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Detection of ERG11 and CDR1 Genes among Fluconazole-Resistant *Candida albicans* Isolated from Women with Vaginitis

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Abstract: Vulvovaginitis caused via *Candida* species is a common fungal infection among adult and pregnant femals especially with *C. albicans*. Fluconazole resistance has been reported more frequently by researchers from around the world. The goals of this study were to determine the prevalence of Candida albicans among suspected vaginitis women, evaluate their ability to form biofilm, and the susceptibility profiles of isolates to fluconazole, as well as the molecular detection of some fluconazole resistance genes.The study included 250 women ages 18 to 45 who were Candida attending specialist hospitals in Baghdad, Iraq. *Candida* spp. was identified using standard methods and biochemical tests after Vaginal smears were cultured on selective HiChrom Candida Differential agar and (SDA).Out of the 250 vaginal smears collected, 150 (60%) isolates of fungal were isolated, with 50 (33.3%) were *Candida albicans* and 100 (66.6%). Out of 50 *Candida albicans* isolates which tested by microtiter plate assay, 21(42%) isolates form a strong biofilm, while 19 (38%) isolates were the moderate producer, and only 7(14%) isolates were weak a biofilm formation. The identification of the *Candida* spp. by PCR confirmed the primary identification, using the primers from *18S rRNA* gene, the PCR assays exhibited the detection of fluconazole resistance genes *(CaERG11*, and *CaCDR1*), where these genes were found in all flucanazole resistant isolates with strong biofilm formation ability. It was concluded that high prevalence of *Candida* isolates with high antifungal resistance among Iraqi females patients with Vulvovaginitis indicates the importance of regular screening and routine examination for candidiasis in Iraqi hospital.

Keywords: *Candida albicans*, Vaginitis, Fluconazole-resistant genes.

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Introduction

Vulvovaginal candidiasis (VVC) defined as a condition characterized by signs and symptoms of vaginal inflammation when the *Candida* spp. are identified an ongoing problem that affecting 70–75% of female of reproductive age at some point in their lives (1). Vulvovaginal candidiasis is considered as one of the more common *Candida albicans* infection. Pathogenicity has been attributed to avariety of virulence factors as well as growing antifungal resistance (2). One of the most prevalent yeast illnesses in hosipital is candidiasis. This infectious disease could be caused by at least Twenty different Candida species (3). *Candida albicans* is the most common etiologic agent, accounting for more than ninety-five percent of candidiasis in the last 3 decades, by *Candida*

*glabrata*e, *Candida tropicalis* ,*Candida parapsilosis,* and *Candida krusei* (4). Studies conducted worldwide have reported the underlying mechanisms of azole resistance among Candida spp., such as mutations in or overexpression of the ERG11 gene or upregulation of the CDR1 and MDR1 genes (5). One of the key reasons of antimicrobial resistance is the erroneous and excessive use of antibiotics due to the lack of time and precision in the identification of the pathogenic microorganism causing the infection(6). Explorations of the vaginal microbiota began over 150 years ago, which can contribute to the risk of femal infertility, one of them its *Candida spp.* (7). Antifungal resistance is frequently the result of a mix of processes,including cellular target including point mutations, target molecules overexpression, and antifungals efflux pump extrusion (8). Many studies have found that biofilm formation plays an important role in the histopathogenesis of vaginal candidiasis and the disease's resistance to antifungal treatment, with biofilm growth on the vaginal epithelium also leading to high resistance to antifungal therapy and promoting the (9). The goals of this study are investigation the incidence of *Candida albicans* among Iraqi female, evaluation the antifungal susceptibility especially against Fluconazole, and the molecular detection of Fluconazole resistance genes among the resistant isolates.

Materials and methods

Candida isolates were collect from 250 vaginal mucosa swabs taken from patients with symptoms of Vulvovaginal candidiasis at three hospitals in Baghdad, Iraq (Kamal AL-Samarrai Hospital (Infertility and IVF Center), AL-Elwiya Educational Hospital and Fatima EL-Zahra and Children. between Octobers to December 2022. A gynecologist collected samples, and the infection criteria were itching, edema, erythema in the vulva and vagina and the presence of lumpy vaginal flow adhering to the vaginal walls. Swabs of vaginal mucosa were cultured for 48 hours at 37^0C from SDA and Hi-Crome Candida Differential Agar (Himedia, India).Candida species were identied utilizing the colors and features of colonies, microscopic examination, and biochemical testing using the VITEK-2 system.

