

Determination of Azole Resistant Gene (ERG11) in Candida Species Isolated from Non-Pregnant Women Attending Amassoma Community Hospitals, Bayelsa State, Nigeria

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Abstract: *Candida species are dimorphic organism in which they can exist as moulds and yeast. They have the ability to cause infection in individuals with impaired immunity. This study was carried out between January and August, 2018 to determine the presence of azole resistant gene (ERG11) in Candida species isolated from non-pregnant women between ages 18 and 36 attending Amassoma Community hospitals, Bayelsa state, Nigeria. A total of 150 higher vagina swabs were collected from 150 subjects and immediately transported to the laboratory for culture using Sabouraud Dextrose Agar. Pure isolates were characterised and identified on the basis of Gram Staining, Germ tube test, molecular analysis for the presence of ITS (Internal Transcriber Spacer) gene and CHROMagarCandida. Antifungal susceptibility testing was conducted by standard procedures and molecular analysis using polymerase chain reaction to reveal the resistant gene ERG11. A total of 92 (77%) of Candida species were isolated; Candida albicans 72 (78%), Candida krusei 15 (16%), Candida glabrata 5 (5%). Most of the Candida species isolated were susceptible to Ketoconazole (92.8%) followed by fluconazole (66.6%) and the least itraconazole (27%). Resistant strains among the Candida species expressed ERG11 genes. Observation showed that women of ages 18 to 27 years had the highest Candida infection this is followed by ages of 28 to 33 years and the least women with Candidiasis were of ages 34 to 36 years. In conclusion, this study has revealed ketoconazole as the most potent antifungal that can be prescribed for clinical use. Also antifungal resistant gene was common among the Candida species isolated in the study. Further study should be carried out to combat this effect of resistance.*

Keywords: Candida infections, Non- Pregnant women, Resistance genes, Antifungal Susceptibility Testing, Higher Vaginal Swab

1. Introduction

Candida is a dimorphic organism, meaning it can exist in two shapes and forms simultaneously. One form is a yeast-like state that is a non-invasive, sugar fermenting organism the other is a mould form that produces very high very long root-like structures, called rhizoids that can penetrate the mucosa and is invasive (Bailey *et al.*, 2014).

Healthy immune system prevents this yeast from becoming an infectious fungus. It is when our bodies lose their proper immune protection or the intestinal pH is altered unfavourable, that the organism can change from the yeast form to the fungal form. When this happens, the now parasitic fungal form penetrates the gastrointestinal mucosa and breaks down the boundary between the intestinal tract and the rest of the circulation in our bodies. It has been officially estimated that about 80% of the population may have candidiasis that is out of control (Ashman *et al.*, 2012)

The immune system is attacked by *Candida* as a result of the prolonged use of antibiotics, taking steroids or oral contraceptives on a regular basis, or due to high sugar diet. It is also known that *Candida* increases its number during periods of stress and lowered immune states also one major reason for the increase in *Candida* infections is the development of its resistant strains to azole drugs, such as fluconazole used in prophylaxis and treatment of candidiasis (Shahid *et al.*, 2013).

Fungal infection of the vagina is sometimes called thrush. Candidiasis is also known as yeast infection. It is a common fungal infection that occurs when there is overgrowth of the fungus called *Candida* which is always present in the body in small amount. However, when an imbalance occurs, such as when the normal acidity of the vagina changes or when hormonal balance changes, *Candida* can multiply. When this happens, symptoms of candidiasis appear. This may include depression, itchy, flaky skin, anxiety, recurring irritability or mood swings, heartburn, indigestion, lethargy, food and environmental allergies, joint soreness, chest pain or other skin problems, recurring cystitis/vaginal infections, premenstrual tension and menstrual problems. Immune suppression, AIDS, diabetes and thyroid disorder may also cause candidiasis (Sudbery *et al.*, 2014). *Candida* is a genus of yeasts and is the most common cause of fungal infections worldwide (Manolakak *et al.*, 2010). Many species are harmless commensals of hosts including humans; however, when mucosal barriers are disrupted or the immune system is compromised they can invade and cause disease. When *Candida* is grown in the laboratory, it appears as large, round, white or cream colonies, which emit a yeasty odour on agar plates at room temperature. (Kourkourmpetis *et al.*, 2011). The genus *Candida* are about 154 species, among these, six are most frequently isolated in human infections, while *Candida albicans* is the most abundant and significant specie. *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida lusitanae* are also isolated as causative agents of *Candida* infections.

