Detection and Identification of Candida auris and Candida kefyr from Different Clinical Samples by Conventional and Molecular Methods with Determination of Their Antifungal Responses Using Sanger Sequencing

Lecturer Arjan A. Zeynel[1*], Professor Dr. Azhar A. F. Al-Attraqchi[2] and Professor Dr. Hameed M. Jasim[3]

[1]*Medical Laboratory Technology Department/ Health and Medical Technical College-Kirkuk/ Nothern Technical University*
[2]*Department of Microbiology/ College of Medicine/ Al-Nahrain University*
[3]*College of Biotechnology/ Al-Nahrain University*

KEYWORDS

Candida auris, Candida kefyr, Antifungals, Amphoterecin B, Ergosterol

ABSTRACT

Background: Candidiasis is an opportunistic fungal disease causing local or systemic invasive infections, especially in hospitalized and immunocompromised patients. Some of Candida species exhibit greater resistance to antifungals and their misidentification by available laboratory diagnostic methods lead to difficulty in control and treatment.

Aim of the study: To search for pathogenic C. auris and C. kefyr isolates from specimens taken up from inpatients and outpatients from different hospitals or private laboratory in Kirkuk city, to test their susceptibilities to various antifungals and the genetic background for their resistance pattern to the tested antifungals.

Materials and methods: The study was carried out in the period of March/ 2022 to August/ 2023, comprising the collection of 1047 samples from four major hospitals and a private laboratory in Kirkuk city. Samples were cultured on Sabouraud dextrose agar plates, then all isolated Candida species were identified morphologically by direct and indirect methods, and the detection confirmed by molecular methods. Antifungal susceptibility testing was performed using disc diffusion method, Etest and Vitek 2 system.

Molecular techniques that included conventional polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing were accomplished by targeting internal transcribed spacer region 1 (ITS1)-ITS2, domain 1 (D1) -D2 regions of 28S ribosomal DNA (rDNA), and ITS2- 28S rDNA for C. auris recognition from pink-colored Candida species. Futherly, ITS1-ITS4 region was amplificated for analysis of all isolates that had been identified as C. kefyr previously by phenotypic techniques. Ergosterol2 (ERG2), ERG3, ERG6 and ERG11 genes were subjected for studying the resistance profile of C. Kefyr isolates genotypically.

Results: Among the total samples, 215 were positive for Candida species. The pink-colored isolated species by Vitek 2 system were: C. rugosa 6/15 (40%), C. lipolytica 5/15 (33.3%), and C. kefyr 4/15 (26.7%). Finally after application of molecular analysis, C. auris has not been detected, and four isolates of C. kefyr were found, which represent 1.9% of the total 215 Candida isolates; two isolates from urine samples, and the others from wound swabs.

The disc diffusion method displayed amphotericin resistance of all C. kefyr isolates. Regarding azoles, all isolates were sensitive excluding one isolate that was found to be voriconazole and 5-flucytosine resistant.

The molecular analysis demonstrated that ERG3, ERG6 and ERG11 genes were expressed in all isolates. The ERG2 was not found to be expressed in isolate number 3, contrary to the FUR1 gene expression which was noted only in this isolate. On the other hand, isolate number 4 harbored one and two mutations in ERG2 and ERG6 genes separately. The ERG3 gene was highly mutated and included two, three and five mutations in isolates number 1, 2 and 4 respectively; while one and two mutations were found in isolates number 2 and 1 independently in the ERG11.

Conclusion: C. auris was not present. The incidence of Candidiasis caused by C. kefyr is low. There is extreme resistance of all four C. kefyr isolates to amphotericin B, which occurred by the contribution of mutations of at least more than one gene of the following: ERG2, ERG3, ERG6 and ERG11. Voriconazole and 5-flucytosine were inactive against one isolate; the expression of the FUR1 gene was responsible for resistance against the last antifungal.

1. Introduction

Candida species are the most prevalent fungal pathogens in humans, as most people carry Candida yeasts (1). They are part of the natural microorganisms found on the skin, mucous membranes, and digestive system. They establish themselves on the mucous surfaces of all individuals shortly after birth, and the potential for internal infection is constant (2).

Candida species' resistance to antifungal medications is becoming a growing concern, as there are only

a limited number of antifungal classes available and Candida species have developed a wide variety of resistance mechanisms with diverse molecular origins (3). The primary cause of antifungal drug resistance seems to be point mutations in either drug targets or transcription factors that regulate the expression of resistance-related genes (4).

Multi-drug resistant (MDR) Candida species have grown in significance for treating invasive fungal infections. These infections are linked to high rates of illness and death and may be connected to transmission within healthcare settings (5).

C. auris is a recently emerged invasive pathogen, frequently MDR pathogen, capable of spreading within healthcare facilities. It has the potential to lead to severe infections and poses challenges in terms of identification using conventional yeast detection techniques (6). C. kefyr is an infrequently isolated Candida species from clinical samples (7). After being exposed to antifungals, there have been reports of C. kefyr development of MDR, particularly in patients with hematologic malignancies (8).

Aims of the Study

1- Isolation and identification of pathogenic C. auris and C. kefyr from variants leftover clinical samples by phenotypic and molecular methods.

2- Studying the antifungal susceptibilities of the isolates using disk diffusion method, Etest, and VITEK 2 compact system.

3- Determination of the Ergosterol2 (ERG2), ERG3, ERG6, ERG11, and fluorouidine 1 (FUR1) genes responsible for the antifungal-resistant profile of the mentioned isolates by conventional polymerase chain reaction (PCR) and sequencing

Patients, Materials and Methods

Patients

The Institutional Review Board of the College of Medicine/ Al-Nahrain University and the Ethics Committee of the Kirkuk Health Office approved the current study. These were confirmed in documents presented to the college council and committee. The written informed consent was obtained from the patients.