Testing for antifungal susceptibility

Candida albicans colonies were injected into 5 mL of sterile salined and adjusted visually McFarland standards of 0.5 to remove excess fluid,a sterile was cotton wool swab soaked appropriate inoculum suspension and rolled the inside of a tube above fluid surface. The lawn of isolate made by streaking the surface (MHA) agar. Antifungal susceptibility was tested by using disk diffusion method, the clinical laboratory Standared Institute CLSI (10) Recommends using antifungal disks (MAST), Fluconazole disk (10µg), Nystatine (50IU), and AmphotericinB(100IU). Before being read, the plates were incubated in ambientair in$35C^0$ for 24 hrs. The diameters of the zone of inhibition on each antifungal disk were measured (mm) using a ruler. CLSI standards were used to interpret all antifungal susceptibility (susceptible S, susceptible dose dependent SDD, and resistant R) (Table 1).

Table (1): Interpretation of varyingzone antifungal zone sizes for disk diffusion method.

Anti-fungals discs	Disk concentration	Zone diameter (mm)		
		S	SDD	R
Amphotericin B	100 U	≤10	10-14	≥15
Fluconazol	10 µg	≥17	14-16	≤10
Nystatin	50 U	≤14	18-15	≥13

SDD stands for susceptible dose dependent, S sensitive, and R stands for resistant

Biofilm formation detection

Candida albicans colonies were cultured on Sabouraud broth at 35ºC for 18 hours. The *Candida albicans* suspension was then made using Muller-Hinton broth at the concentration (10^6/ml), each well was washed in (200 l) phosphate buffer and air-dried in 45 minutes before being stained at (110 l), 0.4% crystal violet solution. Following four washes at 350 liters of sterile distilled water, (200l) of 95% was added and left for fourty-five minutes. The visual adensity was measured in 595 nm using a microplate reader,after 100 µl of the solution in each well was transferred to a new well, The values of absorbance for the controls specimens were subtracted from an absorbance values for test wells(11). "Based known established optical density cut-off values (ODc), which were derived for a mean value of Negative controls' (mean ODnc) added to Negative controls' three standard deviations (3 x SD_{nc}): OD_c = averageOD_{nc} + (3 xSD_{nc}) The following are a biofilm density categories: OD≥ODC=negative for biofilm, 2xODC≥OD=mildly positive for a biofilm, 2XODC<ODC = moderately positive for a biofilm, OD>4Xodc= intensely positive for a biofilm"(12).

Molecular study for the isolated *Candida albicans*

DNA Extraction from *Candida*

DNA was extracted for *Candida albicans* isolate throughe Promeg DNA extraction kit according to manufacturer's instructions (Promega, USA).

Identification of *C. albicans*

Table (1): Component of PCR Master Mix Reaction

PCR Master mix reaction components	Volume	
Go Tag green master mix		12.5µl
DNA template		2 µl
Primers	Forward	1µ
	Reverse	1µ
Nucleases free water		8.5 µl
Total volum		25 µl

Candida albicans-specific primers were used amplify an extracted DNA. Table 1 lists the primers used for the 18S *rRNA* gene. The amplification procedures were carried to exactly describe previously (13).

Detection fluconazole resistance genes by PCR

Table (2): Real Time PCR.

Steps	C^0	m:s	Cycle
RT. Enzyme Activation	95	5:00	1
Initial Denaturation	95	00:30	30
Denaturation	60		
Annealing	72	01:0 0	
Extension	72	0500	1

The amplification protocols of all genes under consideration were fllowed exactly as described previously, and the amplification concentrations used were from Promega, along with (2.5 µl) of each primer and, 5 µl of extract DNA (13). Electrophoresis on 1.5% agarose gels for 1.5hrs at 80v was used to (100base per) DNA ladder from (Promega, USA).

