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Analysis of Antifungal Resistance Genes in *Candida albicans* Isolates Using Next Generation Sequencing

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Abstract

Background *Candida albicans* (*C. albicans*) is determined as the major human pathogen in the genus Candida. The presence of virulence genes and the increasing prevalence of resistance to antifungal therapy have contributed to the pathogenicity of *C. albicans*. Next-generation sequencing (NGS), also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern sequencing technologies. These technologies allow for sequencing of DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, and as such revolutionized the study of genomics and molecular biology.

Objective To determine the mutations in the genes responsible for multidrug resistance among isolates of pathogenic *C. albicans* using NGS technique.

Methods This study comprises a total of (1596) samples collected from different body sites and lesions (blood, wounds swabs, ear swabs, bronchoalveolar lavage, high vaginal swabs, sputum, threat swabs, and urine); after diagnosis the *C. albicans* isolates DNA extraction was done and final using NGS technique.

Results Only 209 of 1596 samples that were taken from the patients and cultured on Sabouraud's dextrose agar were found to be positive. Candida isolates were cultured on Chrom Candida Agar the results revealed that 59 of the isolates were *C. albicans*. ERG11 gene in this study found to cause 12 silent mutations, while missense mutation was just two compared with reference sequences standard strains SC5314. ERG11 gene in this study found to cause 12 silent mutations, while missense mutation was just two. One deletion mutation was found in the YOR1 gene in this investigation, this gene alone has 2 silent mutations and 1 missense mutation, the PDR16 gene contained 15 missense mutations and 6 silent mutations.

Conclusion This study showed that next generation sequencing allows the thorough investigation of isolates more cost efficient and faster than another technique.

Keywords *Candida albicans*, NGS technique.

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List of abbreviations: AmB = Amphotericin B, ERG = Ergosterol, NGS = Next generation sequencing, PCR = Polymerase chain reaction, QC = Quality control, SDA = Sabouraud's dextrose agar

Introduction

Candida infections have a significant rise in morbidity and mortality, especially in recent years due to the continuous increase in the number of immunosuppressive patients [(1)]. *Candida albicans* (*C. albicans*) is

determined as the major human pathogen in the genus Candida [2]. *C. albicans* is classified as commensal fungi that present in many anatomical sites of the human body [3]. *C. albicans* can cause oral and vaginal infections as well as systemic diseases [4]. The rise in the incidence of Candida infections is complicated by the antimicrobial resistance and the limited number of available antifungal [5]. The presence of virulence genes and the increasing prevalence of resistance to antifungal therapy have contributed to the pathogenicity of *C. albicans* [6]. The widespread use of a limited number of antifungal agents, particularly azoles drugs, has led to the development of drug resistance in the treatment of *C. albicans* infections, a problem of growing importance [7]. Azoles, polyenes, and echinocandins are the three main antifungal classes, being the last considered first-line therapy in many hospitals for the treatment of invasive candidiasis [8]. Amphotericin B is available as a systemic therapy, it comprises an amphophilic, monocyclic polyene lactone ring, which is linked to mycosamine [9].

The mechanisms action of Amphotericin B (AmB) acts by binding to ergosterol (ERG) in the cell membrane of most fungi. After binding with ergosterol, it causes the formation of ion channels leading to loss of protons and monovalent cations, which results in depolarization and concentration-dependent cell killing [10]. Several mutations in genes of the ERG biosynthesis pathway (ERG genes) have been associated with this mechanism. In *C. albicans*, the loss of function of ERG11 and ERG3 genes (lanosterol 14α-demethylase and C-5 sterol desaturase, respectively) leads to the exchange of ergosterol for alternate sterols such as lanosterol, eburicol and 4,14-dimethyl-zymosterol in the membrane [11,12].