A descriptive cross-sectional study was extended from March/ 2022 to August/ 2023 in following cites of Kirkuk City: General Kirkuk, Azadi Educational, Pediatric, Al-Nasr Pediatric and Gynecological hospitals, and Dr. Dalia's private laboratory.

This study involved the collection of 1047 samples from inpatients admitted to medical, surgery, medical emergency, surgery emergency and ICU wards of both General Kirkuk and Azadi Educational hospitals, in addition to various wards of Pediatric and Al-Nasr Pediatric and Gynecological hospitals. Furthermore, other specimens with their demographic and medical archives were obtained from outpatients who came to Dr. Dalia's private laboratory. The samples included urine, skin swabs (axilla, groin, and wounds), vaginal and oral swabs.

2. Methodology

Conventional Methods

Isolation and Identification

The impregnated cotton end of each swab was spread onto sabouraud dextrose agar (SDA), and all urine specimens were inoculated on SDA and incubated at 37 °C for 48 hours. Any isolate characterized by smooth, pasty, opaque, and creamy white-coloured colonies on SDA was identified as *Candida* spp. and subjected to Gram staining (9).

Once the Gram stain confirmed the identification of the Candida isolate as Gram-positive budding yeast cells, the SDA plates were further subcultured onto CHROMagar Candida at 37 °C for 1-4 days to differentiate Candida isolates by their colour and morphology of colonies (2).

The incubation of CHROMagar Candida took for 48 hours, and the colonies of each isolate appeared in a specific colour based on the *Candida* spp. To isolate *C. auris* and *C. kefyr*, all fungal isolates that appeared pink were accounted for and were subsequently cultured on Pal's media at 42 °C for 48 hours, considering that it is possible through Pal's media to differentiate *C. auris* from closely related species (10).

The suspected *C. kefyr* and *C. auris* isolates were preserved at −70 °C in the SDA slants with 10 % glycerol until they were further identified by biochemical profile and molecular methods (11).

All the preserved isolates were subcultured on yeast extract peptone dextrose (YEPD) broth at 44 °C for 48 hours. Then, a loopfull from broths was transferred on YEPD agar and incubated at 44 °C for 48 hours to obtain yeast colonies (12). For precise identification by carbohydrate assimilation reactions of API system represented by KB006 HiCandida Test kit (13). Finally, all isolates were subjected to species confirmation using the VITEK 2 Compact System Version 07.01 following the manufacturer's instructions (14). At the end of biochemical reactions, sabouraud dextrose broth (SDB) preservation at −70 °C with 10 % glycerol was made for the isolates diagnosed as *C. kefyr* to conduct molecular methods (11).

Antifungal Susceptibility Testing (AST)

Antifungal susceptibility tests were applied to colonies of *C. kefyr* isolates on YEPD agar that appeared on subculturing of YEPD broth obtained from SDA slants preservative cultures, as mentioned previously in biochemical tests (12). Disc diffusion method was performed as described in the National Committee for Clinical Laboratory Standards Institute (CLSI) document, 2018 (15). ETes**t** was performed according to the manufacturer's instructions. Yeast inoculum suspensions were prepared as described in (16); The profile of the antifungal drug sensitivity was classified as sensitive (S), dose-dependent sensitivity (SDD), intermediate (I), and resistant (R). The breakpoints used to evaluate S, I, and R for each isolate against fluconazole, itraconazole, voriconazole and caspofungin were those defined by interpretive susceptibility criteria recommended by CLSI, 2022 (17) or European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2020 (18). The VITEK 2 compact system was used for AST for amphotericin B, 5-flucytosine, fluconazole and voriconazole. The test was carried out according to the manufacturer's instructions (14). The AST of the isolates was interpreted as S, I and R according to the CLSI, 2022 or EUCAST, 2020 interpretative breakpoints criteria (17, 18**).**

Molecular Identification of *Candia auris* and *Candia kefyr* with Their Antifungal Resistance Genes

For identification of *C. auris*, isolates were taken from the −20 °C frozen SDA slants and revived on a YEPD broth for 48 hours at 37 °C. For each isolate, a loopfull of yeast suspension was transferred from YEPD broth to fresh YEPD agar plates and incubated at 37 °C for 48 hours. For identification of *C. kefyr*, the yeast cells were retrieved and subcultured onto potato dextrose agar (PDA) for 48 hours at 37 °C after transferring a loop full of −20°C frozen SDB storage to PDA (12).

Genomic DNA (GD) was extracted from each fungal isolate using previously described method (19). This was done through an application of Presto™ Mini gDNA Yeast and Quick-DNA™ Fungal/Bacterial Miniprep kits for *C. auris* and *C. kefyr* separately.

Based on the data received in Sambrook and Russell, 2001 (20), using (agar gel electrophoresis) AGE after GD extraction serves as a quality check to ensure that the DNA has been successfully isolated and remains in good condition for downstream applications of PCR and sequencing.

Amplification of molecular identification genes and antifungal resistance genes was achieved by using nucleotide primers; ITS1, ITS2, D1, D2 regions of 28S rDNA were amplified for identification of *C. auris* (21-23), through employment of Maxime™ PCR PreMix (i-Taq) kit. To ensure the accuracy of *C. kefyr* identification, the PCR was performed on the ITS1 and ITS4 regions in the rDNA (24), in other hand primers for amplifying *C. kefyr* antifungal-resistant genes were *ERG2*, *ERG3*, *ERG6*, *ERG11* (25, 26) and *FUR1* (27); the preparation kit was *AccuPower*® PCR PreMix.

Sequences of all primers were equated with sequences in public databases using the Basic Local Alignment Search Tool algorithm from Gene Bank (www.ncbi.nlm.nih.gov/genbank/) for primer design.

PCR was carried out in total volume of 25 µl. The reaction components based on the information of (Moody, 2007) (28).

