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Original Article **Open Access**

Antifungal susceptibility and detection of mutant *ERG11* gene in vaginal *Candida* isolates in University of Uyo Teaching Hospital, Uyo, Nigeria

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Abstract:

Background: *Candida* vulvovaginitis is an important cause of morbidity among women. Fluconazole and other azoles are among the commonest antifungal agents used for the treatment of this condition. Azole resistance among *Candida* species is an increasing problem, and mutations in the *ERG11* gene is the commonest cause of fluconazole resistance in *Candida*. The objectives of this study are to determine antifungal susceptibility of vaginal *Candida* isolates and detect carriage of mutant *ERG11* gene by them.
Methods: High vaginal swabs obtained from 260 participants were cultured on Saboraud's Dextrose agar (SDA) for isolation of *Candida*, and identified by growth on CHROMagar *Candida*, germ tube and carbohydrate fermentation tests. Antifungal susceptibility to fluconazole, voriconazole, nystatin and flucytosine was determined by the Kirby Bauer disc diffusion method on supplemented Mueller Hinton agar. *ERG11* gene was detected by conventional singleplex polymerase chain reaction (PCR) assay.
Results: *Candida* was isolated from 126 of 260 (48.5%) participants, and the identified species were *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilopsis* and *Candida famata.* There were 112 (88.9%) isolates susceptible to fluconazole, 122 (96.8%) to voriconazole, 111 (88.1%) to nystatin, and 16 (6.6%) to flucytosine. The mutant *ERG11* gene was detected in all four fluconazole-resistant isolates but not from any of five randomly selected fluconazole susceptible dose dependent (SDD) isolates.
Conclusion: Azole resistance among *Candida* in this environment is associated with mutant *ERG11* gene expression.

Keywords: antifungi, fluconazole, *Candida*, *ERG11*, PCR

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Sensibilité antifongique et détection du gène *ERG11* mutant dans des isolats vaginaux de *Candida* à l'hôpital universitaire de Uyo, Uyo, Nigéria

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Abstrait:

Contexte: La vulvovaginite à *Candida* est une cause importante de morbidité chez les femmes. Le fluconazole et d'autres azoles sont parmi les agents antifongiques les plus couramment utilisés pour le traitement de cette condition. La résistance à l'azole chez les espèces de *Candida* est un problème croissant, et les mutations du gène *ERG11* sont la cause la plus fréquente de résistance au fluconazole chez *Candida*. Les objectifs de cette étude sont de déterminer la sensibilité antifongique des isolats vaginaux de *Candida* et de détecter le transport du gène *ERG11* mutant par eux.
Méthodes: Des écouvillons vaginaux élevés obtenus auprès de 260 participants ont été cultivés sur gélose Dextrose de Saboraud (SDA) pour l'isolement de *Candida*, et identifiés par croissance sur CHROMagar *Candida*, tube germinatif et tests de fermentation des glucides. La sensibilité antifongique au fluconazole, au voriconazole, à la nystatine et à la flucytosine a été déterminée par la méthode de diffusion sur disque de Kirby Bauer sur de

la gélose Mueller Hinton complétée. Le gène *ERG11* a été détecté par un test classique de réaction en chaîne par polymérase (PCR).
Résultats: *Candida* a été isolé sur 126 des 260 participants (48,5%), et les espèces identifiées étaient *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilopsis* et *Candida famata*. Il y avait 112 (88,9%) isolats sensibles au fluconazole, 122 (96,8%) au voriconazole, 111 (88,1%) à la nystatine et 16 (6,6%) à la flucytosine. Le gène *ERG11* mutant a été détecté dans les quatre isolats résistants au fluconazole, mais pas dans aucun des cinq isolats dépendants de la dose (SDD) sensibles au fluconazole sélectionnés au hasard.
Conclusion: la résistance à l'azole chez *Candida* dans cet environnement est associée à l'expression du gène *ERG11* mutant.

Mots-clés: antifongiques, fluconazole, *Candida*, *ERG11*, PCR

Introduction:

With a global prevalence of 5-20%, *Candida* vulvovaginitis is an important cause of morbidity among women. This condition affects up to 75% of reproductive age women at least once (1). *Candida* occurs as a normal commensal of the vagina in about 48% of women. There are about 200 species of *Candida*, of which more than 20 species are associated with human infections, with only four causing vast majorities of the clinical infections, *C. albicans, C. glabrata, C. parapsilopsis* and *C. tropicalis*. While *Candida* vulvovaginitis can occur in otherwise healthy women, it is more prevalent in women who are pregnant, diabetic, taking oral contraceptives or on prolonged antibiotic treatment.