Importantly, there has been a recent increase in infections due to *non-albicans Candida* spp., such as *Candida glabrata* and *Candida krusei* (Arif et al., 2014). *Candida albicans* are budding and diploid fungi (a form of yeast). Like other types of *Candida*, it is an opportunistic causal agent for candidiasis in human; a type of fungal yeast infection. In fact, according to Clinical Microbiology Reviews (CMR), *Candida albicans* is also the most common causal agent of candidiasis. *Candida albicans* are commensals and normal flora of the human oral and gastrointestinal tract. Under normal circumstances, they colonize 80% of the human population with no harmful effects, although sometimes overgrowth occurs resulting in candidiasis (Pfaffer and Diekema, 2010). To be pathogenic, the usual unicellular yeast-like form of *Candida albicans* reacts to environmental cues and switches into an invasive, multi-cellular filamentous forms (Vendettuoli et al., 2012). Infection is often observed in immune compromised individuals such as HIV/AIDS patients. These characteristics are also expressed by non-*Candida albicans* which include *Candida krusei*. *Candida krusei* has a high natural resistance to standard antifungal treatments (Pfaffer and Diekema 2010); *Candida glabrata* is associated with oral candidiasis (Marr et al., 2011); *Candida parapsilosis*, the causative agent of sepsis and tissue infections (Levy et al., 2010); *Candida tropicalis* which is found in digestive tract and the skin (Jain et al., 2012). These yeast infections include thrush, which is an oral infection; vaginitis, an infection of the vagina; *Candida onychomycosis*, a fungal nail infection; and diaper rash. *Candida* septicemia can also occur in the blood and in the genital tract in severely immune compromised individuals.

Virulence factors of *Candida* species

The ability of *C. albicans* to cause infections in their host victims are due to some virulence factors and fitness attributes; these include the morphological transition between yeast and hyphal forms, the expression of adhesins and invasins on the cell surface, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes are considered virulence factors (Weems et al., 2014). This study aims at determining the prevalence of *Candida* species in non-pregnant women between ages 18-36 and subject the isolates to antifungal susceptibility testing to commonly prescribed azole drugs in the hospitals, Fluconazole, Ketoconazole and Itraconazole thereby evaluating the presence of Azole resistant gene (ERG11) from the multi-resistance expressed strains to the test azole drugs.

2. Materials and Methods

Study Area

Bayelsa state is located south-south of Nigeria and share common boundary with two other states; Rivers state to the east and Delta state to the west. This state has a population of 1.7 million people based on preliminary 2006 census figures. It has eight Local Government areas which include Sagbama, Brass, Yenagoa, Ekeremor, Ogbia, Nembe, Southern Izon Local Government Areas and three senatorial zones and one out of the eight local government area is urban while the remaining seven are rural. The study was carried out in one private hospital and one public hospital

within Amassoma Bayelsa State; General Hospital Amassoma and Tantua Memorial Hospital Amassoma,

Ethical Consideration

Ethical approval was requested from the medical director of each hospital and approval was given.

