PRESENCE OF CDRI EFFLUX GENE AMONG MULTI-DRUG RESISTANT *Candida albicans* FROM CLINICAL ISOLATES IN BENIN CITY, NIGERIA

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ABSTRACT

Background*:Candida albicans* are the most common cause of yeast infections worldwide. They are known to cause opportunistic infections being able to colonise the vaginal area, gastrointestinal tract and respiratory tract when theimmune system is compromised. The antimicrobials of choice for the treatment of yeast infections are antifungal agents. Most of these antifungal agents have developed resistance over the years due to inappropriate use.

Aim: This study was done to determine the presence of CDR1 genes in Benin City Nigeria.

Methods: A total of 161 consecutive non-repetitive clinical isolates of *Candia albicans* were used in this study. A disc susceptibility test was performed on all isolates with fluconazole, ketoconazole, amphotericin B and nystatin.

Results:Isolates resistant to two or more agents were used for molecular studies to detect CDR1 genes. Fluconazole (42.85%) ketoconazole (34.78%), Amphotericin B and nystatin (0.62% each) were not active against *Candida albicans*. Eighty percent (80%) (32/40) of representative *Candida albicans* harbored CDR1 gene. The distribution of the CDR1 gene was not affected by gender, age, specimen type and source of patients ($P<0.005$).

Conclusion: The resistance to fluconazole was prevalent in this study, Eighty percent of the reprentative Candida isolates haboured the CDR1 genes.

Keywords:CDR I Efflux Gene, Multi-Drug Resistant, *Candida albicans*

INTRODUCTION

Candida species are the most common cause of fungal infections worldwide (Saranya *et al*., 2004). They are known to be normal microbiota within the gastrointestinal tract, respiratory tract, vaginal area and the mouth. It is also known to cause sexually transmitted diseases (Prescot *et al*., 2008). The genus *Candida* is composed of a heterogeneous group of organisms, and over 17 different *Candida* species are capable of causing human infections. However, more than 90% of invasive infections are mainly caused by *Candida albicans, C. glabrata, C. parasilopsis, C. tropicalis, C. krusei, C. dubliniesis* and *C. lusitanae* (Ali *et al*., 2011). *Candida* causes infections when the host becomes immunocompromised as a result of diseases like the human immunodeficiency Virus (HIV) (Lopez, 2010), and advances in medical management such as antineoplasm, chemotherapy, organ transplantation and hemodialysis has led to an expanding population of susceptible host (Mikulska *et al*., 2012). The yeast begins to invade and colonise the body tissues by releasing potent toxins/chemicals into the bloodstream, causing varying symptoms such as lethargy, chronic diarrhoea, yeast vaginitis, bladder infections, muscle and joint pains, menstrual problems, constipation and severe depression (Kim and Sudbery, 2000; Adard *et al*., 2001).

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The most prevalent pathogen amongst the *Candida* species is *Candida albicans.* It ranks as the fourth leading cause of nosocomial infections worldwide,with a mortality rate of about 50% (Kent, 1991, Simoes *et al.*, 1998; Fermer, 2000; Derosa and Primel, 2004; Aci *et al.,* 2011).

Most yeast infections caused by *Candida albicans* are commonly treated with azole drugs, although treatment is hampered by problems of solubility, stability absorption and high level of resistance (Hoffman *et al.,* 2000). The search for new antifungal drugs is highly prioritised because drug resistance is increasing (NCCLS, 1997).

The widespread and prolonged use of fluconazole therapy in the 1990s has led to an increased frequency of treatment failure due to fluconazole-resistant *Candida albicans* (Ruhnke *et al.,* 1994; Boschma *et al*., 1998). The primary mechanism responsible for high-level azole resistance in *Candida albicans* isolates is the over-expression of plasma membrane efflux pumps (Sanglad and Bill, 2002; White, 2002; Roger and Bakes, 2003; Holmes *et al.,* 2008). There are two prominent families of efflux pump protein. The ATP binding cassette (ABC) superfamily transporters encoded by *Candida* drug resistance genes (CDR) and multifamily efflux transporter encoded by the multi-drug resistance gene (MDR) (Prassid *et al*., 1995; Sanglad *et al.,* 1997; Perea and Peterson, 2002).