Azole resistance among *Candida* species is an increasing problem especially against the backdrop of opportunistic *Candida* infections in immunocompromised patients (2,3). Azoles, polyenes and antimetabolites such as flucytocine are among the classes of antifungals used to treat *Candida* infections. With increasing resistance among *Candida* isolates, it is important to monitor the susceptibility pattern of these organisms to antifungal agents in order to guide empirical therapy (4). Fluconazole is a triazole antifungal drug with good activity against *Candida* as well as low toxicity and is a drug of choice for *Candida* vulvovaginitis. Fluconazole acts prim- arily on ergosterol biosynthesis by targeting 14-α-lanosterol demethylase encoded by *ERG11* gene resulting in the inhibition of cytochrome P450-dependent conversion of lanosterol to ergosterol. The resulting ergosterol depletion interferes with the bulk functions of ergosterol as a membrane component, but more importantly, severe ergosterol depletion may also interfere with the "sparking" functions of ergosterol, affecting cell growth and proliferation. The blocking of 14α-demethylase results in the accumulation of toxic methylated sterols leading to membrane stress (5).

While some *Candida* species such as C. *krusei* have intrinsic resistance to fluconazole, the current increase in resistance is being driven by widespread continuous use of fluconazole as prophylaxis. Mutations in the *ERG11* gene is the commonest cause of fluconazole resistance in *Candida* (6). Point mutations in *ERG11* may lead to fluconazole resistance in two ways; first, by reducing the affinity of the target to azoles, and second, by enhancing transcription of gene or chromosomal amplification (7). The aim of this study was to determine antifungal susceptibility of vaginal *Candida* isolates and to detect carriage of mutant *ERG11* gene by the isolates.

Materials and methods:

Study setting and design

This was a descriptive, cross sectional, hospital-based study of women of childbearing age attending the Obstetrics and Gynaecology clinics of the University of Uyo Teaching Hospital, a tertiary hospital in Uyo, Akwa Ibom State, South-South Nigeria between April and May 2017.

Subject participants and sampling method

A sample size of 236 was calculated using the formula by Godden (8) and an expected prevalence of 18.9% (9). Participants were randomly selected from women aged 18 years and above attending the Obstetrics and Gynaecology clinic of the hospital. Women on menstrual period were excluded.

Data and sample collection

All eligible participants were interviewed using a 12-point semi-structured questionnaire which covered bio-data, genital hygiene, prior history of antibiotic use and medical history. A pair of high vaginal swab (HVS) samples was collected aseptically from each participant by trained personnel in the Obstetrics and Gynaecology clinics.

Using a sterile disposable speculum, a sterile cotton swab was introduced 4-5 cm into the vagina and turned gently 3-4 times, removed and returned to the swab tube. The swab was labeled appropriately with participant's name and study number and transported to the Microbiology Laboratory of the University of Uyo Teaching Hospital for analysis.

Sample processing

One HVS sample from each participant was used for microscopic examination by wet mount preparation for the presence of yeast cells and gram staining for the presence of budding cells, yeast hyphae and beaded appearance. The second swab stick was streaked on Sabouraud's dextrose agar (SDA) (Lab M, UK) supplemented with chloramphenicol to inhibit bacterial contamination. The inoculated plates were incubated aerobically at 37^{o}C for 24 hours. All soft, moist white-cream colonies on the plates after 24 hours were stained by Gram's method and if budding yeast morphology was present, further identification was carried out.

Identification of isolates

Candida isolates were identified by culture on CHROMagar *Candida* (HK Media, China), germ tube test and carbohydrate fermentation test using glucose, sucrose, maltose and lactose. *Candida* species were identified on CHROMagar *Candida* based on the color of the colony and morphology (rough/smooth) as stated by the manufacturer. Purple-pink colonies were identified as *C. glabrata*, iron blue colonies as *C. tropicalis*, and Beige white as other species. Germ tube test as described elsewhere was used to confirm identification of *C. albicans*.

Carbohydrate fermentation test was used for complete specie identification of all the different *Candida* species isolated. The fermentation test medium was prepared as described by Mackie and McCartney (10). The test organisms were inoculated by adding one drop of 0.5 McFarland turbidity suspensions of each isolate into each tube and incubated for 48 hours at 30^{o}C. The ability of the yeast to ferment a sugar was shown by the presence of acid indicated by change of the yellow indicator to pink colour.

