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Original Article

Fluconazole susceptibility and *ERG11* gene expression in vaginal *candida* species isolated from lagos Nigeria

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Abstract: Fluconazole resistance is an important type of resistance in *Candida* because in most countries, fluconazole is the drug of choice for vulvovaginal candidiasis. *Candida* species resist fluconazole by various mechanisms but there is paucity of data on these in our environment. Such mechanisms include among others, over-expression of the *ERG11* gene, which codes for synthesis of the target enzymes in the fungus. The aim of this study was to screen *Candida* spp. resistant to fluconazole for the expression of *ERG11* gene. Fluconazole susceptibility test was performed on 28 clinical strains of *Candida* species previously obtained from students of a School of Nursing in Lagos, Nigeria. They were identified by API Candida, CHROMagar candida and germ tube test. Using 25 mcg discs, fluconazole susceptibility was determined by the disc diffusion method and results were interpreted in accordance with the Clinical Laboratory Standard Institute (CLSI) criteria; sensitive (S), resistant (R) and susceptible dose dependent (SDD). The R and SDD isolates were subsequently evaluated for the presence of *ERG11* gene. Of the 28 clinical isolates, 14 were identified as *C. albicans* and six as *C. tropicalis*. The remaining isolates were identified as *C. glabrata (2)*, *C. famata* (2) *C. kefyr* (2) one each of *C. parapsilosis* and *C. guilliermondii* respectively. In this study, 18 were susceptible (S) to fluconazole, eight were SDD and two were resistant to the antifungal agent. Out of the 14 *C. albicans* isolates, 12 were susceptible, one showed high level resistance and similar number showed susceptible dose dependence. *ERG11* was detected in three susceptible dose dependent *Candida* species. This analysis demonstrates that susceptible dose dependence should not be overlooked as it may be associated with the presence of *ERG11* gene and resistance to fluconazole. There is a need to consider routine antifungal susceptibility testing for *Candida* species causing vulvovaginitis.

Keywords: Fluconazole, *ERG11* gene, *Candida* species, antifungal susceptibility

Introduction

Candida is a yeast fungus found as part of the muco-cutaneous flora of humans. There are approximately 200 species, among which are *Candida albicans, glabrata, tropicalis, stellatoidea, parapsilosis, catemilata, ciferri, guilliermondii, haemulonii, kefyr* and *krusei* [1]. *Candida albicans* is the most common species and is found in the vagina of 20-50% of healthy and asymptomatic women [1]. Non-albicans such as *C. glabrata* and *C. tropicalis* are also found in vaginal specimens [2]. Although *Candida* species occur as normal vaginal flora, opportunistic conditions such as diabetes, pregnancy and other immune depressants in the host enable them to proliferate and cause infection. About 75% of adult women experience at least one episode of vulvovaginal candidiasis during their lifetime among which approximately 40-50% would experience further episodes and 5% will develop recurrence, with at least three symptomatic episodes in one year [2-4]. The number of episodes tends to be more in women who are sexually active, pregnant, immunocompromised or on contraceptive pills [5-8].

A number of antifungal agents especially azoles are available to treat vulvovaginal candidiasis.

However, fluconazole is a triazole introduced in the 1990's for the treatment of infections due to *Candida* and other opportunistic yeasts [9]. Currently, it is recommended in various guidelines as the first drug of choice because it is less toxic and can be taken as a single oral dose [9, 10]. Ergosterol is an essential component of the fungal plasma membrane which maintains the integrity of fungal cell wall. Lanosterol 14α-demethylase enzyme is a key enzyme in the synthesis of ergosterol. This enzyme is the target of fluconazole which it inhibits with a resulting impairment of ergosterol synthesis and depletion of ergosterol in the fungal cell membrane.

Production of lanosterol 14α-demethylase is encoded by the *ERG11* gene. Over-expression of *ER11* gene results in production of a large amount of lanosterol 14α-demethylase and this favours continuous synthesis of ergosterol and maintenance of the integrity of the cell wall which enables *Candida* to resist fluconazole [11-14]. This type of resistance has been associated with widespread and continuous usage of fluconazole as prophylaxis [11]. In addition, mutations in *ERG11* gene exist and contribute to resistance in about 65% of fluconazole-resistant *Candida* species [11, 15]. Genetic variation due to mutation in *ERG11* gene among strains of *Candida* species is responsible for significant differences observed in their susceptibilities to fluconazole and itraconazole [16].

