Detection of cyclohximide resistant gene in selected pathogenic fungi

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Objective To detect the genes that play a role in resistance to cyclohximide by some pathogenic fungi.
Methods Isolates of dermatophytic fungi (*Trichophyton rubrum, Microsporum canis, Candida albicans and Aspergillus* spp.) were isolated from patients with fungal infection attending the AL-Diwaniya Teaching Hospital and identified based on the microscopic and macroscopic characteristics. The effect of cycloheximide and ammonium hydroxide in isolation of these fungi in sabourauds dextrose agar were tested by the quantative transcrptive assay of gene expression (CYH1, CYH2, CDR1, CDR2 and ABC gene) using real-time PCR.
Results The result of this estimation revealed that the amplified DNA (PCR product) were 463 bp for CYH1; 265 bp for CDR1 ; 160 bp for CDR2; 359 bp for CYH2 in the pathogenic fungi *C. albicans , T. rubrum, M. canis and A. niger*.
Conclusion The tested pathogens had resistance genes to cycloheximide such as (CHY1, CHY2, CDR1, CDR2 and HKG).
Keywords cycloheximide, cycloheximide resistant fungi, antifungal activity, cycloheximide resistance genes

Introduction

The present work aims to detect the genes that play a role in resistance to cyclohximide in some pathogenic fungi.

Cycloheximide is used in a range of media for the isolation of pathogenic fungi to inhibit certain non-pathogenic fungi similar to saprophytic moulds and yeasts. It is especially useful in the isolation of dermatophytes. Since the pathogenicity of fungi and the immune status of patients vary, care should be taken when a medium with cyclohximide is used for the isolation of fungi because certain opportunistic fungi might be missed. Chloramphenicol is a broad-spectrum antibiotic, which is inhibitory to a wide range of gram-negative and gram-positive bacteria but may have an inhibitory effect on several pathogenic fungi.[1]

The Cycloheximide (actidione), which is an antibiotic that is produced by *Streptomyces griseous*, is highly active against a large number of yeast. But have no marked antibacterial activity.[2] The use of cycloheximide in culture media is to facilitate the isolation of numerous pathogenic fungi from medical samples and the environment.[3]

ATP-binding cassette (ABC) transporters are integral membrane proteins that can be found in all kingdoms of life. Some members of the super family are involved in processes like DNA repair, translation or regulation of gene expression, but most couple the binding and hydrolysis of NTPs, usually ATP, to transport a large variety of substrates across cellular membranes.[4]

The drug efflux pump encoded by candida drug resistance genes (*CDRI*) of *Candida albicans* was the first ABC efflux pump implicated in conferring resistance to cycloheximide in a PDRS disruptant hypersensitive strain of *Saccharomyces cerevisiae*.[5]

The present work was designed to evaluate the efficacy of ammonium hydroxide in isolation of pathogenic fungi instead of cyclohexamide and study the molecular basis of the resistant of these fungi to cyclohexamide.

Methods

Patients

A total of 103 patients [males (71) and females (31)] were clinically diagnosed as cases of dermatophytosis by the dermatologist after attended the outpatient clinic of the Department of Dermatology at AL-Diwaniya Teaching Hospital from the beginning of December 2014 to the end of February 2015.

Mycological Examination

The evidence of infection established on demonstration of fungal elements [branching septate hyphae and spore (arthrospores)] by the direct microscopically examination of hair, scales from the lesion by KOH mounting. Enough materials of sample was taken to permit repeated direct examination, then, a single preparation may not provide enough evidence for the existence of an infecting fungus.

Potassium Hydroxide (KOH) Mounts

Skin scrapings and hair fragments were placed on a surface of clean slide swamped with drops of 10% KOH, heated mildly (30°C) for about 5–10 min, after which the slide was allowed to cool. After cooling, the cover slip was applied and the slide was examined under the low (10×) and high (40×) microscope lens. Gentle warming of the preparation speeded up the reaction.[6]

Fungal Genomic DNA Extraction

Fungal genomic DNA from of *C. albicans* isolates were extracted using EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit, and done according to company instructions.

