, 2006-2011. Mycoses. 2014;57:602–11. doi: 10.1111/myc.12206. [PubMed] [Google Scholar]
61. Hamad M, Kazandji N, Awadallah S, Allam H. Prevalence and epidemiological characteristics of vaginal candidiasis in the UAE. Mycoses. 2014;57:184–90. doi: 10.1111/myc.12141. [PubMed] [Google Scholar]
62. Whaley S G, Berkow E L, Rybak J M, Nishimoto A T, Barker K S, Rogers P.D. Azole Antifungal Resistance in Candida albicans and Emerging Non-albicans Candida Species. Front Microbiol, 2017; 7:2173. doi: 10.3389/fmicb.2016.02173
63. Alvarez-Rueda N., Fleury A., Logé C., et al. The amino acid substitution N136Y in Candida albicans sterol 14 α-demethylase is involved in fluconazole resistance. Med Mycol. 2016;54(7):764–775. [PubMed] [Google Scholar]
64. Manastir L., Ergon M. C., Yücesoy M. Investigation of mutations in Erg11 gene of fluconazole resistant Candida albicans isolates from Turkish hospitals. Mycoses. 2011;54(2):99–104. doi: 10.1111/j.1439-0507.2009.01766.x. [PubMed] [CrossRef] [Google Scholar]
65. Heilmann C, Schneider S, Barker KS, Rogers PD, Morschhäuser J. An A643T mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and increased fluconazole resistance in Candida albicans. Antimicrob Agents Chemother. 2010;54(1): 353–359
66. Cannon R D, Lamping E, Holmes A R, Niimi K, Tanabe K, Niimi M, et al. Candida albicans drug resistance another way to cope with stress. c 2007;153(10): 3211-3217.

Figures and Tables:

Table 2: Age distribution and recovery of Microorganisms from Genitourinary clinical specimens of patients from whom Clinical Specimens were collected

Age (Years)	HVS	Urine	Total (%)
15-19	4	2	6(5.88)
20-24	15	7	22(21.57)
25-29	21	10	31(30.39)
30-34	21	14	35(34.31)
35-39	3	1	4(3.92)
40-44	2	2	4(3.92)
Total	66(64.71)	36(35.29)	102(100.00)

Key: HVS, Higher vaginal Swab

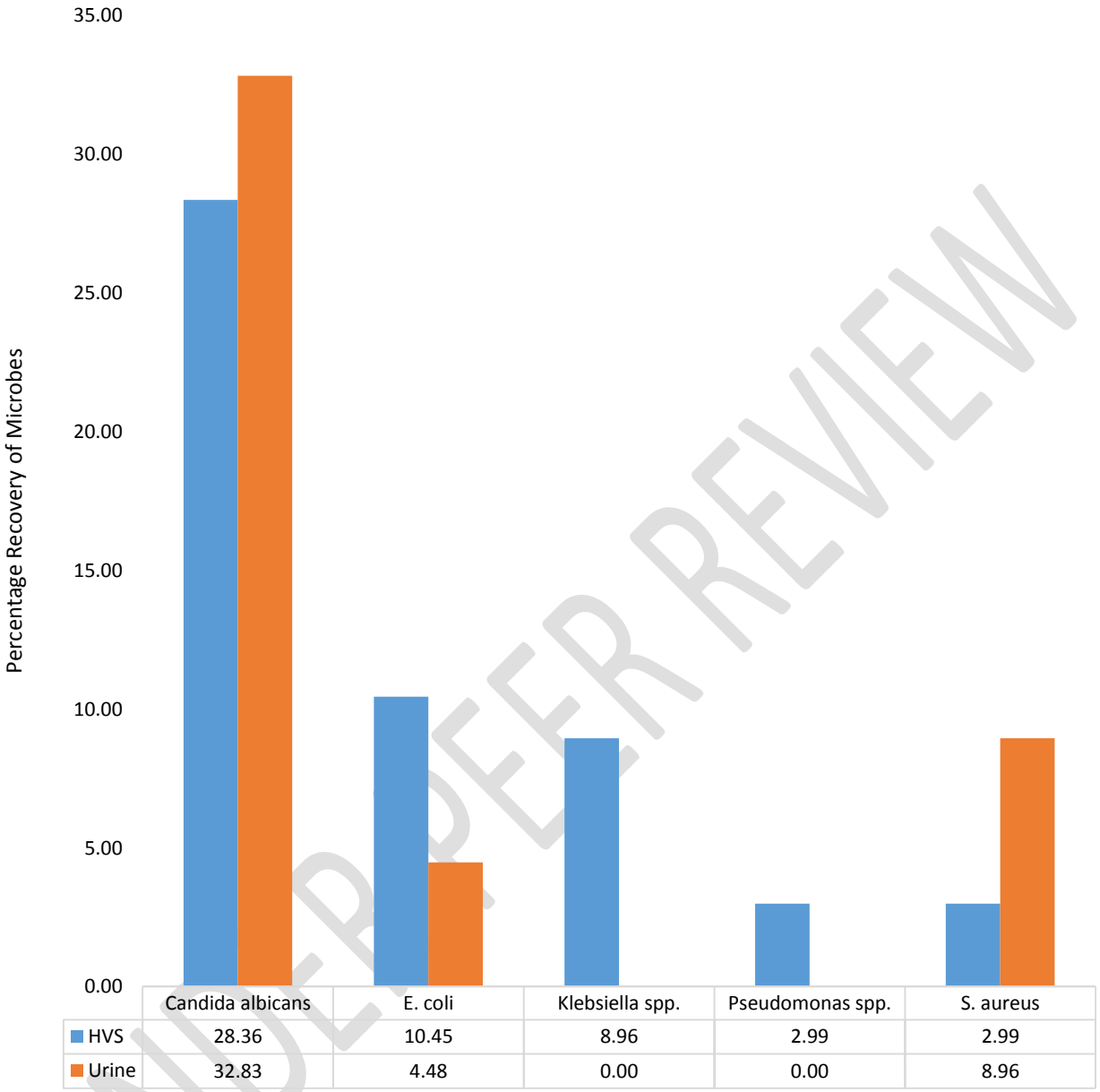
447 Table 3. Susceptibility and Resistance of *Candida albicans* strains isolated to antimycotic drugs.