Go taq master mix thawed at room temperature, then mixed by vortex and volume of the reaction mixture were completed to 25 µl using nuclease free water. According to (Najafov and Hoxhaj, 2017) (29).

PCR amplification products were sent to Macrogen Company/ South Korea for sequencing according to Sanger method (30), and then sequences were aligned online with the same sequences of the related genes indicated in NCBI using Bioedit software (31).

3. Result and Discussion

Conventional Methods

Isolation and Identification

Out of the 1047 samples incubated on Sabouraud dextrose agar, 215 yielded Candida species. The 215 Candida isolates were directly inoculated on CHROMagar Candida medium. The number of pink-colored Candida isolates observed on CHROMagar Candida after 48 hours of incubation was 15/215. The all pink-colored Candida isolates also produced pink colonies on Pal′s agar after 2 days of incubation of colonies subcultured from CHROMagar Candida, figure (4-1).

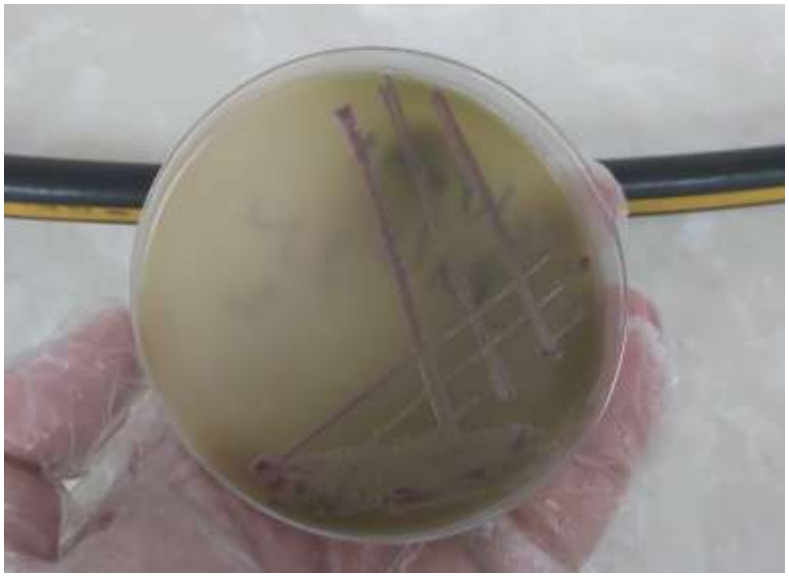


Figure (4-1): Pal's media plates showing pink color of Candida species

Candida isolates that displayed pink-colored colonies on Pal′s media were subjected into the Vitek 2 system for further identification. The range of species-level identification of pink-colored colonies with the Vitek 2 system was: *C. rugosa* 6 (40%), *C. lipolytica* 5 (33.3%) and *C. kefyr* 4 (26.7%) as illustrated in figure (4-2).

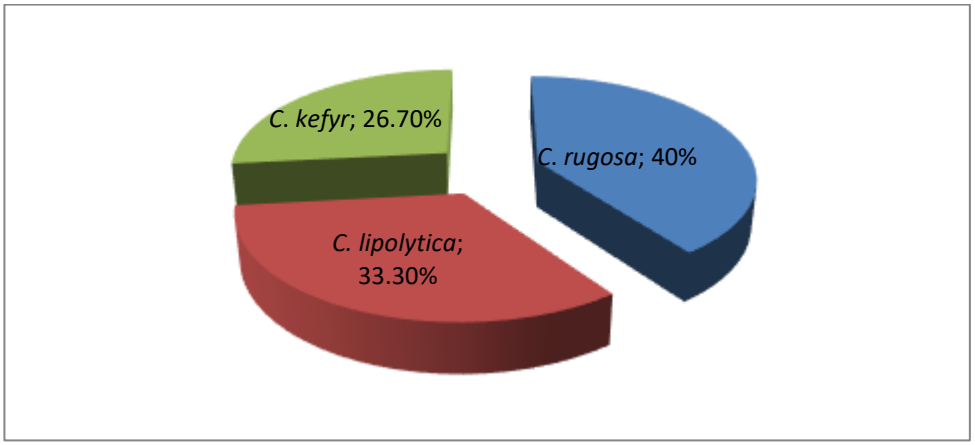


Figure (4-2): Proportions of the pink-colored Candida species identified by Vitek 2 system

In the current study, the API system correctly diagnosed all of *C. kefyr* with "excellent" identification, as displayed in figure (4-3).

Figure (4-3): API Candida system results obtained from all isolates of *C. kefyr*

Patient data and clinical characteristics of candidiasis caused by *C. kefyr* isolates are summarized in table (4-1).

Table (4-1): Clinical characteristics of candidiasis caused by *C kefyr* isolates

Isolate Symbol	Sample	Sex	Age	Signs and Symptoms	Risk Factors
CK[a]-1	Wound Swab	Male	44 year	Chest pain, back pain and chronic illness	TPN
CK-2	Wound Swab	Male	67 year	Sweeting, loss of appetite and cough	Immunosuppression due to neutropenia
CK-3	Urine	Female			Hypertension and DM

			72 year	Sudden loss of conscious	
CK-4	Urine	Female	35 year	Shortness of breath	Pregnancy and DM

[a] *C. kefyr*

Antifungal Susceptibility Testing

Disc diffusion method indicated that all *C. kefyr* isolates were resistant and susceptible to amphotericin B and azole antifungals respectively except voriconazole resistance which was observed in isolate number 3, table (4-2).