Sample Size Estimation

The samples were collected using probability sampling technique and the sample size was worked out mathematically with a formula that was developed by the Research Division off the National Education Association (NEA) 1960 in the United States using the formula

$S = X^2NP(1 - P)/a^2(N - 1) + x^2P(1 - P)$ where S= Sample size; x^2 =chi-square table value for one degree of freedom at the desired level of confidence which is 3.84; P= population proportion, assumed to be 0.5 as it provides maximum sample size; N= population size; a= level of significance, usually 0.05 at which decision errors (type1 and type 2) are reduced to barely minimum. One hundred and fifty (150) samples were collected and analysed in this study. One hundred (100) higher vagina swabs were collected from General Hospital Amassoma and 50 samples collected from Tantua Memorial Hospital from women who came to the various hospitals with any of the following complaints; vaginal itching, vaginal discharges, swelling around the vagina, redness, soreness, rash, pain during sex.

Research Area

The research area covered hospitals in Amassoma community; Amassoma General Hospital and Tantua Memorial Hospital, Bayelsa State. Women of ages 18 to 36years were clinically assessed. They were chosen because they are within the range of high risk group, are sexually active and as well, are the most vulnerable because of the nature of their immune system.

Materials

Glass ware, equipment and media used

Standard and recommended materials were used in this study which include; autoclave, centrifuge, dryer, incubator, beakers, Petri dishes, cotton wool, wire loop, sterile water, distilled water, perforator, sterile cotton buds, Mc-Cartney bottles, sterile swab sticks, microscope, human serum. Sabouraud Dextrose Agar, Chrom agar *Candida* (CaC), nutrient agar, Mueller-Hinton with 2% glucose and 0.5µg/ml Methylene Blue for antimicrobial sensitivity testing, Luria Bertani (LB) media.

PCR equipment

Micro tubes, Sterile aerosol pipette tips 0.1-10microliter, Eppendorf research plus 10-100microliter, Ecopipette by CAPP 100-1000micro liter, Eppendorf research plus 0.5-10microliter, XH-B Vortex eltech, Gene Amp PCR System 9700, Desktop computer/printer, Boiler (Wealtec Corp. HB-2), Microwave, Electrophoretic chamber, UV transilluminator- NIPPON Genetics, Electrophoresis machine (Elite 300 plus Wealtec), WISS 66 Zentrifuge 3200, Micro centrifuge tubes, Normal saline, 5ml syringe, TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA),

Agarose gel, Ethidium bromide, Casting tray Quick-load purple 100bp DNA ladder.

PCR components;

- 1) Tag. (*thermos aquaticus*) polymerase; bacterium from where the enzyme is being obtained
- 2) Magnesium chloride; helps the enzyme to anneal to the DNA strand
- 3) Buffers
- 4) DNTPs (deoxynucleoside triphosphate) bases; used by the enzyme to synthesis the new DNA
- 5) Dyes; loading and tracking dyes
- 6) Primers; short oligonucleotides between 18-30bp, they are specific and unique and are in pairs (forward and backward primer)
- 7) Template; genomic DNA that has been extracted
- 8) Nuclease free Water

Every PCR mixture must have Tag polymerase, magnesium chloride, buffers, DNTPs, and dyes. These are compounded in a master mix (MM) X2 (Faoud et al., 2013; Goshorn et al., 2012; Kanagal et al., 2014).

Method

Sample Collection

Higher vaginal swabs were aseptically collected from 150 women with the aid of sterile swab sticks attending Amassoma general hospital and Tantua memorial hospital. The swab sticks containing the samples were immediately transported to the Pharmaceutical Microbiology laboratory for culture and analysis.

Isolation, Characterisation and identification of the microbial isolates

Samples were streaked on Sabouraud Dextrose Agar (SDA) and labelled appropriately, then incubated for 72hrs. After incubation period discrete colonies of were picked and sub-cultured for more yield on SDA. Pure cultures were transferred into slants containing Sabouraud Dextrose Agar for further laboratory identification. Pure culture of *Candida* species isolated were characterized and identified on the basis of their cultural, morphological, gram staining, germ tube technique, the Chromogenic agar *Candida* obtained from Oxoid representative company, Lagos, Nigeria for colour differentiation and molecular analysis evaluating the presence of ITS gene.