Estimation of DNA yield and quality: The extracted genomic DNA was checked using Nanodrop spectrophotometer (THERMO. USA), that checks and measures the purity of DNA by reading the absorbance at 260/280 nm.

Preparation of Primers

The primers were prepared according to the manufacturer's instructions by dissolving the lyophilised primers with TE buffer to stock solution with counteraction of 100 pmol/µl, after spinning-down and allowing it to stay overnight at 4°C, primers working solution with TE buffer, using the equation C1V1 = C2V2 (concentration versus volume) to gel final working solution (20 pmol/µl) for each primer.

Primers

The PCR detection primers were designed using NCBI-Gene Bank database and Primer 3 design online, and supported by Bioneer, Korea.

PCR Primers

Primer		Sequence	Amplicon (bp)
PCR- CHY1	F	CGGTTTCGGTGGTCAAACC	463
	R	TGTCACCACCCAATTCGAAATG	
PCR- CHY2	F	AGGGTGTCGTAAACATACCATTC	359
	R	ACCTTGAGCAAATAAGGAAGCTC	
PCR- CDR1	F	TGCTGCCATGTTCTTTGCTG	265
	R	TGGTCTGGCTTCGAAAAGTG	
PCR- CDR2	F	ACACGTCTTTGTCGCAACAG	160

Polymerase Chain Reaction (PCR)

PCR technique was performed for detection multidrug resistance-related ABC transporter genes groups of study fungus. The method was carried out according to method described in a study by Walker et al.[7]

PCR Master Mix Reaction Preparation

PCR master mix reaction was prepared using AccuPower PCR PreMix Kit according to the manufacturer's instructions. The PCR master mix reaction components (DNA template volume 5 µl, F. primer 10 pmol volume 1.5 µl, R. primer 10 pmol volume 1.5 µl, PCR water volume 12 µl and total volume 20 µl) were placed in standard PCR tubes containing the PCR PreMix, as lyophilised materials containing all other components needed PCR reaction, such as Taq DNA polymerase, dNTPs, Tris–HCl pH: 9.0, KCl, MgCl2, stabilizer and tracking dye. The tube was then placed in Exispin vortex and centrifuged for 3 min and transferred to the Mygene PCR thermocycler.

PCR Thermo Cycler Conditions

PCR thermocycler conditions for each gene were done using conventional PCR thermocycler system Initial denaturation: Repeat1, Temp. 95°C, Time 5 min, Denaturation: Repeat 30, Temp 95°C, Time 30 s, Annealing: Repeat 30, Temp 30°C, Time 30 s, Extension: Repeat 30, Temp 72°C, Time 45 s, Final extenθ sion: Repeat 1, Temp. 72°C, Time 7 min, Hold: -,4C, Forever.

Table 1. **Values of DNA concentration and purity of selected sample of extracted DNA**

Sample no	Concentration ng/µl	Purity 260/280 nm
1	39.4	1.76
2	25.1	1.61
3	24.1	1.7
4	17.8	1.40
5	10.7	1.23
6	31.6	1.47
7	18.1	1.42
8	155.9	1.99
9	40.1	1.77
10	54.1	1.75
11	141.7	1.68
12	72.4	1.4

Gel Electrophoresis

PCR products of each genes were analysed using agarose gel electrophoresis method.

Results

DNA Quality and Purity

The results of evaluating and estimating the DNA extraction were measured using Nano drop spectrophotometer at a wavelength of 260–280 nm. It gave an optimal concentration of DNA for amplification ranging from 10.7 to 155.9 ng/µl and purity ranged from 1.4 to 1.99 nm (Table 1).

Based on the standard values of DNA concentration for amplification, the values of the present study are considered of efficient value and suitable for the establishment of the DNA extracted with target primers or sequences amplification (Applied Biosystems, 2008).