The interpretive breakpoints for *Candida* species tested against fluconazole are based on the analysis of treatment outcomes of infections [17, 18]. In addition to the traditional categories of "susceptible" and "resistant", the interpretive breakpoint includes a novel category, "susceptible-dose dependent" [17, 18]. The SDD category encompasses MICs of 16 and 32 μg/ml (or a width of clearance zone of 15-18 mm as determined by the disc diffusion method. Isolates for which fluconazole MICs were above 64 μg/ml (≤ 14 mm) were termed resistant (R), whereas those inhibited at a lower concentration of 8 μg/ml (> 19 mm) were labeled susceptible (S). This study was conducted to evaluate the susceptibility of clinical isolates of *Candida* to fluconazole and to investigate isolates with resistance and SDD susceptibilities for the expression of *ERG11* genes.

Materials and methods

Study design

Fluconazole susceptibility testing was carried out on clinical isolates of *Candida* spp. obtained from some young women. The resistant isolates and susceptible dose dependent species were subjected to polymerase chain reaction using primers that identify *ERG11* gene expression.

Study population

A total of 28 clinical isolates of *Candida* were obtained from high vaginal swabs collected from students of the school of nursing of the Lagos University Teaching Hospital, Lagos Nigeria between May and August, 2008. All patients gave written informed consent to be recruited for this study, which was approved by the Research and Ethics Committee of the College of Medicine, University of Lagos, Proc. No. CM/COM/8/VOL.XIX and Lagos University Teaching Hospital (LUTH) Idi-Araba Proc. No. ADM/DCST/221/VOL.10. The samples were collected using sterile swab sticks and cultured on sabouraud dextrose agar at 37°C for 24 hours. Isolates were characterized using API Candida kit (Biomerieux, France) CHROM agar Candida (Biolmerieux, France) and germ tube formation. The pure isolates were stored in 10% glycerol in Brain-Heart infusion broth at - 80°C.

Antifungal drug susceptibility testing

Suspension of inoculums was prepared in 5 ml of sterile saline (0.85%) and the turbidity adjusted to 0.5 McFarland standards. Within 15 minutes of adjusting the turbidity, each isolate was plated onto a dried surface of a sterile Mueller-Hinton + Glucose + Methylene Blue agar plate respectively using a sterile cotton swab. Antimicrobial disks containing 25 μg of fluconazole were dispensed onto the surface of the inoculated agar plate. Each disk was pressed down to ensure its complete contact with the agar surface. The plates were incubated at 37°C and examined after 24 hours of incubation. The zones of inhibition were measured in millimeter and the results were interpreted using validated CLSI interpretive breakpoints for *in vitro* susceptibility testing of fluconazole [17]. *Candida* species were reported as suscep-

tible S (zone diameter ≥ 19 mm); susceptible dose dependent SDD, (15 to 18 mm) and resistant R (≤ 14 mm).

DNA extraction

Yeast strains were cultured on Sabouraud Dextrose Agar (SDA) at 37°C for 24 hours. The *ERG11* genes of all fluconazole resistant (< 14 mm zones) and susceptible doses dependent isolates (15-18 mm zones) were amplified using PCR. DNA was extracted from yeast isolates using a method previously described [19] and slightly modified in this study. Briefly, isolates were harvested from culture media into 30 ml sterile de-ionized water and washed at 6,000 rpm for 15 min and washed once in distilled water. The cell pellets were resuspended in 700 µl of lysis buffer (10 mM Tris-HCl [pH 8.0], 250 mM EDTA [pH 8.0], 0.5% Triton X-100 [vol/vol]) supplemented with 3 mg/ml of lyticase enzyme and incubated at 37°C overnight. The spheroplasts were subsequently lysed by incubating the sample with 3 mg/ml of proteinase K at 55°C for 2 h, after which the proteinase K was inactivated at 95°C for 10 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, the tube was mixed by inversion and centrifuged at 12,000 rpm for 15 minutes. The aqueous layer in the eppendorf tube was transferred to a fresh tube, and an equal volume of ice-cold isopropanol was added, the DNA was precipitated by centrifugation at 12,000 rpm for 15 min, while the pellet formed was washed twice with 70% ethanol. Air-dried pellets were re-suspended in 200 µl of TE buffer and stored at - 20°C prior to use. DNA concentrations were estimated against known concentrations of lambda DNA using Nanodrop spectrophotometer (ND 1000). *Candida* DNA was diluted to a working concentration of 10 ng/µl.