Discs and Bioassay Procedure

Antifungi discs for three (3) commonly prescribed azole drugs (Fluconazole (25µg), Ketoconazole 15µg and Itraconazole 10µg) were obtained from the Oxoid Company representative in Lagos, Nigeria. Yeast suspension was standardised equivalent to 0.5 McFarland standard and susceptibility testing was carried out using agar diffusion method. A sterile cottonswab was dipped into the suspension and used to inoculate the already prepared sterile agar plate containing Mueller Hinton with 2% glucose and 0.5µg/ml methylene blue. The sterile cotton swab in the suspension was rotated several times and the excess fluid was removed from the swab by pressing firmly against the inside wall above the fluid level, this was used to inoculate the dried surface of the agar by streaking the cotton swab according to

the standard method for even distribution of the inoculum. The plate was left open for 3 – 5 minutes, allowing excess moisture to be absorbed before dispensing Antifungi discs onto the plate and incubated at 35°C +/- 1°C within 15 minutes after the discs have been applied. The zones of inhibition were measured between 20 and 24 hours after incubation. The mean value of the zone was determined according to the class of antifungi agent on the basis of the CLSI guidelines, M27-A. The zones of inhibition were then recorded and isolates which were resistant to the antifungal agents were taken to the molecular biology laboratory for further analysis on resistant genes using boiling method of DNA extraction.

Analysis of Variance (ANOVA) was done with the means values obtained from the zone of inhibition of susceptible antibiotics.

Extraction Procedure using the Boiling Method of Extraction

Twenty-four (24) isolates that showed complete resistance to the three azoles were picked for molecular typing. The Samples were grown at 37 °C on Luria Bertani (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) media before extracting their DNA. Luria Bertani (LB) media is used because it provides a broad base of nutrient for cells. 24 micro liter centrifuge tubes were brought and numbered appropriately from 1-24. The isolates were transferred into the micro centrifuge tubes, which were then placed in the Eppendorf zentrifuge for one minute at 10,000xg in order to condense the cells, then the supernatants were discarded, after which normal saline was added to the tubes, then vortexed using the XH-B Vortex machine. The tubes were then transferred into a preheated heating block at 95°C for 20mins, after which were centrifuged and vortexed again to ensure that all the components of the cell membrane are broken down to expose the DNA. The tubes were fast freeze for 5minutes, after which 300microliter was pipetted into 1.5ml Eppendorf tubes respectively.

Nanodropping: with the aid of a 10micro litrepipette, 2ml DNA elution buffer was pipetted and placed on the pedestals of the nanodrop to blank, then initialized with 2micro litre of sterile water. Then 2microliter of the isolates were withdrawn using 10microliter eppendorf pipettes and nanodropped, the concentrations of the DNA of each isolate was displayed on the computer screen.

Amplification of ERG11 Gene

Primers eCRG11F (GTTGAACTGTCATTGATGG), Ecrg11R (TCAGAACTGAATCGAAAG) were provided. Primers PCR amplification was performed with a volume of 25µl. Two microliters of each sample was added to the PCR master mixture, which consisted of 5µl of 10× PCR buffer, 4µl of a 25oxynucleoside triphosphate mixture (0.1 mM each dNTP), 0.5µl of each primer (40 pmol of each primer), and 0.4µl (2.0 U) of ExTaq DNA polymerase, with the remaining volume consisting of nuclease free water. Amplification consisted of an initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 4 min; a Gene Amp PCR System 9700

thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.) was used.

Electrophoresis.

Gel electrophoresis with 1% agarose gels was conducted with 1× TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) at 4.8 V/cm for 2 h. A 100-bp DNA ladder (Promega Corp., Madison, Wis.) was run concurrently with amplicons for sizing of the bands. Gels were stained with ethidium bromide-TBE solution and the DNA ladder was added to the gel with the amplicons which were transferred to agarose electrophoresis chamber at 250v for 20 min, after which it was placed in the UV transilluminator and then photographed.