Table (4-2): Susceptibility of *C. kefyr* isolates to different antifungals using disc diffusion method

Drug	Isolate Symbol	Interpretation
AP	CK-1	R[a]
	CK-2	R
	CK-3	R
	CK-4	R
CC	CK-1	S[b]
	CK-2	S
	CK-3	S
	CK-4	S
FLC	CK-1	S
	CK-2	S
	CK-3	S
	CK-4	S
IT	CK-1	S
	CK-2	S
	CK-3	S
	CK-4	S
KT	CK-1	S
	CK-2	S
	CK-3	S
	CK-4	S
MIC	CK-1	S
	CK-2	S
	CK-3	S
	CK-4	S
POS	CK-1	S
	CK-2	S
	CK-3	S
	CK-4	S
VO	CK-1	S
	CK-2	S
	CK-3	R
	CK-4	S

[a] Resistant

[b] Sensitive

Figure (4-4) A and B showed the susceptibility of *C. kefyr* against antifungals: Amphoterecin, clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, and posaconazole.

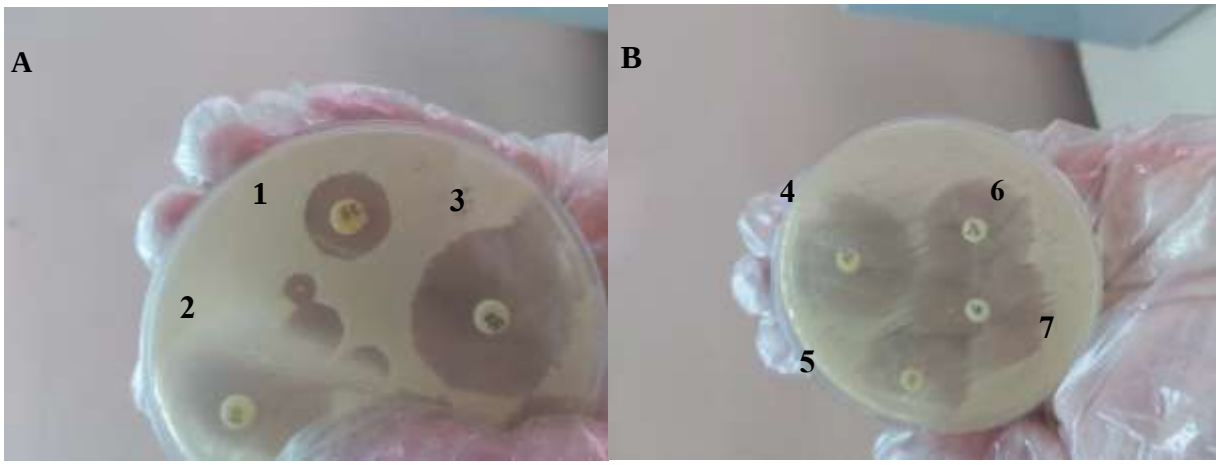


Figure (4-4): Susceptibility of *C. kefyr* against different antifungals by disc diffusion method

A: Agents: 1- Amphoterecin B 2- Clotrimazole 3-Fluconazole

B: Agents: 4- Itraconazole 5- Ketoconazole 6- Miconazole 7- Posaconazole

Using the CLSI or Eucast guideline, the MIC breakpoints were compared and susceptibility profiles were prepared for all *C. kefyr* isolates to ten antifungal drugs through the Etest. Generally, all isolates were uniformly susceptible to azole class but they exhibited resistance against amphotericin B due to presented MIC of 24, 16, 16 and 12 µg/ml respectively, table (4-3).

Table (4-3): Antifungal MIC values for the four *C. kefyr* isolates included in this study

Isolate Symbol	Antifungal MIC (µg/ml)									
	FLU	AP	CLO	FLC	ISA	ITR	KET	MIC	POS	VRC
CK-1	-	24	0.016	0.38	0.047	0.064	0.125	0.032	0.047	0.064
CK-2	-	16	0.012	0.125	0.064	0.064	0.125	0.047	0.032	0.032
CK-3	-	16	0.047	0.032	0.032	0.047	0.016	0.016	0.064	0.25
CK-4	-	12	0.016	0.094	0.032	0.047	0.125	0.125	0.012	0.016

The MIC pattern of fluconazole and amphotericin B Etest strips displayed in figure (4-5).

Figure (4-5): Antifungal susceptibility of *C. kefyr* represented by Etest strips

A: Fluconazole

B: Amphoterecin B

Table (4-4) illustrates the MIC ranges and interpretation of different antifungals against *C. kefyr* isolates employing Vitek 2 system are shown in table (4-6). Drug resistance patterns varied between the classes of test drugs. For 5-Flucytosine; high MIC (≥ 64) in isolate number 3 was encountered, sensitive pattern (≤ 1) was observed in both number 1 and 2 isolates, in other hand, the only isolate number 4 was intermediate sensitive (MIC = 8). Regarding amphotericin B; the number 1 isolate showed sensitivity (MIC = 1), the isolates that exhibited resistance (MIC ≥ 16) were number 3 and 4, while isolate number 2 revealed an intermediate sensitive pattern (MIC = 2). All isolates were susceptible to voriconazole (MIC ≤ 0.12), except isolate number 3 which was resistant (MIC ≥ 8).

Table (4-4): Results of antifungal susceptibility testing by Vitek 2 method

Antifungal	Isolate Symbol	Results	
		MIC	Interpretation
5-Flucytosine	CK-1	≤ 1	S[a]
	CK-2	≤ 1	S
	CK-3	≥ 64	R[b]
	CK-4	8	I
Amphoterecin B	CK-1	1	S
	CK-2	2	I
	CK-3	≥ 16	R
	CK-4	≥ 16	R
Voriconazole	CK-1	≤ 0.12	S
	CK-2	≤ 0.12	S
	CK-3	≥ 8	R
	CK-4	≤ 0.12	S

[a] Sensitive

[b] Resistant

[c] Intermediate

Molecular Analysis

Molecular identification of *C. auris* and *C. kefyr* was carried out, first by extraction of genomic DNA from each isolate, then by amplification, sequencing, and alignment of diagnostic genes (ITS1, ITS2, and 28S rDNA) for 15 clinical Candida isolates validated by Vitek 2 compact system and (ITS1 and ITS4) for *C. kefyr*.