448

Antimycotic drugs	No (%) Resistant	No (%) Sensitive
Fluconazole	27(65.85)	14(34.15)
Nystatin	25(60.98)	16(39.02)
Voriconazole	27(65.85)	14(34.15)

449

450



	Candida albicans	E. coli	Klebsiella spp.	Pseudomonas spp.	S. aureus
HVS	28.36	10.45	8.96	2.99	2.99
Urine	32.83	4.48	0.00	0.00	8.96

Figure 1. Recovery of Microorganisms isolated from genitourinary clinical specimens

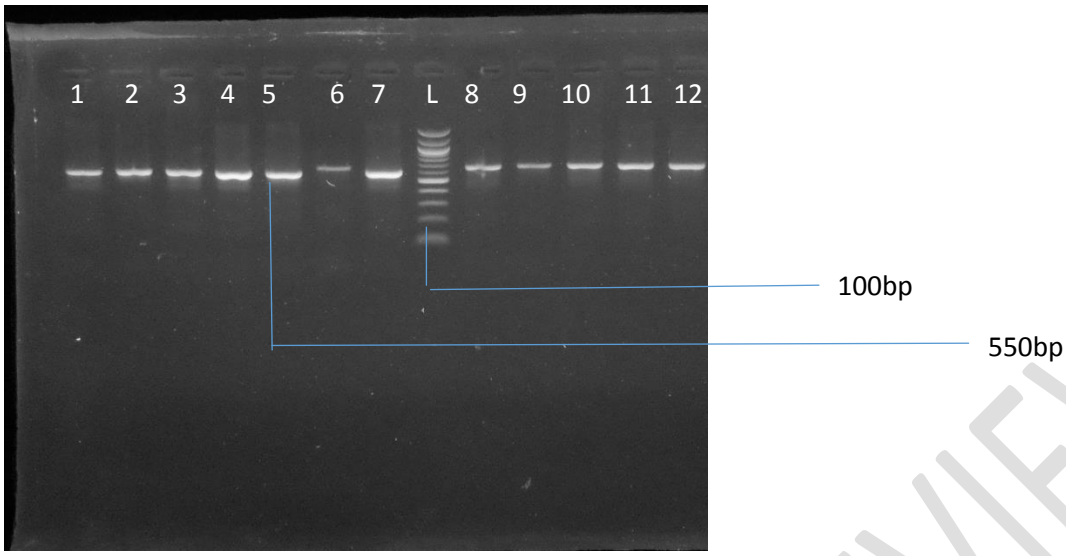


Figure 2. Agarose Gel Electrophoresis showing ITS region of fungi (*Candida* species). Lanes 1-12 represent the isolates while L represent the 100bp molecular ladder.

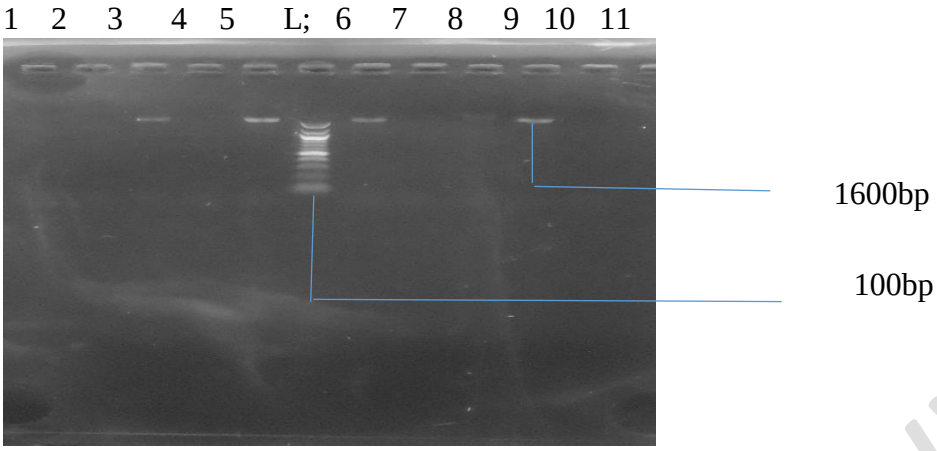


Figure 3. Agarose Gel Electrophoresis showing *ERGII* resistance gene *in Candida albicans*. Lanes 1-10 represent the isolates, Lane 3, 5, 6, 8 and 9 were positive while L represent the 100bp molecular ladder.