In other reports, AmB resistance in *C. albicans* is associated with a substitution in ERG11 and loss of function of ERG5 (C-22 sterol desaturase), again associated with an alternate membrane sterol composition [13]. Azole, a second generation of chemical antifungals was introduced in the 1970s. Azoles are unsaturated aromatic molecules containing at least one nitrogen atom. They are the most used antifungals because of their high efficiency and broad-spectrum activity [14]. The fungal target of azole antifungals is a cytochrome P450 (encoded by ERG11) involved in the 14-demethylation of lanosterol, an essential step in the biosynthesis of ergosterol [15]. Ergosterol is an essential component of *C. albicans* membrane, which influences the membrane permeability and the activity of membrane-bound enzyme membrane [16]. Antifungal azoles can inhibit the growth of fungi through preventing ergosterol synthetic pathway [17]. The alterations and or overexpression of ERG genes, targeting enzyme genes in ergosterol synthetic pathways, are considered as the major azoles-resistance mechanism in *C. albicans* [18]. Several molecular biological methods have been applied to the detection of *C. albicans*, such as polymerase chain reaction (PCR) [19]; Real-time PCR [20], mass spectrometry [21], and immunoassay [20]. These techniques are relatively complex and require expertise and expensive instruments. Thus, a simpler, more cost-effective method is needed [22].

Recently, next-generation sequencing (NGS) has provided an improvement over conventional culture techniques, allowing rapid comprehensive analysis of oral fungal biodiversity [23]. NGS provides a large number of sequences reads in a single rapid run and detects not only the most dominant community but also the low-abundance [24]. The NGS is a new laboratory detection technology that directly extracts the nucleic acids from all the pathogens in the samples of the patient for high-throughput sequencing; the NGS is able to detect the thousands of fragments in a laboratory test, therefore this technology can reduce the number of tests and period of detection [25,26].

This study aimed to determine the mutations in the genes responsible for multidrug resistance

among isolates of pathogenic *C. albicans* using NGS technique.

Methods

Samples Collection

This prospective study comprises a total of (1596) one thousand five hundred and ninety-six samples collected from different body sites and lesions (blood, wounds swabs, ear swabs, branchoalveolar lavage, high vaginal swabs, sputum, throat swabs, and urine.) of in and outpatients with different illness from both sexes who attended and admitted Al-Imamein Al-Kadhimein Medical City during the period from January 2021 to October 2021.

Isolation and identification of fungi

Swab sticks were collected from patients from different sites of body. Then streaked directly on labeled Sabouraud's dextrose agar (SDA) plates and incubated at 25°C for (2-7) days. The growth was identified based on their morphological and cultural characteristics and microscopic examination which was done using lactophenol cotton blue staining technique [(27)]. The growing colonies of *Candida spp.* isolates were initially diagnosed on SDA agar then confirmed by Chrome Candida Agar; the plates were incubated under aerobic conditions for 24 hr at 37°C [(28)].

Antifungal susceptibility testing

Inoculum was prepared by picking five distinct colonies of like morphological type were selected from a culture on modified Mueller Hinton Agar to ensure purity and viability, which was incubated at 35°C (±2°C). Colonies were suspended into 5 ml of sterile 0.85% normal saline. Turbidity was adjusted with a spectrophotometer by adding sufficient sterile saline or more colonies to adjust the transmittance to that produced by a 0.5 McFarland standard (which equals $1.5*10^8$ cells\ml). After 24 hr of incubation, the resulting zones of inhibition were uniformly circular with a semi-confluent lawn of growth. The zone diameter was measured to the nearest whole millimeter at the point at which, there was a prominent reduction in growth. Pinpoint micro colonies at the zone edge or large colonies within a zone were encountered frequently hence they ignored [(29)].

Molecular method

Two isolates were selected according to the results of identification tests as a representative of all Candida isolates (resistant isolates) and used for DNA extraction, PCR assays and next generation sequencing.

DNA Extraction

Sample Preparation

Using an inoculating loop to transfer (up to $2*10^8$) from an agar plate to a 1.5 ml micro-centrifuge tube.

Lysis

Six hundred 600 µl of GT Buffer was added to the cell pellet and suspended by vortex mixer. The re-suspended yeast cells and 5 µl of RNase A (50 mg/ml) were transferred to a bead beating tube. Taping with standard vortex was done on bead beating tubes for 10 min at room temperature. Incubation bead beating tubes were incubated at 70°C for 10 min. One hundred µl of PR Buffer was added into these tubes. Bead beating tubes were incubated on ice for 5 min. Tubes were centrifuged at 13,000 rpm for 3 min at room temperature then 450 µl of supernatant was transferred to 1.5 ml micro-centrifuge tube.