Genomic DNA Extraction

Genomic DNA was extracted from *C. kefyr* and Candida species suspected to be *C. auris* using DNA extraction kits. The extracted DNA was of high purity (1.8-2.0), and concentrations (90-100 ng/µl) indicating high DNA quality and integrity for PCR experiments. The extracted DNA solutions were checked by electrophoresis on 1% agarose gel. Results of electrophoresis illustrated in figure (4-6) A and B showed clear and sharp DNA bands visualized under UV ray.

Figure (4-6): Genomic DNA bands for suspected *C. auris*, and *C. kefyr* after electrophoresis on 1% agar at 5 V/cm for 60 minutes.

A: Lane (1-5): Isolates suspected to be *C. auris*

B: Lane (1-4): *C. kefyr* isolates

Molecular Identification of *Candida auris*

Regarding PCR amplification of *C. auris* by using ITS1 and ITS2 universal primers for amplification of the Internal Transcribed Spacer 1 and 2 which were of great importance in distinguishing fungal species by PCR analysis. Results of amplification illustrated in figure (4-7) showed an amplified product of 292 bp represents ITS1 and ITS2 domain. The amplified product was sequenced according sanger method, then sequence was aligned with the reference sequence recorded in NCBI online using Bioedit program. Results of alignment illustrated in figure (4-8) showed up to 99% sequence similarity with corresponding sequence recorded for *Pichia kudriavzevii* by comparing the observed nucleotide sequence of the investigated isolates with retrived reference sequence recorded in Genbank with an accession number of CP039612.1.

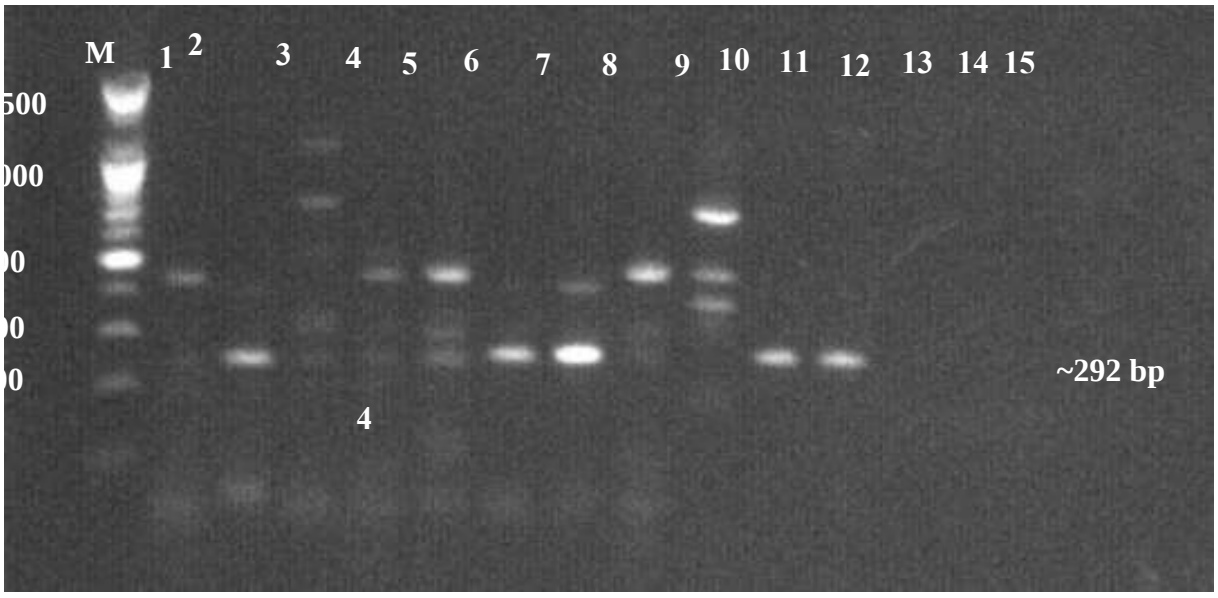
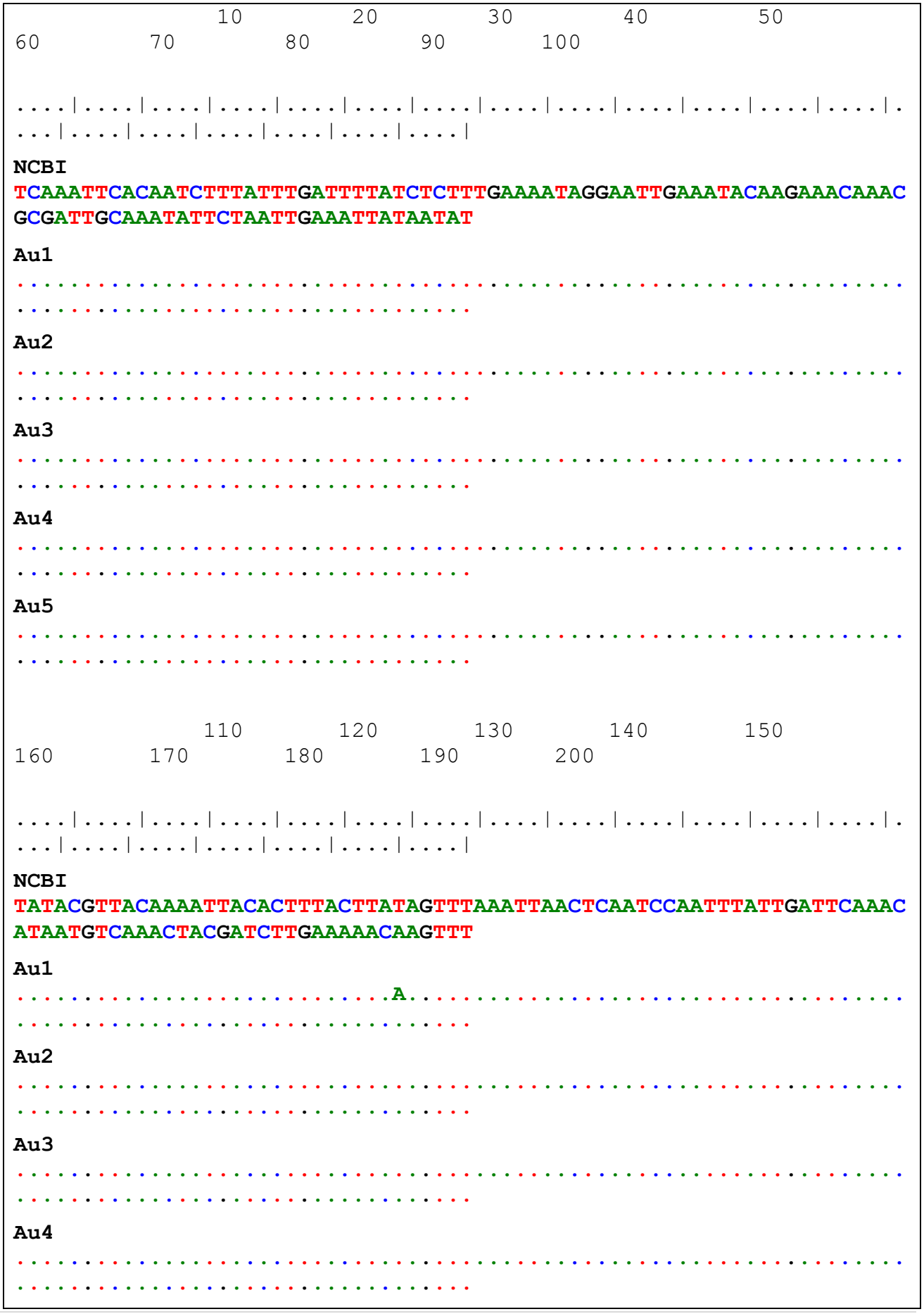


