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

Abstract

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~~in~~ microdilution techniques~~studies~~. 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~~as~~ 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~~d~~ 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.

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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 been~~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

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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

~~The study has been approved by the Research and Ethical Committee of The Federal Medical Centre, Yenagoa. Informed consent was also obtained from all individual participants included in this study.~~

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].

Inclusion and Exclusion Criteria

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Comment [OP3]: If clinical samples were collected by the authors rather than clinical isolates of C. albicans, the I will advise the authors to reframe the manuscript title and abstract (methodology).

Comment [OP4]: Insert Reference number of Ethical clearance please.

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

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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, ~~at the same time,~~ 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~~higher vaginal swab~~ 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^{0}C and was~~tubes were then transferred to a heating block at 95^{0}C 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 ~~1~~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 ~~in accord to the~~ 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^{0}C).

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^{0}C for 4 minutes, denaturation at 94^{0}C for 30 seconds, annealing at 55^{0}C for 30 seconds, extension at 72^{0}C for 1 minute and final extension at 72^{0}C 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

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112 photographed. The polymerase chain reaction (PCR) method was performed for amplification of
113 genes with specific primers shown in table 1.

114

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 has been approved by the Research and Ethical Committee of The Federal Medical Centre, Yenagoa. Informed consent was also obtained from all individual participants included in this study.

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Statistical Analysis

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Results

Sixty-seven (65.69%) of the genitourinary specimens collected from the 102 pregnant outpatients' women attending the facility~~FMC~~ 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 ~~respectively~~, 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~~ly~~ 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 [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 has~~Reasons have it that the high level of reproductive hormones and increase glycogen content of vagina ~~favours~~favors candidiasis in pregnancy [46]. Hence this might be the common predisposing factor associated with vaginal

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

The *C. albicans* strains described in this study were resistant to nystatin (n = 25(60.98%). This is in sharp contrast to reports in other studies [21, 28, 56, 57] that found nystatin to be highly efficacious. This result outcome suggests that nystatin can neither be used as empirical therapy nor as an alternative for the treatment of vaginal infections caused by strains of *C. albicans* which are resistant

to azoles as earlier suggested by Achkar & Fries [45]. There is need to draw the attention of clinicians in our environment to this situation so that they can sought improve treatment via different approaches, which may include the combination (synergistic) of antifungals as evidence has shown that combinatory therapy contributes to reducing toxicity and could be an alternative for treatment of candidiasis due to *C. albicans* [58, 59]. However, the possibility of some system bias cannot be excluded due to the potential reasons of the different specimen, test method, and regional disparity [60, 61].

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

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

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Conclusion

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~~In conclusion,~~ T~~t~~his 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

221 that regimens should be adjusted according to local surveillance and *in vitro* susceptibility results, as
222 high-level azole resistance is a problem of critical importance in our setting.

223 **Conflict of interest statement**

224 We declare that we have no conflict of interest

225 All the authors read and approved the final manuscript.

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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

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

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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)