DNA Binding

An equal volume 450 µl of GB Buffer and absolute ethanol were prepared previously and added to the sample and mixed immediately by shaking vigorously for 10 sec. An equal volume of GB Buffer and ethanol mixture 900 µl to the sample then mix by shaking vigorously after that placed a GD column in a 2 ml collected tube. A 700 µl of the sample mixture was transferred to the GD Column and centrifuged at 18,000 rpm for 1 min at room temperature, the flow-through was discarded; the remaining

sample mixture was transferred to the GD column and centrifuge at 18,000 rpm for 1 min at room temperature.

Wash

Four hundred µl of W1 buffer was added to the GD column, then centrifuged at 18,000 rpm for 30 sec at room temperature. The flow-through was discarded then the GD column was re-backed in the 2 ml collection tube. A 600 µl of washing buffer was added to the GD column, then centrifuged at 18,000 rpm for 30 sec at room temperature. The flow through was discarded and the GD column was re-back in the 2 ml collection tube, and centrifuged at 18,000 rpm for 3 min at room temperature.

Elution

Dried GD column was transferred in to a new 1.5 ml microcentrifuge tube. A 100 µl of preheated elution buffer1, water was added into the center of the column matrix, left to stand for at least 2 min to allowed elution buffer, water to been completely absorbed. Tubes were centrifuged at 18,000 rpm for 2 min at room temperature to elute the purified DNA.

Sample Preparation for Library Construction

DNA was extracted from a sample. After performing quality control (QC), passed sample was preceded with the library construction.

Library Construction

The sequencing library was prepared by random fragmentation of the DNA sample, followed by 5' and 3' adapter ligation.

Sequencing

For cluster generation, the library was loaded into a flow cell where fragments are captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment was then amplified into distinct, clonal clusters through bridge amplification. When cluster generation was completed, the templates are ready for sequencing.

Illumina sequencing-by-synthesis (SBS) technology utilizes a proprietary reversible terminator-based method that detects single bases as they are incorporated into DNA template strands. As all 4 reversible, terminator-bound deoxynucleoside triphosphates (dNTPs) are present during each sequencing cycle; natural competition minimizes incorporation bias and greatly reduces raw error rates compared to other technologies. The result was highly accurate base-by-base sequencing that virtually eliminates sequence-context-specific errors, even within repetitive sequence regions and homopolymers.

Raw data

Sequencing data was converted into raw data for the analysis.

Programs used in analysis

1. BWA; (Burrows-Wheeler Aligner) http://bio-bwa.sourceforge.net/ BWA was an alignment program that aligns short reads to a reference genome.
2. Picard; http://broadinstitute.github.io/picard/ Picard was a collection of Java-based command-line utilities that manipulate SAM files, and a Java API (SAM-JDK) for creating new programs that read and write SAM files.
3. SAMTools; http://samtools.sourceforge.net/ SAMTools can handle sam/bam file to read, write, modify and index.

Statistical analysis

The statistical analysis of this prospective study was performed with the statistical package for social sciences (SPSS) 16.0 Software and Microsoft Excel 2016. Sensitivity, specificity, positive predictive value and negative predictive value were calculated.

Results

Only 209 of 1596 samples that were taken from the patients and cultured on Sabouraud's dextrose agar were found to be positive. The

results were 202 isolates of Candida (96%); 3 isolates of Aspergillus (2%); 2 isolates of Mucor (1%); and only 2 isolates of Cryptococcus. Following that, Candida isolates were cultured on Chrom Candida Agar the results revealed that 59 of the isolates were *C. albicans*, while the remaining 143 were not, as shown in figure (1).