DNA amplification

The reaction solution consisted of 2.5 ml of PCR reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 10 ng of target genomic DNA, 200 mM of dNTPs, 1 mM of each primer, 0.5 U Taq polymerase, and sterilized distilled water to a final volume of 25 ml. PCR was carried out by using primers that span the entire *ERG11* open reading frame: 5'-GTT GAA ACT GTC ATT GAT GG (forward) and 5'-TCA GAA CAC TGA ATC GAA AG (reverse) [13, 15]. The PCR was performed in a 25 well thermocycler (Epperndorf, Germany). The system was programmed to 3 min of denaturation at 92°C and this was followed by 30 cycles, each consisting of 1 min of denaturation at 92°C, 1 min of annealing at 43°C, and 1 min of extension at 72°C. At the final cycle, an additional 10 min of incubation at 72°C was performed for complete extension. For each PCR run, a negative control was also included containing the reaction buffer, dNTPs, Taq polymerase without the target DNA. Reference strain *C. parapsilosis* ATCC 22019 was included in each run. The amplicon was detected by electrophoresis on a 2% agarose gel performed at 70 V for 2.5 h. The gel was stained with ethidium bromide (0.5 µg/ml) and photographed under UV light with a Photo documentation system (GenoSens 1500). A DNA ladder of 100 bp (Promega, USA) was used as molecular weight marker.

Table 1. *In vitro* susceptibility pattern of *Candida* species to fluconazole

Species	Number of isolates tested (%)	S (%)	SDD (%)	R (%)
C. albicans	14(50)	12(85.7)	1(7.1)	1(7.1)
C. tropicalis	6(21.4)	3(50)	3(50)	-
C. glabrata	2(7.14)	1(50)	1(50)	-
C. kefyr	2(7.14)	1(50)	1(50)	-
C. famata	2(7.14)	1(50)	-	1(50)
C. guilliermondii	1(3.57)	-	1(100)	-
C. parapsilosis	1(3.57)	-	1(100)	-
Total	28(100)	18(64.3)	8(28.6)	2(7.14)

S: susceptible; SDD: susceptible dose dependent; R: resistant.

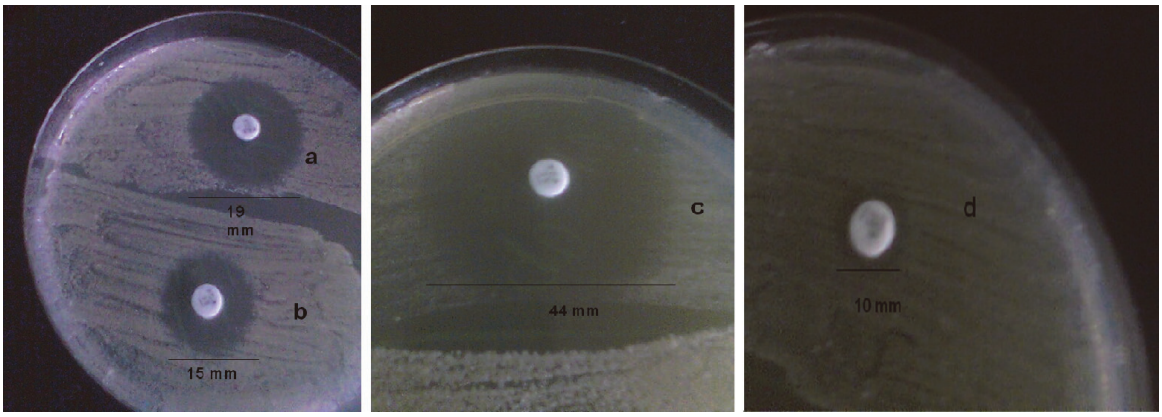

Figure 1. Susceptibility pattern of *Candida* specie to of Fluconazole (25 µg) showing different sizes of inhibition zone. **A.** zone size of 19 mm (S); **B.** 15 mm (SDD); **C.** 44 mm (S); **D.** 10 mm (R).

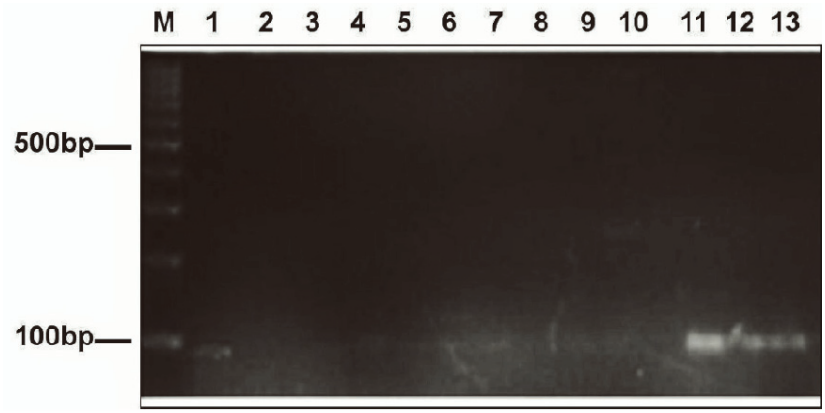

Figure 2. Agarose gel electrophoresis of the amplicon lane M: DNA marker, lane 2 (+) positive control, lane 3 (–) negative control and lanes 11, 12, 13 showing visible amplification of *ERG11* gene with band size of 92 bp for SDD isolates; *C. albicans, C. parapsilosis* and *C. tropicalis.*