3. Results

Out of a total of 150 HVS samples, a total of 92 (77%) *Candida* species were isolated. These were identified as *Candida albican* 72 (78%), *Candida krusei* 15 (16%), *Candida glabrata* 5 (5%).

Candida species among the age bracket 18-22years (34%) had the highest frequency of *Candida* infection, this is followed by ages 23 to 27years with a total of (30%) while the least age bracket 28 to 37 years had (17%).

Table 1.0: Percentage Susceptibility Test Result for various Antifungal agents.

Figure 1.0: shows the bar chart of Antifungi susceptibility testing of *Candida* isolates to Azole antifungi drugs using Disc Diffusion method, figure 2.0: is a bar chart depicting percentage prevalence of *Candida* isolates. Figure 3.0: depicts Agarose Gel Electrophoresis ITS Region of fungi (*Candida* species), while figure 4.0: is the Agarose Gel Electrophoresis showing ERG11 Resistance gene in *Candida* species.

Table 1: Percentage Susceptibility Test Result for Various Antifungal Agents

Antifungal Agent	Organisms	% Susceptibility
Fluconazole	<i>C. albican</i>	85.70%
	<i>C. glabrata</i>	68%
	<i>C. krusei</i>	46.20%
Ketoconazole	<i>C. albican</i>	100%
	<i>C. glabrata</i>	86%
	<i>C. krusei</i>	92.30%
Itraconazole	<i>C. albican</i>	20%
	<i>C. glabrata</i>	28.60%
	<i>C. krusei</i>	23.10%

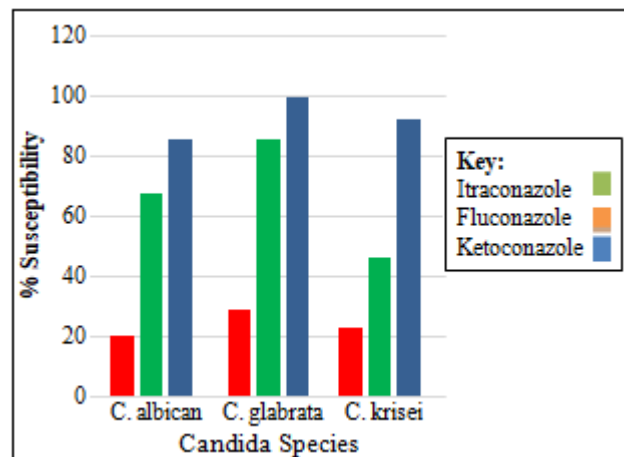


Figure 1.0: Bar chart showing Antimicrobial Susceptibility testing of *Candida* isolates to azole antifungal drugs using Disc Diffusion method