Table (3): Sequences of the primers use to amplify *CaERG11, CaCDR1* and *18S rRNA* Genes fragments.

Gene Name		Sequence 5′-3′	Annealing temperature (^{0}C)	Product size base per	Reference
CaERG11b	**F**	TTTGGTGGTGGTAGACATAGAT	60	128	(Zhang et al.,2016)
CaERG11b	**R**	TAATCAGGGTCAGGCACTTT			
CaCDR1	**F**	GATTCTCAAACTGCCTGGTC		158	(Zhang et al.,2016)
CaCDR1	**R**	CCAAAATAAGCCGTTCTTCCAC			
18S rRNA	**F**	TCTTTCTTGATTTTGTGGGTGG		150	(Zhang et al.,2016)
18S rRNA	**R**	TCGATAGTCCCTCTAAGAAGTG			

Revelation of PCR products

Electrophoresis on (1.5%) agarose gel prepared in 1× tris base-borate EDTA solution at 80 V for 1 hour 30 minutes was separated the amplicons. A molecular weight was DNA marker a (100bp) DNA marker underUV illumination, The PCR products were visualized with ethidium bromide (BET) (0.5 µg/mL) under UV.

Results and dicussion

Isolation and identification of *Candida* isolates Species

Isolation and identification of *Candida* Species were done following their morphology in direct microscopic method and, biochemical properties. HiChrom Candida Differential agar and (SDA) were used to culture the swabs. Because various Candida Species create distinct colors on medium,HiChrome agar Candida medium was shown to be extremely helpful in routine clinical mycology service,facilitating the detection of mixed cultures of yeasts and allowing directly identification of C.albicans(14). These strains identified using the germ tubes test,morphology on Cornmeal agar and Hi-Chrome agar,which revealed that all Candida isolates grew well after 48 hours of incubation (Figure 1).

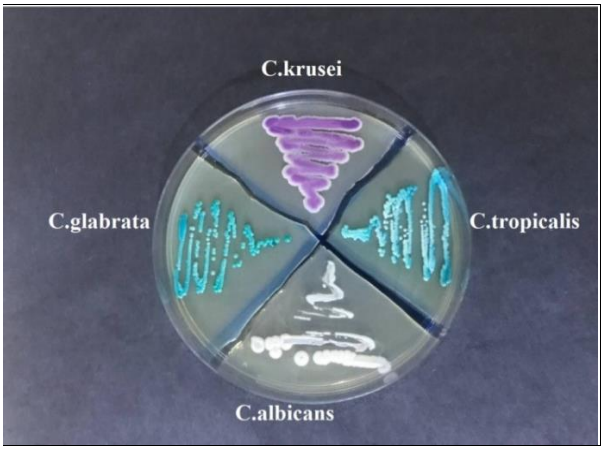

Figure (1): Candida Species Colonies on HIChrom Candida differential agar. *Candida albicans* (Green),*Candida glbrata* (white),*Candida krusei* (purple) and *Candida tropicalis* (blue) after 48 hours of incubation at 37 ºC.

In the results of classic germ tube test, the production of germ tubes are usually of diagnostic *Candida albicans* (Figure 2). It is advised that at least five well-defined germ tubes are observed befor the isolate is called positive. This is due to many similarities between Candida *dubliniensis* and its close relative *Candida albicans* share many features in common like Microscopic morphology and ability to form germ tubes in serum, as well as the generation of blastoconidia with pseudohyphae, true hyphae, and chlamydospores, which are routinely by average clinical mycology laboratory to identify Candida albicans (15).

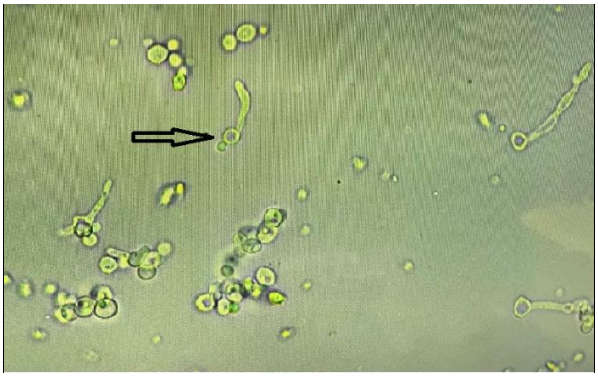

Figure (2): *Candida albicans* positive test in germ tube (direct microscopic examination at40x).