These proteins act as molecular pumps that actively translocate drugs throughthe plasma membrane using energy from ATP hydrolysis (Lorena and Pierre, 2014). The emergence of drug resistance in several *Candida albicans* isolates from patients undergoing azole treatment is mostly from previously susceptible strains. The step-wise acquisition of resistance has been associated with the over-expression of specific MDR genes such as CDRI and CDR2 (Prasad *et al*., 2011).One central mechanism for high-level azole resistance is the over-expression ofthe plasma membrane efflux pump. (Sanglad and Bill, 2003; Roger and Bakers, 2003; and Holmes *et al.,* 2008). Therefore, This study aims to determine the presence of CDR1 genes amongst azole-resistant *Candida albicans* from clinical specimens.

MATERIALS AND METHODS

Study Location

This study was conducted in the University of Benin Teaching Hospital, Benin City, Edo State: South-South geopolitical zone, Nigeria. The Hospital is a tertiary institution with a referral status. It has about 900-bed space and is within the rain forest zone, serving as a referral hospital for 6-10 neighbouring States in Nigeria. The Ethics and Research Committee of the University of Benin Teaching Hospital approved the protocol for this study.

Candida Isolates

The clinical isolates of *Candida albicans* were retrieved from the medical microbiology laboratory from various clinical specimens; gender, age and other information were also retrieved from the laboratory records. The candida isolates were identified with standard techniques and speciated using CHROMAgar Candida as described by (Paripokee *et al.,* 2005).

Antifungal Yeast Susceptibility Testing

Disc antifungal susceptibility testing was performed on 161 *Candida albicans* isolates using agar diffusion methods as previously described (CLSI, 2009; CLSI, 2012). The antifungal agents were fluconazole (25mg) ketoconazole (10mg), amphotericin B 25mg) and nystatin 10 units. Briefly, each isolate was emulsified in sterile water, and the turbidity was matched with 0.5 MacFalandStandard (this was prepared by adding 0.5ml of 1% barium chloride to 99.5ml of 1% sulphuric acid). Once matched, a sterile cotton swab was dipped into the suspension, and excess liquid was removed by squeezing the swab on the side of the test tube. The entire surface of the Muller-Hinton agar (containing 2% glucose and 0.05ml of methylene blue) was seeded by swabbing in three different swabs. The antifungal disc was placed on the surface of the agar plate using sterile forceps.

Each disc was pressed down to ensure complete contact with the agar surface. The plates were incubated at 37ºC for 24 hours, and the zone of inhibition was measured in millimetres. A standard chart was used to determine if the isolates were sensitive or resistant to the antifungal agents (CLSI, 2009). The isolates resistant to the above antifungal agents were subjected to DNA extraction, PCR amplification, gel electrophoresis and sequencing of PCR products. The molecular analysis was done atthe Lahor Research Laboratoryin Benin City, Nigeria and Inqaba Laboratory in South Africa.

DNA Extraction of the Candida Isolates

The DNA of the 40 *Candida albicans* isolates resistant to two of the azoles antifungal agents was extracted using ZR fungal/bacterial DNA miniprep. Tm^{kt}Zymo Research Cooperation, USA) following the manufacturer's instructions. Briefly, overnight cultures of the *Candida albicans* isolates from Sabouraud DextroseAgar (SDA) were emulsified in nuclease-free water. Two microliters of the candida suspension were added to the ZR bashing bead ^{TM} lysis tube, and 750µl lysis solution was added to the tube. This was centrifuged at 10,000g for 1minute in a zymo-spin IV spin filter into a collection tube; 400µl of the centrifuged supernatant was added and centrifuged at approximately 7,000g for 1minute. The zymospin IV spin filter was snapped off, and 1,200µl of the fungal/ bacterial DNA binding buffer was added to the filtrate in a collection tube. From this mixture, 800µl was collected and added to the zymo spin ^{TM} IIC in a collection tube and centrifuged at 10,000g for 1minute. The collection and the flow through were removed and discarded. The zymo spin IIC column was placed into a new collection tubecontaining the mixture's candida DNA and the fungal DNA binding buffer. Eight hundred microliters were added to the zymo-spin TMIIC column in a fresh collection tube and centrifuged at 10,000g for 1minute. To this zymo spin,^{TM}IIC was added 200µl of the DNA prewash buffer in a fresh tube and centrifuged at 10,000g for 1minute. This was followed by adding 500µl of fungal DNA buffer and centrifuging at 10,000g for 1minute. The zymo spin ^{TM}IIC column was then transferred to a 1.5ml microcentrifuge, and 100µl of the DNA elution buffer was added directly to the column matrix and centrifuged at 10,000g for 30 seconds to elute the DNA.