Antifungal susceptibility testing

Antifungal susceptibility test was performed on Mueller Hinton agar (Oxoid, UK) supplemented with 2% glucose and methylene blue (25 ml) by the Kirby Bauer disc diffusion method. The following antifungal agents; fluconazole (25µg), nystatin (100 iu), voriconazole (1µg) and flucytosine (1µg) disks (Oxoid, UK and Abtek, UK) were used on a 90 mm plate to test the susceptibility pattern of the isolates. For fluconazole and voriconazole, the Clinical and Laboratory Standards Institute M44-A document (11) guidelines and interpretative criteria were followed. For nystatin and flucytosine, the diameter of zone of inhibition obtained were compared with the standard zones interpretative breakpoints published by Abtek biological in a leaflet included in the disk pack. The control organism was *C. albicans* ATCC 90028 strain.

DNA extraction:

DNA from all four fluconazole-resistant isolates and five randomly selected intermediately-susceptible isolates was extracted using the ZymoResearch™ (ZR) Fungal DNA Mini prep extraction kit (Inqaba, South Africa). Fungal DNA quantification was done on a Nano-drop-1000 spectrophotometer (SN 1844 ND-1000UV/VIS spectrophotometer, USA) for purity check of the extracted DNA.

Amplification and detection of mutant *ERG11* gene by singleplex PCR:

The mutant *ERG11* gene was amplified using the forward primer sequence 5'-GTTG AAACTGTCATTGATGG-3' and revese primer sequence 5'-TCAGAACACTGAATCGAAAG-3'. A micropipette adjusted to the required volume was used to pipette 7.68 µl of the PCR water into 9 Eppendorf tubes, 0.16 µl each of the forward and reverse primers were added into each of the tubes, and 10 µl of the PCR master mix (Taq polymerase, DNTPs and $MgCl_2$, from Inqaba, South Africa) was added to each of the tubes and 2 µl of the extracted DNA as template. A final volume of 20 µl was obtained in each of the 9 tubes (0.5 ml PCR tube).

The tubes were placed on the micro titer tray of the thermal cycler (ABI 9700 Applied Biosystems), which had been programmed for 30 cycles to run at the following conditions; initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 43°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 7 minutes. The amplified product was resolved on a 1.5% agarose gel at 120 V for 15 minutes and visualized on a UV transilluminator (Analytic Jena, Germany) for the expected band size of 1650 bp.

Statistical analysis

Demographic variables and frequency of occurrence of *Candida* species was analyzed using SPSS (Statistical Package for the Social Science) version 20.0. Descriptive results were expressed in frequency and percentage. Measure of association of the categorical variables was analyzed using Pearson Chi-square. Association between variables were considered statistically significant at *p*-value less than 0.05.

Ethical consideration

Ethical approval was obtained from the Ethical Review Board of the University of Uyo Teaching Hospital, Uyo where this research was conducted before the commencement of the research work. Written informed consent was obtained from all participants prior to specimen collection. No information that could directly identify participants was included and all information from participants was treated with utmost confidentiality.

Results:

Study participants

A total of 260 women participated in the study. The ages of participants ranged from 18-46 years while the mean age was 28 years. Of the 260 women, 77 (29.6%) were single, 181 (69.6%) were married, 1 (0.4%) divorced and 1 (0.4%) widowed. One hundred and twenty-nine (49.6%) participants were pregnant.

Prevalence of *Candida* among participants

Candida was isolated from 126 (48.5%) of women examined. The most frequently isolated specie was *C. albicans* (77.8%), followed by *C. glabrata* (15.1%), *C. tropicalis* (4.8%), *C. parapsilopsis* (1.6%) and *C. famata* (0.8%).

Susceptibility of *Candida* species to antifungal agents

The antifungal susceptibility of *Candida* species isolated to antifungal agents tested is shown in Table 1. Out of the 126 isolates, 112 (88.9%) were susceptible to fluconazole, while 10 (7.9%) were intermediate and 4 (3.2%) were resistant; 122 (96.8%) were susceptible to voriconazole, while 1 (0.8%) was intermediate and 3 (2.4%) were resistant; 111 (88.1%) were susceptible to nystatin, 10 (7.9%) were intermediate and 5 (4.0%) were resistant; 16 (6.6%) were susceptible to flucytosine, 30 (24.2%) were intermediate and 78 (69.2%) were resistant.