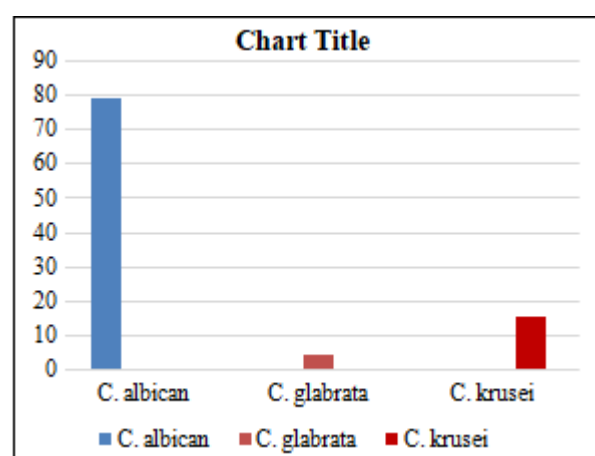


Figure 2: Bar Chart Showing Percentage Prevalence of *Candida* Isolates

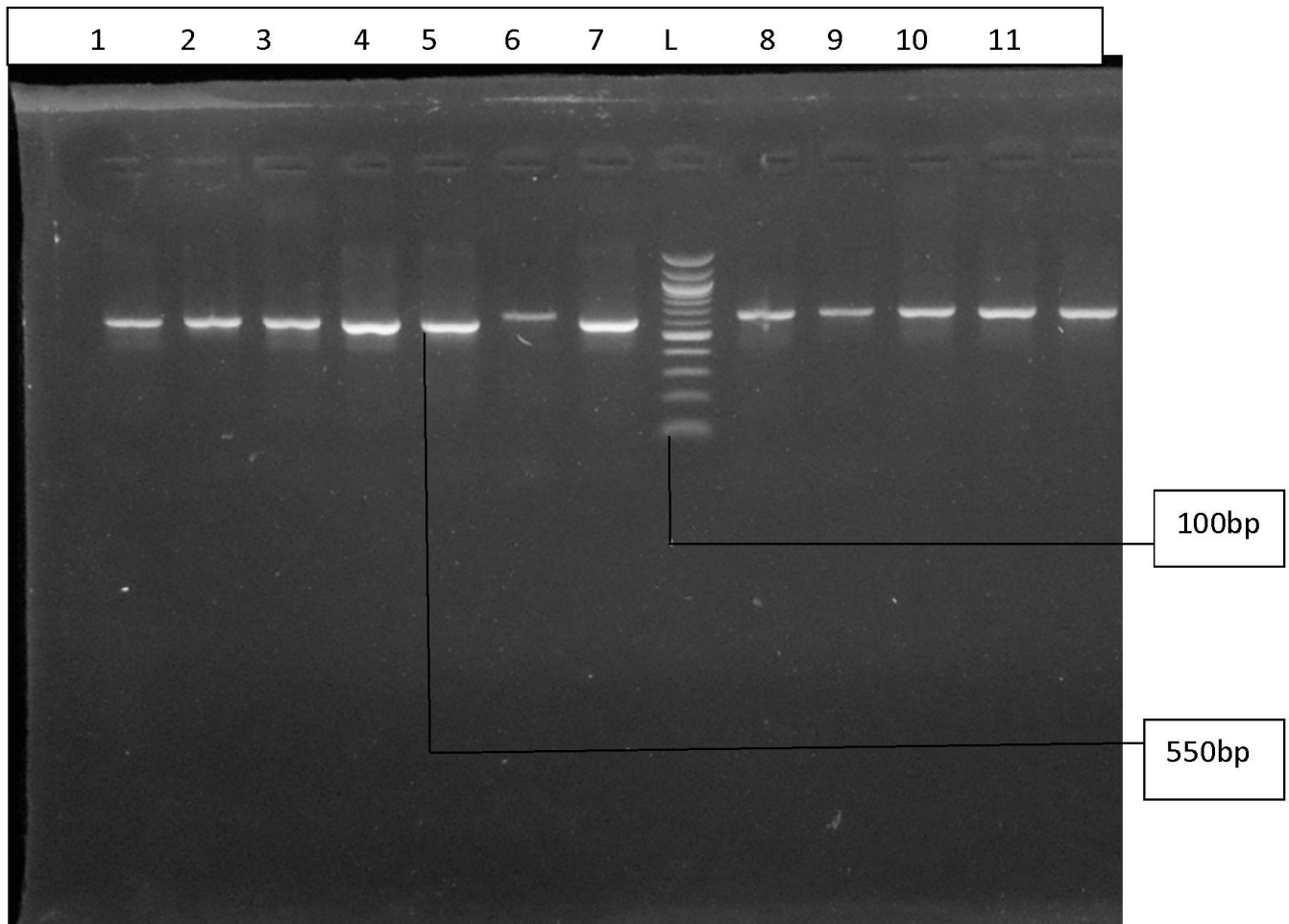


Figure 3: Agarose Gel Electrophoresis showing ITS region of fungi (*Candida* species).
Lanes 1-12 represent the isolates while L represents the 100bp molecular ladder