Biochemical tests

The isolates were identified using growth -based conventional techniques and API 20. API 20C Aux (BioMerieux / France) *was used to* identifiy the *Candida albicans*.The API 20C Aux *Candida* identification system uses sugar assimilation patterns to identify *Candida* species.

The results revealed the identification of 150 isolates of *Candida* species (Table4), including 50 *Candida albicans*, 30 *Candida krusei*, *35 Candida glabrata* and 35 *Candida tropicalis* isolated from clinical specimens.

Table (4): The prevalence of *Candida* species among 250 vaginal swabs from femals patients.

Candida Spp.	No. of Isolates	%
Candida albicans	50	33.3%
Candida glabrata	35	23.3%
Candida Krusei	30	20%
Candida tropicalis	35	23.3%
Total	150	100%

Different species of yeast can behave as opportunistic pathogens in people with weakened or suppressed immune systems (16). In one of the local investigations, 67 of 97 vaginal swab samples collected from probable women with vaginitis were positive, whereas 30 were negative. *Candida* isolates include following species: *Candida albicans* was isolated 46 (68.65 %) *Candida albicans* isolates, 11(16.41) %), Candida tropicales isolates, 7 (10.44 %), Candida parasilopses isolates, and 3 (4.47 %) *Candida kyfer* isolates (17).

Antifungals susceptibility

The disc diffusion method was used to assess resistance to popular antifungal medication in *C. albicans* isolates (50 isolate) *C. albicans* shown to be extremely susceptible to Amphotericin B (74%). The results of Antifungal susceptibility for 50 *C.albicans* isolates were summarized in Table (5).

Table (5): *Candida albicans* antifungal susceptibility patterns.

Antibiotic	Concentration (microgram\disc)	Resistant	Intermediate	Sensitive
Amphotericin B	100 U	8 (16%)	5 (10%)	37 (74%)
Fluconazol	10	18 (36%)	5 (10%)	27 (54%)
Nystatin	50 U	21 (42%)	0 (0%)	29 (58%)

Antifungal resistance is a major issue with alarming increaseb in the treatment of Candida species (18). Azoles are extensively employed in therapeutics,but due to their fungistatic nature, *Candida* sp. evolvelps resistance to polyenes and echinochandins in addition to azoles (19). *C. glabrata* khown to be the most resistant strain and was clased as a multidrug-resistant infection in the research of Saudi women, but *Candida albicans* and *Candida tropicalis* were both extremely susceptible to terbinafine .Candida albicans was resistant to fluconazole,clortimazole,and nystatin,but Candida tropicalis,the most sensitive strain, was responsive to all the antifungal medications tested except nystatin (20).

Biofilm formation

Microtiter plate method was conducted for detection of biofilm formation among all *C. albicans* isolates as showed in figure (3).

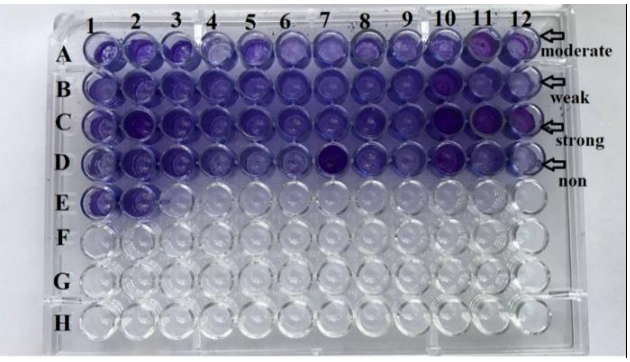

Figure (3): Biofilm formation detection of *Candida albicans* isolates by microtiter plate assay.