Table 1: Antifungal susceptibility pattern of *Candida* species isolated (n=126)

Antifungal agents	FCA			VOR			NYST			FLU		
Species	S (%)	SDD (%)	R (%)	S (%)	SDD (%)	R (%)	S (%)	SDD (%)	R (%)	S (%)	SDD (%)	R (%)
C. albicans (n=95)	87 (91.6)	4 (4.2)	4 (4.2)	94 (98.9)	1 (1.1)	0	84 (88.4)	7 (7.4)	4 (4.2)	13 (13.8)	22 (23.4)	59 (62.8)
C. glabrata (n=19)	15 (78.9)	4 (21.1)	0	8 (88.9)	0	1 (5.3)	18 (94.7)	0	1 (5.3)	3 (16.7)	3 (16.7)	12 (66.7)
C. tropicalis (n=9)	7 (77.8)	2 (22.2)	0	18 (94.7)	0	1 (11.1)	6 (66.7)	3 (33.3)	0	0	3(33.3)	6 (66.7)
C. famata (n=1)	1 (100)	0)	0	1(100)	0	0	0 (0)	1(100)	0	0	1 (100)	0
C. parapsilopsis (n=2)	2 (100)	0	0	2 (100)	0	0	1 (50)	1(50)	0	0	1 (50.0)	1 ((50.0)
Total	112	10	4	123	1	2	109	12	5	16	30	78

Key: FCA=Fluconazole, VOR=Voriconazole, NYST=Nystatin, FLU=Flucytosine, S=Sensitive, R=Resistant, SDD= susceptible dose dependent

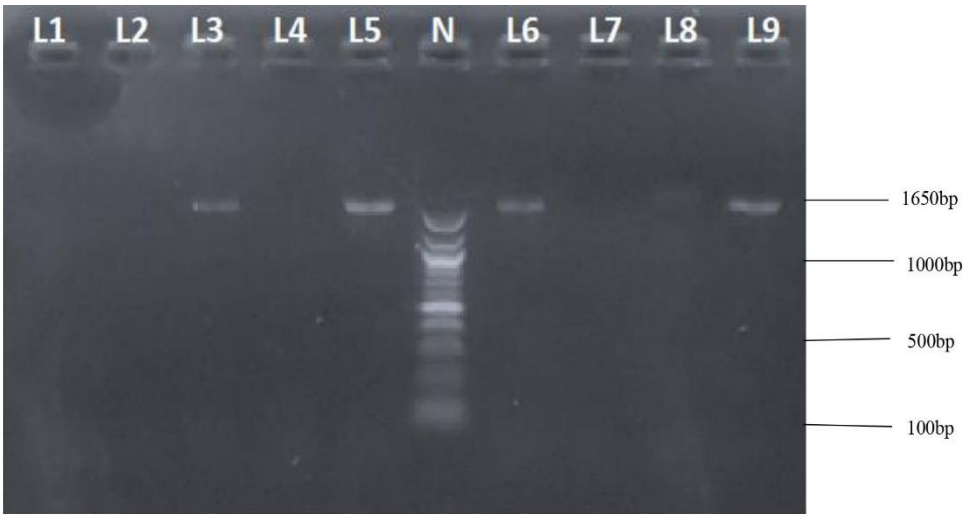

Fig 1: Gel electrophoresis picture of amplified mutated *ERG11* gene from 4 fluconazole-resistant and 5 selected intermediate-dose isolates

Lanes 3, 5, 6 & 9 show the amplified *ERG11*mutated gene bands (1650bp), lane N shows a 1000bp quick-load molecular ladder

Discussion:

Vaginal discharge is one of the most frequent gynaecological problems encountered in females especially during their reproductive age and most women regard any secretion from the vagina as abnormal discharge. The first task of primary health care providers is to ascertain whether this discharge is pathological or physiological (12). The presence of *Candida* in the vagina does not necessarily indicate infection, but colonization of the vagina usually precedes infection. Colonization rate of *Candida* species is believed to vary with geographical location, studied population and, probably, microbiological sampling methods (13).

The prevalence of vaginal *Candida* in this study was 48.5%. This prevalence is comparable to 40.8% and 42.7% obtained from similar studies in Zaria (14) and by Feyi and Amadi in Abia (15), Nigeria respectively. Higher prevalence of 70% and 64.7% have been reported in similar studies by Oyewole et al., (16) in Minna and Lennox et al., (17) in Calabar respectively. The disparity could be due to differences in populations studied and also due to the fact that the study included only participants with symptoms of vaginitis. Lower prevalence rates have been reported from similar studies by Anorlu et al., (18) who reported 22.1% in Lagos and Usanga et al., (19) who reported 21.5% in Calabar.