DNA Amplification

The result of amplification was performed on the DNA extracted from all the studied specimens and confirmed by electrophoresis analysis. In this analysis, the strands of DNA resulted from the successful binding between specific primers of target gene and specimen extracted DNA. The successful binding appeared as single compact band under the UV light using ethidium bromide as a specific DNA stain. The electrophoresis was also used to estimate DNA molecular size depending on DNA marker (100-bp DNA ladder) and the result of this estimation revealed that the amplified DNA marker (100-bp DNA ladder) and the result of this estimation revealed that the amplified DNA (PCR product) were 463 bp for CYH1 (Fig. 1); 265 bp for CDR1 (Fig. 2); 160 bp for CDR2

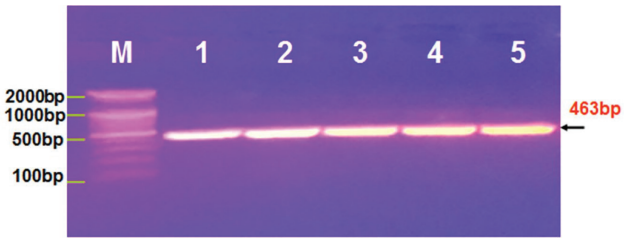

Fig. 1 **Agarose gel electrophoresis image that show the PCR product (463 bp) analysis of CHY1. Where M: marker (100–2000 bp), lane 1: *C. albicans*; lane 2: *T. rubrum*; lane 3: *M. canis*; lane 4 and 5: *A. niger*; lane 5: *C. albicans* standard strain (ATCC 1032).**

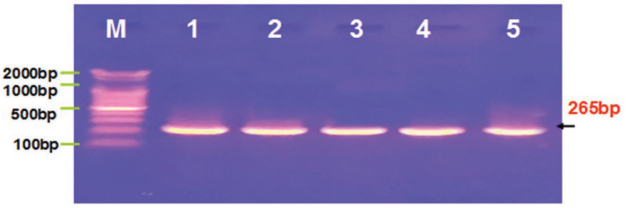

Fig. 2 **Agarose gel electrophoresis image that show the PCR product 265 bp analysis of CRD1. Where M: marker (100–2000 bp), lane 1: *C. albicans*; lane 2: *T. rubrum*; lane 3: *M. canis*; lane 4 and 5: *A. niger*; lane 5: *C. albicans* standard strain (ATCC 1032).**

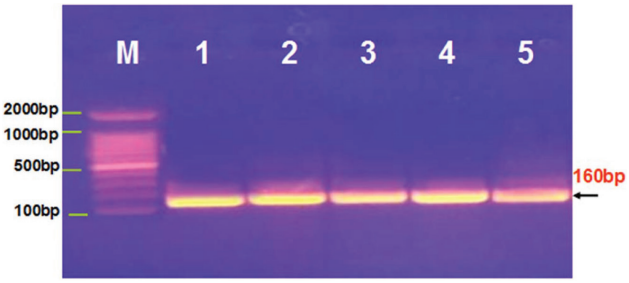

Fig. 3 **Agarose gel electrophoresis image that show the PCR product 160 bp analysis of CRD2. M: marker (100–2000 bp), lane 1: *C. albicans*; lane 2: *T. rubrum*; lane 3: *M. canis*; lane 4 and 5: *A. niger*; lane 5: *C. albicans* standard strain (ATCC 1032).**

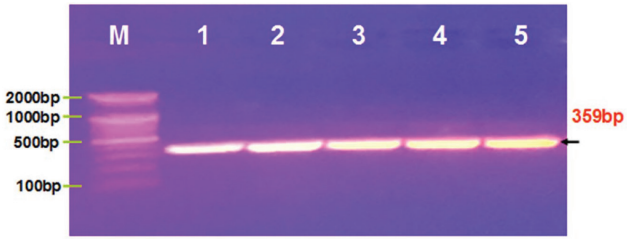

Fig. 4 **Agarose gel electrophoresis image that show the PCR product 359-bp analysis of CHY2. Where M: marker (100–2000 bp), lane 1: *C. albicans*; lane 2: *T. rubrum*; lane 3: *M. canis*; lane 4 and 5: *A. niger*; lane 5: *C. albicans* standard strain (ATCC 1032).**

(Fig. 3); 359 bp for CYH2 (Fig. 4) in the pathogenic fungi *C. albicans*, *T. rubrum*, *M. canis* and *A. niger.*

Light-cycler PCR was set up with fast start DNA master SYBR Green including heat activatable Taq polymerase with each primer and extracted DNA template. The product of PCR is measures once each cycle immediately.