Out of 50 *Candida albicans* isolates, 21(42%) isolates strong biofilm formers, while 19(38%) isolates were moderate producer, and only 7(14%) isolates were weak biofilm formers. also it was found that 3 (6%) isolates don't have the ability to form the biofilm (Table 6).

Table (6): Distribution of biofilm formation ability among *Candida albicans* Isolates.

Candida Albicans	Biofilm formation			
	Weak	**Moderate**	**Strong**	**Negative**
Total no. of Isolates =50	7	19	21	3
%	14%	38%	42%	6%

Biofilms are communities of cells embedded in a polymeric extracellular matrix that can contain microorganisms of various species. This condition enables sessile microbial cells to perform a variety of functions efficiently, including nutrition, excretion, growth, communication, and protection.Previons study (21).Fungi communities are composed of a dense network of interconnected hyphae that are covered and buried by an extracellular matrix that can be thin or thick, and shape is frequently regulated by chemicals used for cell communication via quorum sensing (21). Biofilms of Candida spp. contribute to the persistence or worsening of a variety of chronic inflammatory illnesses, as well as acute deep systemic Candida infections. C albicans is still the most common pathogen responsible for fungal biofilm infections (22). The majority of antifungals in the market are either ineffective against Candida biofilms or only effective at high concentrations with severe side effects (23). Demonstrate the role of Aspirin and EDTA as antibiofilm agents when used with Nystatin which have the ability to hinder Candida albicans growth a patients with vulvovaginitis.

Diagnosing of *C. albicans* by *18S rRNA* gene

Eighteen of *C. albicans* isolates that were the most strong biofilm and eighteen resistant to Flucanazole used in this study and detected by PCR technique using *18S rRNA* gene for diagnosing of *C. albicans*. All the tested*C. albicans* clinical contain *18S rRNA* gene (150bp) (Figure 4).

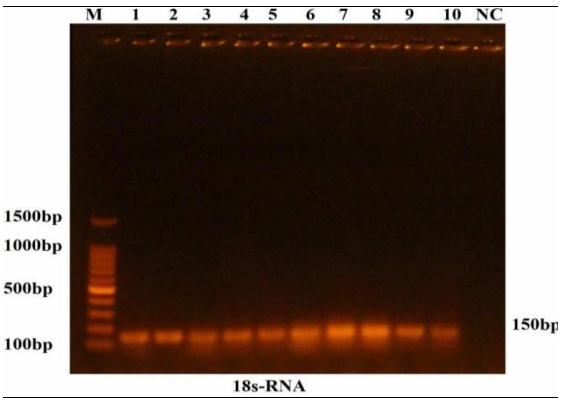

Figure (4): Electrophoresis Agarose gel electrophoresis for the amplification of *18S rRNA* gene of *Candida albicans* had been fractionated on 1.5% electrophoresis gel agarose stained in Eth.Br. M: 100base per ladder marker. Lanes 1-10 resemble 150base per PCR products. (1.5hours, 80 volts).

One of the most powerful and widely used tools for detection and identification of pathogenic fungi,including Candida species,is PCRdetection and identification of fungal DNA (24).Garcia-Salazar research (*25*) Based on sequence analysis of the [18S-ITS1-5.8S-ITS2-28S] region of the rDNA, it was determined that the sensitivity (73%), specificity (96%), and positive (94 %) and negative (80%) predictive values of the PCR assay with the designed oligonucleotides justify their reliable use in diagnosis. When compared to standard laboratory techniques for detecting diseases caused by Candida and fungal pathogens, PCR based on the efficacy of the 18S rRNA gene is a faster, more sensitive, and more useful method for detecting fungal aetiology(26).

Detection of genes that involved in Flucanazole resistance

Tow genes (*CaERG11*, and *CaCDR1*) that seem to be involved in Flucanazole resistance of *Candida albicans* clinical were detected by PCR method. The gel electrophoresis of amplified PCR product for *CaCDR1* showed in figure (5) and CaERG11 figure (6). The results showed that these genes (*CaERG11*, and *CaCDR1*) were found in 100%, of isolate that produce strong biofilm resistant to Flucanazole.