Figure (4-7): The amplified products of ITS1 and ITS2 regions after electrophoresis on 1.5% agarose gel for 60 minutes at 5 V/cm.

Lane (M): DNA ladder marker;

Lane (1-15): Fifty pink colored Candida species on Vitek system

Lane (2, 6, 7, 10 and 11): Suspected *C. auris* isolates


10 20 30 40 50
60 70 80 90 100
NCBI
TCAAATTCACAATCTTTATTTGATTTTATCTCTTTGAAAATAGGAATTGAAATACAAGAAACAAAC
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Au1
Au2
Au3
Au4
Au5
110 120 130 140 150
160 170 180 190 200
NCBI
TATACGTTACAAAATTACACTTTACTTATAGTTTAAATTAACTCAATCCAATTTATTGATTCAAAC
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Au1
A
Au2
Au3
Au4

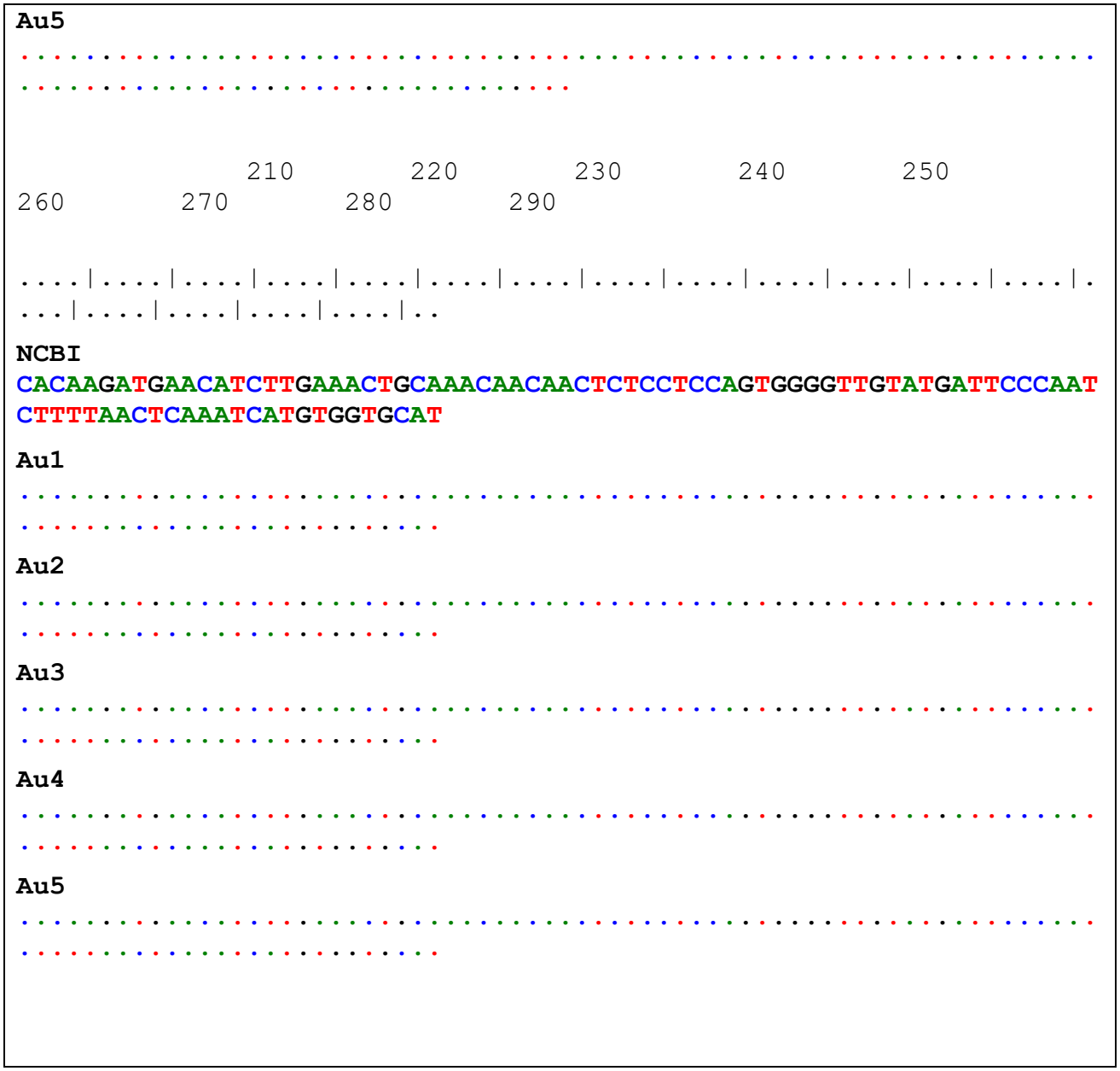

Figure (4-8): Nucleotide sequence alignment of five *C. auris* suspected isolates with their corresponding reference sequences of the amplified 297 bp amplicons. The letters "Au", followed by a number refer to the sample number