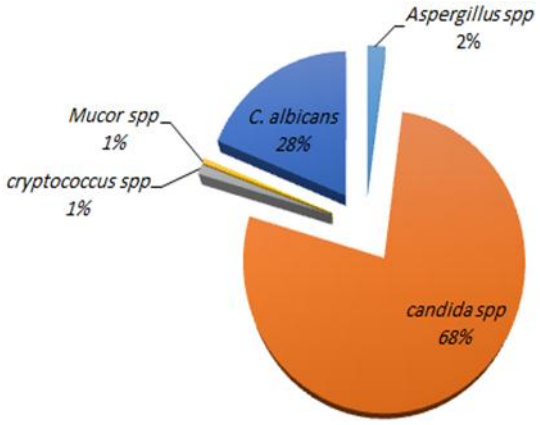

Figure 1. Frequency of fungal isolates detected by culture methods

Molecular Identification of *C. albicans*

Selection of *Candida spp.* for molecular identification was based on their antifungal susceptibility that was resistant upon diagnosis of these isolates (152), as indicated in table (1).

Table 1. Sensitivity test for each isolated *Candida albicans*

Antifungal disc	Number of isolates	
	24	52
Miconazole 10 mg	R	R
Amphotericin B 20 mg	R	S
Fluconazole 10 mg	S	R
Nystatin 100 units	R	S
Clotrimazole 10 mg	S	R
Econazole 10 mg	R	R
Ketoconazole 10 mg	S	R

Next generation sequence analysis

Two samples (24, 52) were retained for the NGS stage following QC step, because samples (24, 52) more resistant to antifungal.

Raw data statistics

Calculations are made for the 2 samples' combined bases, reads, GC (%), Q20 (%), and Q30 (%) percentages. For instance, in (24) 19,917,172 reads are generated, and the total number of read bases is 3.0 G bp. 34.80% of the GC and 91.76% of the Q 30 are present, table (2).

Table 2. Raw data Stats

Library name	Total read bases (bp)	Total reads	GC (%)	Q20 (%)	Q30 (%)
24	3,007,492,972	19,917,172	34.80	96.99	91.76
52	3,077,406,274	20,380,174	34.85	96.80	91.52

Potentially causal resistance mutation

Isolate number 24 resistant to Miconazole, AmB, Nystatin, and Ketoconazole. While isolate number 52 resistant to Miconazole, Fluconazole, Clotrimazole, Econazole, and Ketoconazole. In table (3), ERG11 gene in this study found to cause 12 silent mutations, while missense mutation was just two compared with reference sequences standard strains SC5314.

Table 3. Types of mutation and ERG11 gene sequence in the two studied *Candida albicans* isolates

Genes	Genomic variation-codon 52	Genomic variation. Protein 52	Genomic variation-codon 24	Genomic variation. protein 24	Type of Mutation
ERG11	c. * 148499 A>G	p. Tyr 401 Tyr	c. *148499 A>G	p. Tyr 401 Tyr	synonymous-variant
ERG11	c. * 148592 G>A	p. Leu 370 Leu	c. *148592 G>A	p. Leu 370 Leu	synonymous-variant
ERG11	c. * 148706 A>G	p. Val 332 Val	c. *148706 A>G	p. Val 332 Val	synonymous-variant
ERG11	-	-	c. *148619 T>C	p. Ser 361 Ser	synonymous-variant
ERG11	-	-	c. *148418 A>G	p. Asp 428Asp	synonymous-variant
ERG11	c. *148240 C>T	p. Val 488 Ile	-	-	missense-variant
ERG11	c. *148400 A>G	p. Ala 434 Ala	-	-	synonymous-variant
ERG11	c. * 148406 G>A	p. Ala 432 Ala	-	-	synonymous-variant
ERG11	c. * 148676 T>C	p. Lys 342 Lys	-	-	synonymous-variant
ERG11	c. *148904 T>G	p. Glu 266 Asp	-	-	missense-variant
ERG11	c. * 149044 G>A	p. Leu 220 Leu	-	-	synonymous-variant
ERG11	c. *149291 G>A	p. Ser 137 Ser	-	-	synonymous-variant
ERG11	c. * 149387 A>G	p. Phe 105 Phe	-	-	synonymous-variant
ERG11	c. *149486 G>A	p. Phe 72 Phe	-	-	synonymous-variant

One deletion mutation was found in the YOR1 gene in this investigation. When compared to the reference sequences of the standard strain SC5314, this gene alone has 2 silent mutations and 1 missense mutation, as indicated in table (4).