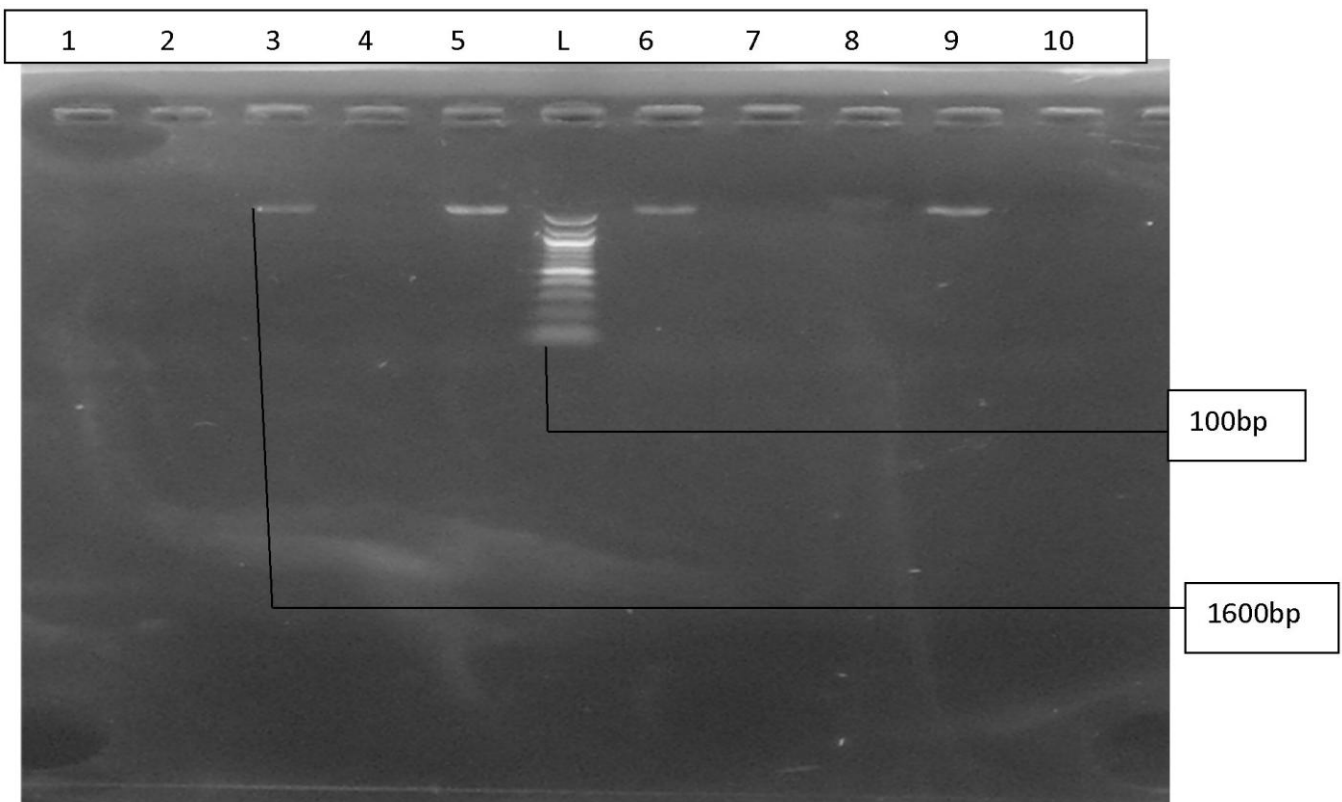


Figure 4: Agarose Gel Electrophoresis showing ERG11 resistance gene in *Candida* species.
Lanes 1-10 represent the isolates, Lane 3, 5, 6, 8 and 9 were positive while L represents the 100bp molecular ladder.

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4. Discussion, Conclusion and Recommendation

4.1 Discussion

Candida infection of the vagina is a common problem that causes significant morbidity and affects the health of women (Arif et al., 2014). It has been suggested that high incidence of *C. albicans* among young women may be due to increased physiological changes in oestrogen and rich glycogen content of the vaginal mucosa thereby providing adequate supply of utilizable sugar that support its proliferation (Barry et al., 2010). Hence under certain favourable conditions such as use of vaginal douching, broad spectrum antibiotics or corticosteroids and other risk factors increases the incidence of vulvovaginal candidiasis.