ITS2 and 28S rDNA genes were also tested using specific primers for rapid detection and identification of *C. auris*. Results of amplification illustrated in figure (4-9) showed negative results for molecular identification of *C. auris* because no amplicon was obtained with DNA isolated from *C. auris* during PCR amplification with ITS and 28S rDNA specific primers from pink colored colonies on CHROMagar Candida Media. Same results were obtained after amplification of D1 and D2 regions of 28S rDNA illustrated in figure (4-10).

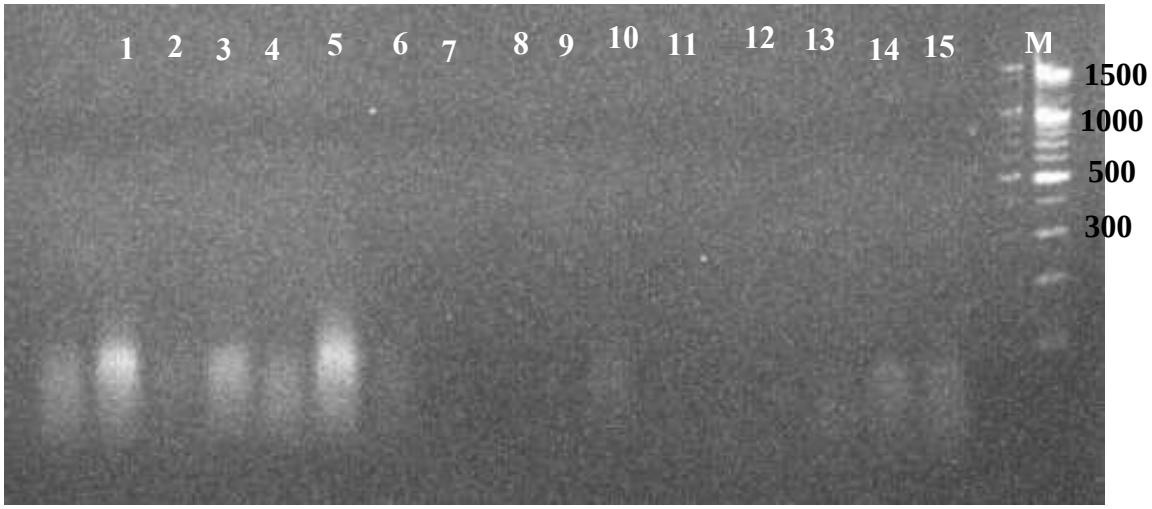

Figure (4-9): The amplified products of ITS2 and 28S rDNA regions after electrophoresis on 1.5% agarose gel for 60 minutes at 5 V/cm.

Lane (M): DNA ladder marker

Lane (1-15): Fifty pink colored Candida species on Vitek 2 system

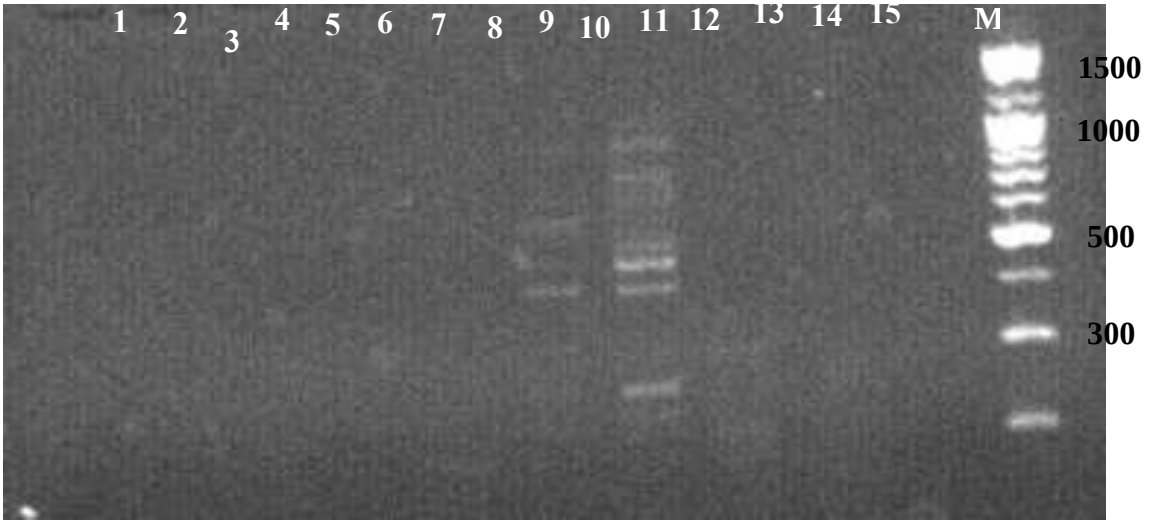

Figure (4-10): PCR amplification of D1 and D2 regions of 28S rDNA

after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm.