Table 4. Types of mutation and YOR1 gene sequence in the two studied *Candida albicans* isolates

Genes	Genomic variation-codon 52	Genomic variation. Protein 52	Genomic variation-codon 24	Genomic variation. protein 24	Type of Mutation
YOR1	c.* 2045766 c.* 1532 TCATCACCATCACCATCACCATCA	p. Asp511fs	c.*2045766 c.*1532 TCATCACCATCACCATCA	p. Asp511fs	deletion-variant
YOR1	-	-	c.* 2044187 T >C	p. ser1037ser	synonymous-variant
YOR1	c.* 2047231 A >G	p. Leu23Leu	-	-	synonymous-variant
YOR1	c.*2044833 G>A	p. Pro822Leu	-	-	missense-variant

Table (5) shows that the PDR16 gene contained 15 missense mutations and 6 silent mutations when compared to the reference sequences of standard strain SC5314.

Table 5. Types of mutation and PDR16 gene sequence in the two studied *Candida albicans* isolates

Genes	Genomic variation-codon 52	Genomic variation. Protein 52	Genomic variation-codon 24	Genomic variation. protein 24	Type of Mutation
PDR16	c.* 801822 C>T	p. Thr32Ile	-	-	missense-variant
PDR16	c.* 801847 G>A	p. Ala41Thr	-	-	missense-variant
PDR16	c.* 801859 A>G	p. Thr45Val	-	-	missense-variant
PDR16	c.* 801860 C>T	p. Thr45Val	-	-	missense-variant
PDR16	c.* 801868 A>G	p. Ile48Val	-	-	missense-variant
PDR16	c.* 802006 C>T	p. His94Tyr	-	-	missense-variant
PDR16	c.* 802019 G>A	p. Arg98Gln	-	-	missense-variant
PDR16	c.* 802086 G>A	p. Leu120Leu	c.* 802086 G>A	p. Leu120Leu	synonymous-variant
PDR16	c.* 802122 T>C	p. Asp132Asp	-	-	synonymous-variant
PDR16	c.* 802296 G>A	p. Glu190Gln	-	-	synonymous-variant
PDR16	c.* 802410 A>T	p. Ala228Ala	-	-	synonymous-variant
PDR16	c.* 802443 T>A	p. Pro239Pro	-	-	synonymous-variant
PDR16	c.* 802698 T>A	p. Asp324Glu	-	-	missense-variant
PDR16	c.* 802707 A>G	p. Ile327Met	-	-	missense-variant
PDR16	c.* 802752 A>G	p. Glu342*	-	-	missense-variant
PDR16	-	-	c.* 801784 G>A	p. Gly20Ser	missense-variant
PDR16	-	-	c.* 801810 AATCA>AA	p. Glu28Glu	synonymous-variant
PDR16	-	-	c.* 801879 T>C	p. Ser51Thr	missense-variant
PDR16	-	-	c.* 802551 G>A	p. Leu275Lys	missense-variant
PDR16	-	-	c.* 802564 C>T	p. Pro280Phe	missense-variant
PDR16	-	-	c.* 802821 C>T	p. Pro365Val	missense-variant

Discussion

Isolation and identification of *Candida spp.*

Figure (1), shows that only 209 of the 1596 patient-derived samples were positive. Candida isolates were cultured on Chrom Candida Agar major clinically significant *Candida spp*. Among Candida species, *C. albicans* was the most dominant species of Candida (28%), similar findings (Kmeid et al, 2020) [30], have been reported in other study that *Candida spp*. were the most prevalent fungal pathogens, especially in critically ill people. Furthermore, (Panizo and Moreno, 2022) [31], reported that *C. albicans* was responsible for (63%) of all candidiasis infections in their study.