PCR Amplification of CDR1 Gene for 7 minutes

The eluted DNA was stored at 4^0C and sent to Inqaba Laboratory South Africa for PCR amplification and sequencing of the CDR1 gene. The *Candida albicans* CRDI genes were amplified using the CDRI forward primers CTCTTTTCCTCTCACCTCCAGA reverse primers TGAGACCACCAGGGGAACTAA CTGT by a modification of the method described by (Young *et al.*,2006). Briefly, 7µl of the PCR grade water, 1µl each of the forward and reverse primers, 10µl of the PCR master mix containing the deoxy-nucleoside triphosphate,, magnesium chloride, Taq polymerase and 1µl of extracted candida DNA were put together in a tube. The PCR protocol involved an initial denaturation step that was carried for 5 minutes, followed by denaturation 94ºC for 30 seconds, which was followed by annealing at 55- 45ºC for 1 minutes (Touchdown PCR) and extension at 72ºC for 1 minute. The product was subjected to a second round of PCR with the following conditions; denaturation; 94ºC for 30 seconds, annealing ; 45ºC for 1 minute and extension; 72ºC for 1 minute. There was a final extension at 72ºC for 7 minutes. This second round of PCR was done for 35 cycles.

Gel Electrophoresis

The agarose gel was placed in the electrophoresis tank, to the first well, 10µl of 50bp to 100000bp fast DNA ladder (New England Bio Labs Inc, England) mixed in loading dyes (ethidium bromide) was placed. The PCR product (10µl) for each metallo- β- lactamase positive was placed alongside with 2µl loading dye in other labelled wells of the agarose gel.

A 90 volt curremt was passed through the gel for it to run for 60 minutes. After 60 minutes the gel was viewed under trans- illumination and photographed with the aid of a computer program.

Sequencing of PCR Products

The PCR products were purified by adding 10ul of the PCR products to 25ul of the exocarp mix, and the mixture was incubated at 37^0C for 30 minutes. This was followed by heating the mixture at 45^0C for 5 minutes to stop the reaction. The clean PCR product was then sequenced using ABI by dye v3.1 following the manufacturer's instructions.

The sequence chromatograph obtained was edited using the software Finch TV, available at http.//.genespoxa.com. int.html. The sequenced data was compared with published CDRI gene sequences available in the Gen-bank using the NCBI blast program (National Centre for Biotechnology Information).

Statistical Analysis

The data obtained were analysed with the Chi-square (X^2) test Fisher's exact and odd ratio analysis using the statistical software INSTAT (Graph Pad Software Inc. La Jolla CA USA).

RESULTS

The susceptibility testing results showed that ketoconazole and fluconazole had high activity levels against the *Candida albicans* isolates, as shown in Figure 1.

The polymerase chain reaction (PCR) and the gel electrophoresis results are shown on plate 1. Of the 40 multi-drug resistant *Candida albicans* whose DNA was extracted and subjected to gene detection, 32 (80%) harboured the CDRI gene. The CDRI gene was not detected from isolates 13, 17, 20, 22, 23, 27, 29 and 31 among the 40 multi-drug resistant *Candida albicans* isolates.

In the table, specimen type did not significantly affect the prevalence of the CDRI gene. However, the distribution of the CDRI gene among the isolates recovered from various specimen type, between in – patients and out-patients, between gender and age of the patients. clinical isolates recovered from stool and aspirates harboured the highest CDRI genes. (100.00% each). Followed by isolates recovered from HVS/ECS 85.71%), the clinical isolates recovered from urine specimens harboured the least CDRI genes (50.00%). All *Candida albicans* recovered from in-patient tested for CDRI gene harbouring the CDRI gene 4 (100.00%). While 28(77.78%) also harboured the CDRI gene among *Candida albicans* isolates recovered from out-patients. However, the difference was not statistically significant (P=0.5658).

The prevalence of CDRI genes in relation to gender and age did not affect the prevalence. Still, patients within the age group of 31 – 40 years harboured the highest number of CDRI genes (100.00%), followed by the group of ≥51 years with a prevalence of 87.50%) while the age group of 21 – 30 years harboured the least number of CDRI genes with a prevalence of (50.0%) (P<0.005).