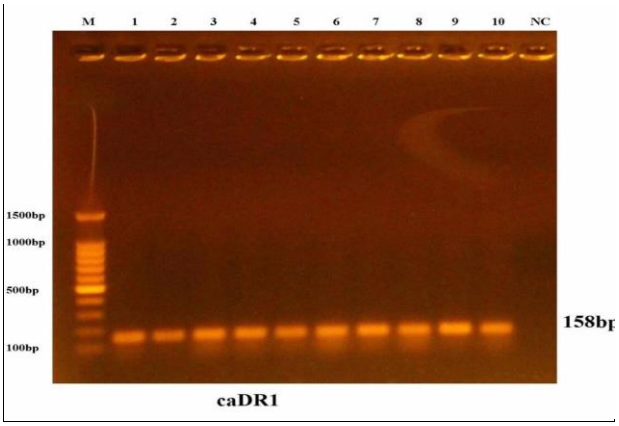

Figure (5): Electrophoresis Agarose gel for the amplification of *caDR1* gene of *Candida albicans* had been fractionated on 1.5% electrophoresis gel agarose stained in Eth.Br. M: 100base per ladder marker. Lanes 1-10 resemble 158base per PCR products. (1.5hours, 80 volts).

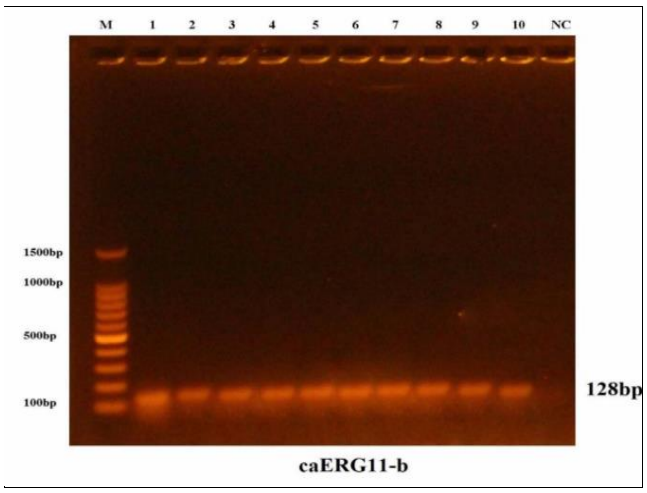

Figure (6): Electrophoresis Agarose gel electrophoresis for the amplification from caERG11-b gene of Candida albicans had been fractionated on 1.5% electrophoresis gel agarose stained in Eth.Br. M: 100base per ladder marker. Lanes 1-10 resemble 128base per PCR products. (1.5hours at 80 volts).

The current study discovered that the genes (CaERG11 and CaCDR1) were found in all Flucanazole-resistant isolates. Previons study (27), Included 100 *Candida* clinical isolates, found that 51% were C. albicans, 31% were *C. glabrata*, 8% were *C. krusie*, 5% were *C. tropicals*, and 5% were C. dupliniens. Fluconazole resistance was 23%, with the ERG11 gene sequence revealing 15 different mutations. Fluconazole resistance was most frequently observed, and mutations in ERG11 are unlikely to be the cause of fluconazole resistance among these isolates. Navarro-Rodrguez research (28) the role of the genes ERG11, CDR1, CDR2, and SNQ2 in voriconazole resistance in investigated in a collection of *Candida glabrata* strains with established in vitro and in vivo susceptibility to this drug, VRC was applied to eighteen clinical isolates of Candida glabrata. Across all strains studied, ERG11 and CDR1 were the most expressed genes, while CDR2 and SNQ2 expression was low.Futhermore, in resistant strains, ERG11 and CDR1 expression increased. Multiple pathways, including those responsible for azole resistance in *Candida* spp., appear to interact.ERG11 mutations were frequently found in *Candida albicans* ,although the role of azoles efflux pumps overexpression appeared to be more prominent (29).

Conclusion

Candida albicans were the most prevalent of *Candida* species among women patients with vaginitis, with high ability to form the biofilm. Vulvovaginitis indicates the importance of regular screening and routine examination for candidiasis in Iraqi hospital.

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