Next generation sequence analysis

Next generation sequence has demonstrated that effectively finding found antifungal resistance mutations in clinically important *Candida spp*. The use of the NGS technology in fungal pathogen detection offers the following benefits: First, NGS technology is suitable for hostile culture and slow-growing microbial infections, such as fungi [32]. Second, NGS provides more accurate identification of fungal species and is even *more specific than other methods [33]. C. albicans* is the easiest to identify by NGS of the nuclear large ribosomal subunit (LSU) and internal transcribed spacer (ITS) Regarding ERG11 gene, it was found to that it can cause 12 silent mutations; there was no change in amino acid codons, while missense mutation were just two single-nucleotide polymorphisms (SNPs); the first missense mutation converted amino acid from valine 488 to isoleucine, and second, once converted from glutamic acid 266 to aspartic acid. Points of mutation make protein non-functional, leading to increased resistance to antifungals. The ERG biosynthetic pathway is interrupted by azoles through inhibition of the enzymatic activity of 14-α-sterol demethylase (also known as CYP51A1), the product of the ERG11 gene. Azoles have basic nitrogen that coordinates with the iron atom of the heme group located in the active site of 14-α-sterol demethylase. Thus, the azole is thus occupied an active site, which acts as a non-competitive inhibitor [34].

Azoles work by inhibiting the biosynthesis of ERG, an indispensable component for maintaining the fluidity in the membranes of eukaryotic cells, which leads to the toxic accumulation of its precursor, lanosterol [35]. *C. albicans* CYP51 (CaCYP51), whose normal substrate is lanosterol, catalyzes the demethylation reaction of lanosterol in a three-step process toward producing ergosterol. Azole antifungal drugs inhibit this enzyme by binding their nucleophilic N–4 atom to the enzyme's heme Fe (iron) at the sixth coordinate position, hence occupying the binding pocket competitively [36]. Sterol 14α-demethylase (CYP51) belongs to the cytochrome P450 superfamily. It is a crucial enzyme in the sterol biosynthetic pathway, where it catalyzes the oxidative removal of the 14α-methyl group from sterol precursors. CYP51 has a high substrate specificity with only five naturally occurring, structurally similar 14α-methylsterols as substrate [37].

Overexpression (CYP51) in the presence of azoles and the point mutation CYP51 is commonly detected in azole-resistant *C. albicans* clinical isolates; this result agrees with (Ruma et al, 2022) [38]. Point mutations in the ERG11 gene in azole-resistant *C. albicans* isolate such mutations can alter the affinity of CYP51A1 for an azole if the resultant amino acid substitutions lead to changes in the enzyme's tertiary structure [39]. The current study, the ERG11 gene was detected in *C. albicans* isolates resistant to azoles. In Nigeria, similar study (Dovo et al, 2022) [40] was carried out showing the presence of the ERG11 gene as (11.18%) in isolated strains of Candida resistant to Fluconazole from vulvovaginitis and (88.89%) in *C. albicans* isolates resistant to Fluconazole and Voriconazole which isolated from pregnant women.

This study discovered a single change from thymine to cytosine at the position 766874 of the FUR1 gene. This change results in an amino acid substitution from Phenylalanine to Leucine at the position 211 in the FUR1 protein. This result agrees with (Dodgson et al, 2004) [41] when they found a relationship between 5-FC

resistance and a decreased susceptibility to azoles, and disagrees with (Hu et al, 2023) [42] who showed that decreased susceptibility and increased resistance mostly correlated with a single change from cytosine to thymine at the position 301 of the FUR1 gene. This change results in an amino acid substitution from arginine to cysteine at the position 101 in the FUR1 protein.

This study showed that the PDR16 gene contained 15 missense mutations, and 6 silent mutations when compared to the reference sequences of standard strain SC5314. Mutation in PDR16 gene led to azole resistance, this result agrees with (Znaidi et al ,2007) [43] who showed that deleting PDR16 in a clinical isolate decreases the azole resistance of the cells while overexpressing PDR16 increases their resistance to azoles, indicating that PDR16 plays a role in clinical azole resistance. This result agrees with (Saidane et al, 2006) [44] who found *C. albicans* PDR16 gene contains a CTG codon that is translated as a serine (position 235) in *C. albicans* not have mutation at this position.

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Author contribution

Dr. Mohammed: samples collection, culturing, diagnoses, DNA extraction and data analysis, wirting manuscrip. Dr. Al-Attraqchi: helped in diagnoses and supervision of all steps of research. Dr. Alrubaye: Consultation of the molecular part of research.

Conflict of interest

The authors declare that they have no competing interests.

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