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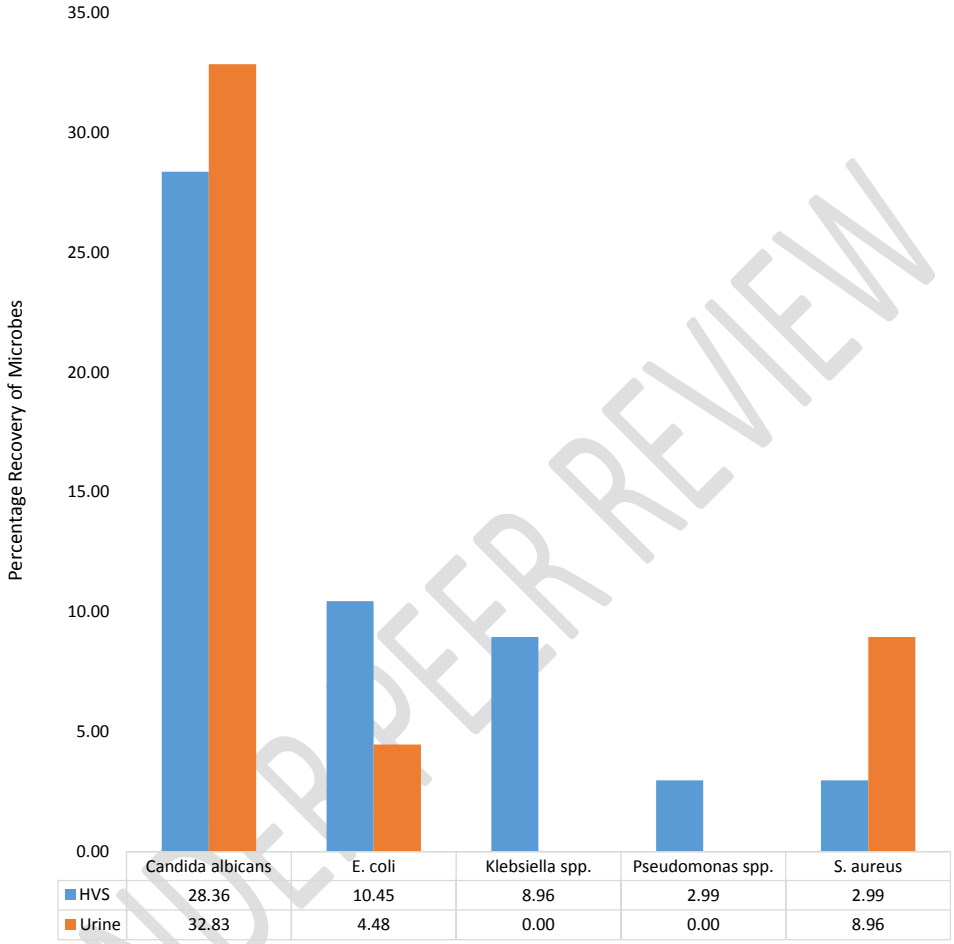

	Candida albicans	E. coli	Klebsiella spp.	Pseudomonas spp.	S. aureus
HVS	28.36	10.45	8.96	2.99	2.99
Urine	32.83	4.48	0.00	0.00	8.96

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445 Figure 1. Recovery of Microorganisms isolated from genitourinary clinical specimens

Comment [OP13]: Rather than using Recovery, why not use occurrences and calculate the percentage occurences?

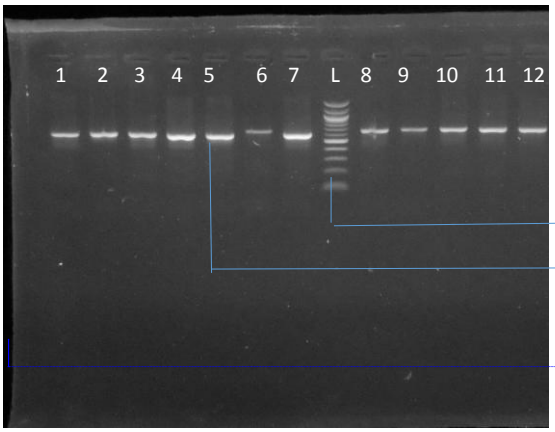

Figure 2. Agarose Gel Electrophoresis showing ITS region of fungi (*Candida* species). Lanes 1-12 represent the isolates while L represent the 100bp molecular ladder.

Comment [OP14]: Marker not showing? Explain the figure briefly below to allow it stand alone.

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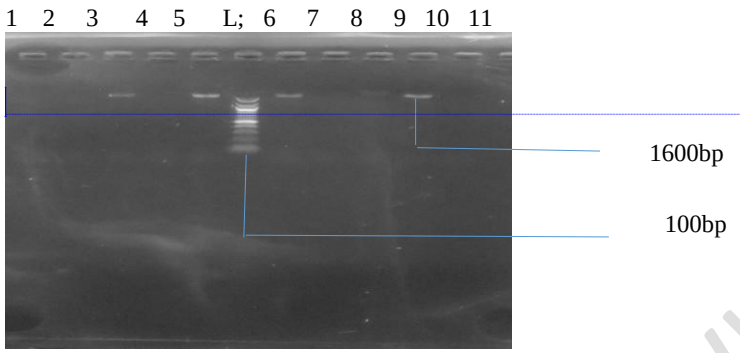

Figure 3. Agarose Gel Electrophoresis showing *ERGII* resistance gene *in Candida albicans*. Lanes 1-10 represent the isolates, Lane 3, 5, 6, 8 and 9 were positive while L represent the 100bp molecular ladder.

Comment [OP15]: Same as above.