Results

Out of the 28 *Candida* species investigated, 14 were characterized as *C. albicans*, six were *C. tropicalis*, two each were *C. glabrata*, *C. famata*, *C. kefyr*, one as *C. guillermondii* and *C. parapsilosis* respectively. The species distribution and *in-vitro* susceptibilities of the *Candida* isolates to fluconazole (**Table 1**). Antifungal susceptibility testing revealed that 18 *Candida* species were susceptible to fluconazole, two were resistant while eight were SDD. Of the 14 species of *C. albicans* tested, one was resistant, another one SDD while the remaining 12 were susceptible. Zones of inhibition showing the interpretive categories in accordance with the disc diffusion technique were presented on **Figures 1A-D**. *ERG11* gene was amplified and detected in two resistant *Candida* isolates which included one *C. albicans* and one *C. famata*. The gene was also amplified in three SDD isolates comprising one each of *C. albicans*, *C. parapsilosis* and *C. tropicalis* respectively (**Figure 2**).

Discussion

Antifungal susceptibility testing of *Candida* is becoming recognized as a useful aid in optimizing the treatment of *Candida* infections as emergence of resistant strains continuously threatens fluconazole therapy. In this study, we provided data on fluconazole susceptibility of vaginal *Candida* species. Overall, most of the 28 isolates investigated were susceptible to fluconazole. *C. albicans* is generally reported to be susceptible to fluconazole [20-24]. Reports emanating from Brazil on MIC ranges of all isolates tested indicated susceptibility to fluconazole [20]. In Benin City Nigeria, 97.2% suscep-

tibility was observed among isolates obtained from patients with genitourinary tract infections, but the study did not define the presence or absence of susceptible dose dependent strains [24]. Although most *C. albicans* studied were sensitive to fluconazole, only 67% were clearly sensitive while 22.2% were susceptible dose dependent. This is noteworthy because increasing the dosage of fluconazole for such susceptible dose dependent strains would likely yield good therapeutic results *in-vivo* [19-22]. *C. tropicalis* is usually susceptible to fluconazole, and in this study three (50%) were susceptible while three (50%) were susceptible dose dependent.

In recent times, most surveillance studies reported fluconazole resistance in both *albicans* and non-*albicans* species [21, 22]. Our study showed that 57.1% of non-*albicans* species were susceptible dose dependent and one isolate of *C. famata* showed high level resistance. An overall resistance rate of 7.14% was noted; this result is similar to that of a previous study that observed resistance in 9.5% of *Candida* species obtained from HIV patients [25, 26] and those with genitourinary tract infection [24] but a bit lower in another similar study [27]. Fluconazole is the first drug of choice for treating vaginitis in most parts of the world in accordance with recommended guidelines [9, 10], however, due to the high cost it is not usually the first drug to be prescribed in Nigeria. It is usually prescribed after cotrimazole has failed to treat vaginitis, so a low resistance rate to it was expected. The level of resistance seen could be due to its widespread use as prophylactic, empiric and definitive therapy of candidal and other fungal infections in HIV/AIDS persons [21-23, 26, 27] that are many in Nigeria [28]. We therefore, highlight the need for routine susceptibility testing before fluconazole is prescribed.

Fluconazole resistance in *Candida* species has been associated with over expression of *ERG11* genes which encodes for lanosterol 14α-demethylase. Over-expression of *ERG11* gene may occur due to genetic point mutation in *ERG11* gene [11]. In this study, PCR amplified *ERG11* gene in three of the isolates that were susceptible dose dependent. Constant exposure of *Candida* species to fluconazole has frequently been associated with resistance. Although *ERG11* gene is present in all species of *Candida*, the primer used in this study was designed to detect point mutation within the *ERG11* gene [13, 15] apparently associated with resistance due to the exposure of these species to azoles hence the detection of *ERG11* gene in some isolates and not in others. The detection rate of *ERG11* gene observed in this study is similar to a previous finding that went further to determine the level of *ERG11* expression [16]. Unfortunately, due to non availability of sequencing machine in our environment, it was not possible to determine the level of expression of the *ERG11* genes.

The *ERG11* genes expression in the fluconazole resistant isolates is noteworthy especially since they were found in susceptible dose dependent isolates. This finding shows that the molecular mechanisms of fluconazole resistance in Nigerian isolates may likely include over-expression of *ERG11* gene. It also demonstrates that susceptible dose dependence should not be overlooked as it may be associated with the expression of *ERG11* gene.

Surveillance of the resistance mechanisms of fluconazole is a potentially valuable tool to track the emergence and spread of fluconazole-resistant *Candida* species and there is need to consider routine antifungal susceptibility testing for *Candida* species causing vulvuvaginitis in our environment.

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