After the 72°C incubation (extension step) by detection of fluorescence related with the binding of SYBR green to the amplification product. Fluorescence curves were established and analysed with the cycler software, version Exicycler real-time PCR.

The analysis of melting curve is performed immediately after the amplification protocol. The peak melting temperature achieved represented the specific amplification product. To guarantee the reliability, the test is considers to have positive results if the signal from the amplified product is clearly positive in both specimens.

Discussion

The resistance mechanism of drugs in microorganisms conventionally may take the pathway of either identifying a cellular determinant that prevents entry of the drug or removes the drug from the cell or inactivates the drug or prevents the drug from inhibiting the target of various combinations of the mentioned pathways. In fungi, mutation in gene encoding target proteins, upregulations of expression of multidrug efflux pumps and drug target themselves, altering the stoichiometry of the inhibitor target ratio in favour of fungus are possible mechanisms.[8] The mechanism of the resistance to the cycloheximide in yeast cells happens by either alteration of ribosomal proteins or expression of certain precise transport systems responsible for a multidrug resistance usually linked to the expression of MDR genes. The results of the PCR is conformational showed that the resistance gene to cycloheximide is found in the pathogenic fungi and this gene responsible for the cycloheximide resistance and several studies show that the first resistance mechanism to cycloheximide, is caused by a single change at ribosomal proteins (rps) L29 or L42 (previously L41) of the amino acid which promotes the cycloheximide cyh (cycloheximide) sensitivity (cyhS)/resistance(cyhr) is a property of the 80S ribosome from *Candida maltosa*[9], *Cryptococcus neoformans*,[10,11] *Schwanniomyces occidentalis*[12] or the *Phaffia rhodozyma*[13] and these results agree that the mutations in CYH2 decrease the affinity of cycloheximide for the ribosome,[14] there is no proof that cycloheximide really binds to L29. The mutation can alter the conformation of an adjacent site. Without a doubt, studies find that cells carrying x2 together with a mutation which alters protein L3, causing resistance to trichodermin, another inhibitor of elongation, are far less resistant to cycloheximide than cells carrying. The second resistance mechanism to the cycloheximide, several yeast MDR genes has been. They are usually connected with either transmembrane proteins, which consist of the ABC or members of MFS (major facilitator system) families. The abundant happening and close similarity of the identified proteins reveal the importance of these protein families From *S. cerevisiae* the genome sequence has revealed the presence of as several as 28 ORFs which is homologous to either the ABC or the major facilitator system.[15] The multidrug transporters of yeast include the ABC's protein CDR1-3 from *C. albicans*,[16] and the MSF (major facilitator system) BENr (CaMDR1) that renders cells resistant to cycloheximide, benomyl, methotrexate, 4-nitro-quinoline-N-oxide or terbinafine.[17] The *C. maltose* CYRR and the *C. dubliniensis* CdMDR1, homologous to CaMDR1 (*C. albicans* multidrug resistance), converse resistance to cycloheximide or to fluconazole, cycloheximide, benomyl or sulphometuronmethyl, respectively,[18] this result agrees with the studies that a second possible mechanism for drug resistance is overexpression of the *Candida drug resistance* (*CDR*)*1* and *CDR2* genes, which code transporters of the ABC family, and the *multidrug resistance (MDR)1* gene, which codes for a main facilitator transporter. Later it was found that the *resistant to 7-aminocholesterol* (*RTA2*) gene might also be involved in the development of azole resistance in a mutant *C. albicans* strain with deletions of *CDR1, CDR2* and *MDR1*.[19]

Conclusion

The tested pathogens had resistance genes to cycloheximide such as CHY1, CHY2, CDR1, CDR2 and HKG. ■

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