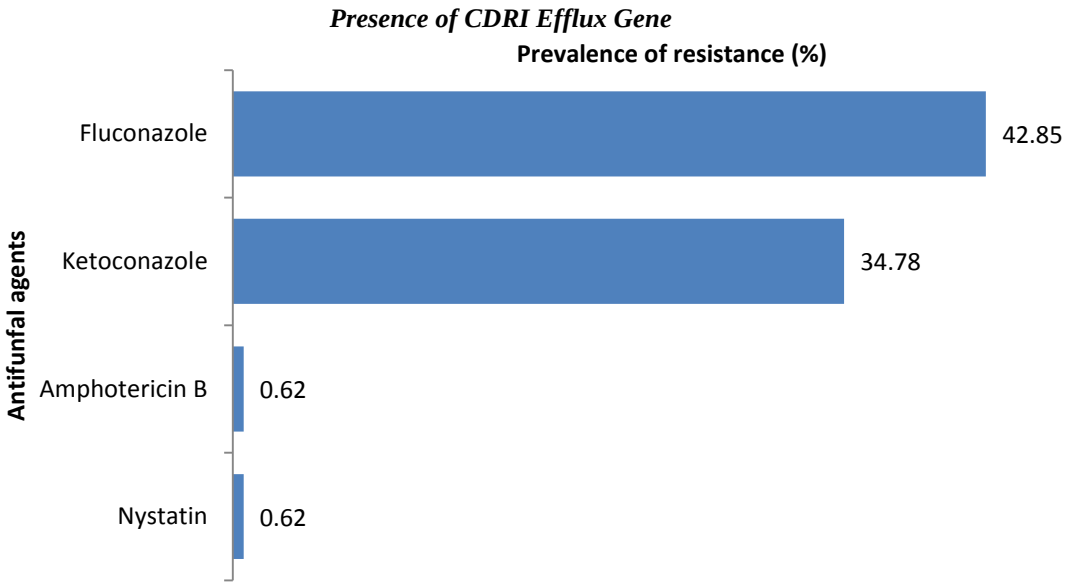

Figure 1: Resistance of *Candida albicans* to the tested antifugal agents

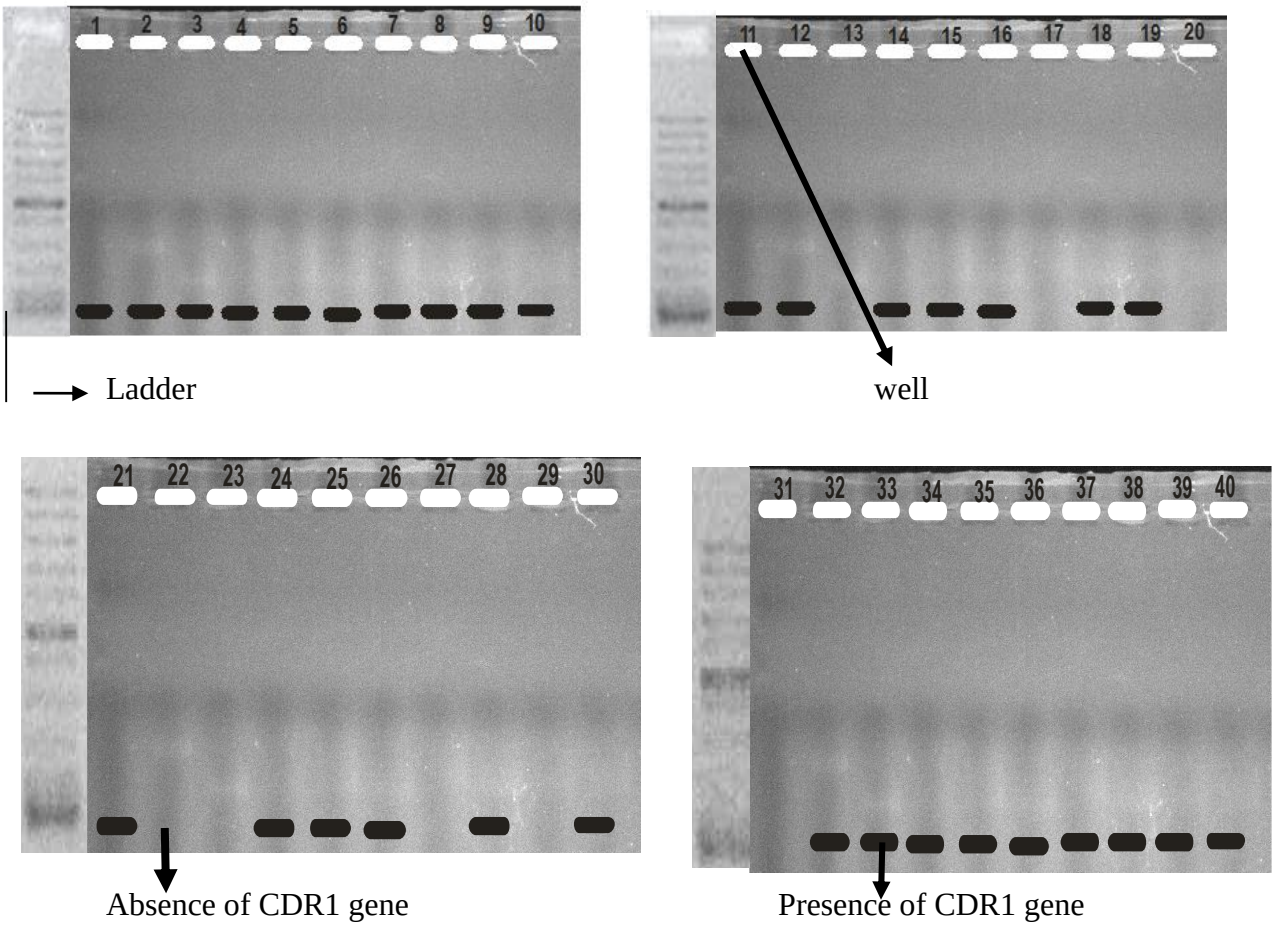

Plate 1: Gel electrophoresis of PCR products to detect CDR1 genes in representative resistant *Candida albicans* isolates

Table 1: Distribution of CDR1 genes

Characteristics	No. tested	No. positive for *CDR1* gene (%)	P value
Gender			1.0000
Male	20	16 (80.00)	
Female	20	16 (80.00)	
Age (years)			0.4418
≤ 1 – 10	5	4	
11 – 20	6	4	
21 – 30	4	2	
31 – 40	5	5	
41 – 50	4	3	
≥ 51	16	14	
Specimen			0.7259
Urine	2	1 (50.00)	
Stool	2	2	
HVS/ECS	7	6	
Sputum	28	22	
Aspirates	1	1	
Source of patients			0.5658
In-patients	4	4 (100.00)	

DISCUSSION

The resistance profile of the candida isolates used in this study showed that the *Candida albicans* were least resistant to the polyenes and most resistant to the azoles, with fluconazole being the highest; this agrees with the work of Panwan and Faujdar (2016). These findings could be because polyenes are not in widespread use as compared to azoles; thus, the rate of development of resistance will be high, while that of the polyenes will be low.

This study selected 40 resistant *Candida albicans* to detect CDR1gene. The selection was based on resistance to at least two antifungal agents (fluconazole and ketoconazole). Of the 40 resistant strains of the *Candida albicans* isolated, 32 (80%) harboured the CDR1 gene. Also, resistance to polyenes had earlier been reported and may be due to mutations in the ERG3 gene and catalase activity (Prasid and Rawa, 2014).