We found that *C. albicans* (77.8%) was the most frequently isolated specie from the study participants, which is similar to what have also been reported from Ogun and Kano States, Nigeria (20,21). The frequency of isolation of non-albicans *Candida* (NAC) species reported in this study are; *C. glabrata* 15.1%, *C. tropicalis* 4.8%, *C. parapsilopsis* 1.6% and *C. famata* 0.8%. NAC species are becoming increasingly more prevalent as reported in previous studies (16,22,23). *Candida* colonization was highest in age groups 18-24 and 25-31 years. This probably could be due to ovarian activity as well as sexual activity which peak in women at 20-30 years of age (24). During this period, the ovary produces adequate amount of estrogen, which favours *Candida* growth by maintaining the acidic pH in the vagina and enhancing the yeast adherence to vaginal epithelial cells (25). The age distribution of *Candida* colonization observed in this study is similar with the findings of Onuorah et al., (26) in Awka, southeastern Nigeria, which reported highest prevalence in the age group 20-30 years. Similarly, studies from Jos and Kano, Northern Nigeria have reported highest prevalence in the age group 21-30 years (22,27). Menza et al., (28) reported a prevalence rate of 60% within the age group 26-35 years in Kenya.

Antifungal susceptibility testing of *Candida* is recognized as a useful aid in optimizing the treatment of *Candida* infections as emergence of resistant strains continuously threatens therapy (29). In this study, vaginal *Candida* isolates showed highest antifungal susceptibility rates to voriconazole, followed by fluconazole and nystatin, and the least susceptibility to flucytosine. This observation is similar to the findings of Efunshile et al., in Lagos (30). *Candida albicans* has been reported in Nigeria (31) and other parts of the world (32) as the specie with the highest susceptibility to voriconazole. The susceptibility of *Candida* species in this study to nystatin is similar to what has been reported in studies from Younde, Cameroon (33) and Kampala, Uganda (34). Other studies have reported up to 100% resistance of *Candida* to nystatin in Yola, Nigeria (35) and 80% in southwest Cameroon (36). The antifungal susceptibility testing in this study showed high resistance rate to flucytosine among all *Candida* species isolated. This is unsurprising as secondary resistance to flucytosine frequently develops especially when used as monotherapy. Similar high rates have been observed in other studies (37,38). Contrariwise, Efunshile et al., in Lagos, Nigeria (30) reported 100% susceptibility of *Candida* to flucytosine.

In this study, PCR amplified mutant *ERG11* gene in all the 4 fluconazole-resistant isolates of *C. albicans* and not in the 5 selected intermediate isolates. Among the molecular mechanisms for azole resistance in *Candida*, the *ERG11* gene plays a prominent role. The *ERG11* gene codes for the enzyme lanosterol demethylase which is the target for azole antifungal agents including fluconazole (29, 39). Resistance to azole may result from overexpression of *ERG11* gene independently or in combination with other resistance mutations. Overexpression of *ERG11* gene has been noted in *C. albicans, C. parapsilosis* and *C. tropicalis*, and studies have shown that this overexpression often involves *Upc2p*, a zinc cluster transcription factor, that is induced upon ergosterol depletion (40). Point mutations in the coding regions of the *ERG11* gene which lead to amino acid substitutions may also impact susceptibility to fluconazole. Over 140 point mutations have been described for *C. albicans* most of which fall into distinct "hot spot" regions within the protein amino acids 105–165, 266–287, and 405–488 (40,41). There appears however to be less variability in the *ERG11* sequence in the NAC species (6).

Resistance to fluconazole in this study was found in 4.2% of *C. albicans* isolates but not in the NAC species. Similar resistance rates have been reported elsewhere in Nigeria and also in the United States (24,42,43). Resistance rates of up to 9.5% has been

reported in Lagos, Nigeria (44) and 11.3% in Xijing, China (38). Relatively high cost of fluconazole in Nigeria leading to reduced usage may be responsible for this low rate of resistance (29).

Our study was limited by inability to carry out full sequencing of *ERG11* gene to determine the actual mutations prevalent in this environment. This should be the aim of future studies. However, it shows the rising incidence of fluconazole resistance among vaginal *Candida* species which could lead to treatment failure where azoles are first line treatment options. In conclusion, we report fluconazole resistance among vaginal *Candida* which is related to mutation in the *ERG11* gene, with consequences for antifungal treatment of vaginal candidiasis in this environment.

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