Out of the 150 samples analysed, 92 (77%) showed positive for *Candida* infection with *Candida albicans* 72 (78%) being the most prevalent, followed by *Candida krusei* 15 (16%) and the least *Candida glabrata* 5 (5%). This highest prevalence of *Candida albicans* showed in this study corroborates with the work of Arif et al. (2014) which stated that *Candida albicans* is the most frequent colonizer as well as responsible for most cases of vulvovaginitis. This prevalence of *Candida albicans* (79%) was higher compared to that reported by Chijioke et al., (2016) to be 2.0%. Observation shows that women between ages 18 to 27 years (30-35%) had the highest incidence of candidiasis; this could be due to the fact that girls between this age range are sexually active and are matured, resulting in hormonal changes and high estrogen content of the vaginal epithelia, thereby making them more vulnerable to vulvovaginal candidiasis. This latter observation correlates with the work done by (Chijioke et al., 2016) which stated that girls within 20-28 years (37-45%) have *Candida albicans* as the most frequent agent.

Three different antifungal agents were used in susceptibility testing and it was found out that fluconazole had the second highest cumulative percentage of 66.6% and *Candida albicans* shows 85.7% sensitivity to it, *Candida glabrata* and *Candida krusei* 68% and 46.2% respectively. This means that fluconazole is less effective in the treatment of infections caused by *Candida glabrata* and *Candida krusei* when compared with *Candida albicans*. Less potency Fluconazole showed against non-albicans correlates with the work done by Chuan et al. (2014) which stated that a high prevalence of non-albicans species is a concern for areas that employ fluconazole as first-line therapy, because some species are intrinsically resistant to azoles (i.e. *Candida krusei*) or susceptible only on high doses (i.e. *Candida glabrata*). Ketoconazole had the highest cumulative percentage (92.8%) on the *Candida* species isolated; this has revealed ketoconazole as the most potent antifungal that could be recommended for clinical use. Itraconazole was the least potent antifungal in this study with a cumulative percentage of 23.9% sensitivity. It was also discovered that azole resistant gene ERG11 was present in four of the isolates that were molecularly analysed, this is because resistance to azole antifungal caused by ERG11 gene occurs as a result of over-expression of the gene.

4.2 Conclusion

This study x-rayed the prevalence of *Candida* infections and the presence of azole resistant (ERG11) in non-pregnant women attending hospitals in Amassoma community. Results from this study have also shown that Candidiasis is prevalent in women between ages of 18 and 27 in Amassoma community. Therefore, there is need for proper hygiene practice in Amassoma community and appropriate use of antibiotics among women in the community. The findings also showed that *Candida albicans* is more prevalent than all the isolated *Candida* species. Ketoconazole had the highest potency of (92.8%), hence recommended as the most effective antifungal drug against *Candida* infections, followed by fluconazole (66.6%) and the least was itraconazole (23.9%).

4.3 Recommendation

One of the leading causes of candidiasis is over use of antibiotics which can lead to the eradication of friendly bacteria that keeps the balance between them and the *Candida* species, therefore, indiscriminate use of antibiotics should be discouraged among youths and proper laboratory diagnosis should be carried out before the commencement of antibiotic treatment. Balanced diet can prevent candidiasis therefore youths should take foods that will boost the immune system.

Proper hygiene should be encouraged to prevent cross-infection

The government in the country should collaborate with the international world to combat antimicrobial resistance existing in the microbial world.

Further study should be carried out to combat the effect of resistance.

Development of new drugs with different mechanisms of action from those of the azoles.

Expansion of the spectrum of activities of the current antifungal drugs to cover a wide spectrum of *Candida* infections.

Based on these findings, ketoconazole is recommended for clinical use against *Candida* infections

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