It is important to note that the isolates were resistant to one or more of the polyenes used in this study; therefore, overexpression of CDRI genes in these isolates may counter resistance to the polyenes. However, further studies are needed to verify this, including studies on over-expression of CDR1, ERG3 mutations and catalase activity in polyenes resistance *Candida albicans*.

A combination of the resistance mechanisms can be responsible for developing multi-drug resistance among fungi isolates (Prasid and Rawa, 2014), which could includeefflux pump, target site alterations, mutations in ERG11, upregulation of target enzyme, development of bypass pathways and chromosomal aneuploidy or isochromosome (Kanafani and Perfect, 2008; Charkrabarti, 2011). These phenomena may be present in our isolates, including those whose CDR1 genes were undetected. Further studies are needed to verify this. The prevalence of CDR1 genes did not differ significantly ($p<0.05$) with specimen type between in-patients and out-patients, gender and age of patients.This may reflect the widespread use of antifungal agents in our environment, as antimicrobial use is unregulated (Ogbolu, 2013). It is important to note that this study did not conduct expression studies of CDR1 genes mRNA.This is necessary to confirm the over-expression of the genes,which was a limitation in this study.

CONCLUSION

Resistance to fluconazole was prevalent in this study; 80% of the representative Candida isolates harboured the CDR1 genes. Prudent measures to stem the tide of high antifungal resistance are advocated.

Conflict of Interest

There are no conflicts of interest.

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