Lane (M): DNA ladder marker

Lane (1-15): Fifty pink colored Candida species on Vitek 2 system

Molecular Identification of *Candida kefyr*

Universal oligonucleotide primers were used for amplification ITS1 and ITS4 domains for identification of *C. kefyr*. Results of amplification illustrated in figure (4-11) showed an amplified product of 650 bp was appeared after electrophoresis on 1.5% agarose gel. The amplified products were sequenced according to sanger method. Results illustrated in figure (4-12) showed the nucleotide sequence of the amplified ITS1 and ITS4 domains were aligned with the reference sequence recorded in NCBI online using Bioedit neocleotide alignment programme. Results of nucleotide sequence alignment for the clinical isolates CK-1, CK-2 and CK-4 showed a similarity of 100 %, 99% and 99% respectively to the nucleotide sequence of *Kluveromyces marxianus* strain Kw3267/17 by comparing

the observed nucleotide sequence of the investigated isolates with retrived reference sequence recorded in Genbank with an accession number of LR738884.1.

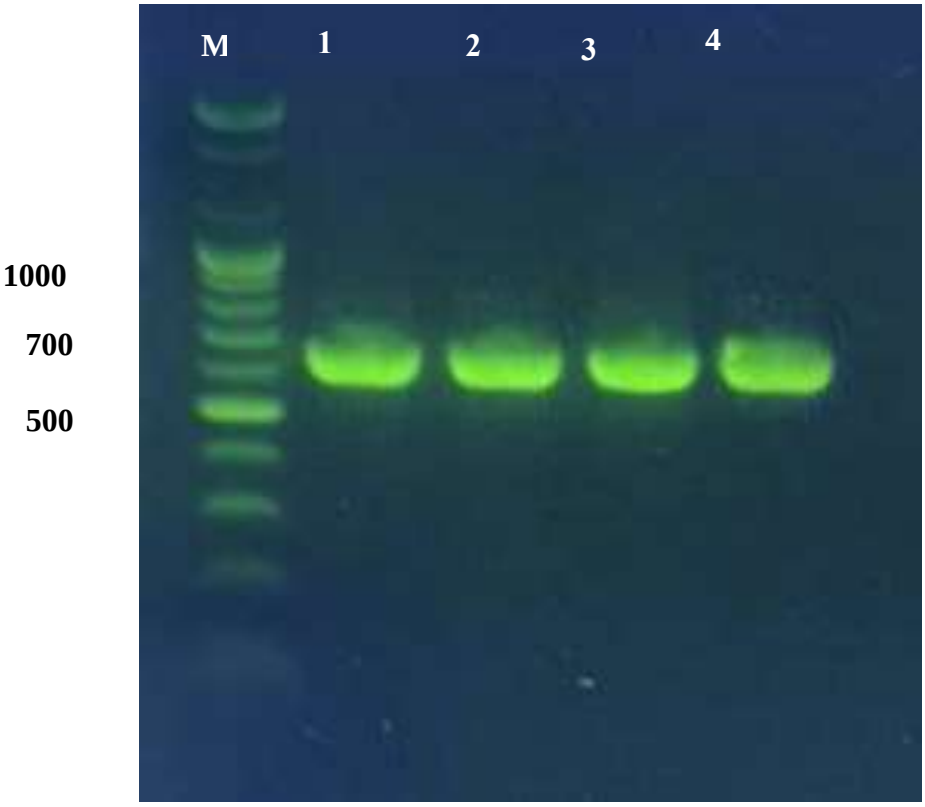

Figure (4-11): PCR amplification of ITS1and ITS4 regions of *C. kefyr* isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm.
Lane (M): DNA ladder marker
Lane (1-4): *C. kefyr* isolates

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Query  61
GAACTACTTCCCTGGAGAGCTCGTCTCTCCAGTGGACATAAACACAAACAATATTTTGTA  120

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Sbjct  163
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Query  121
TTATGAAAAACTATTATACTATAAAATTTAATATTCAAAACTTTCAACAACGGATCTCTT  180

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Sbjct  223
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A

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B

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Query  181
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Query  241
CGTGAATCATCAAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCAGGGGGCATGCCT  300

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  343
CGTGAATCATCAAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCAGGGGGCATGCCT  402
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C

Figures (4-12): Sequence alignment of ITS1 and ITS4 domains of *C. kefyr* isolates. Sequences from the isolate were deposited in GenBank

A: CK-1

B: CK-2

C: CK-4

Molecular Characterization of *Candida kefyr* Antifungal Resistant Genes

***Ergosterol 2* (*ERG2*)**

PCR amplification of the *ERG2* gene resulted in a ~846 bp band in three *C. kefyr* isolates (CK-1, CK-2 and CK-4), while *ERG2* was not detected in CK-3 isolate as shown in figure (4-13). To determine any mutation in *ERG2* in each isolate of *C. kefyr* CK-1, CK-2 and CK-4, the amplified gene products were sequenced and aligned with *ERG2* reference sequence. Results of alignment showed a complete sequence identity between *ERG2* of *C. kefyr* CK-4 and the reference sequence except one site of substitution mutation (transversion mutation C→A) as shown in figure (4-14) (C), while the nucleotide sequence of *ERG2* of the isolates CK-1 and CK-2 was free of genetic mutations as demonstrated in figures (4-14) (A) and (B), with complete identity with the reference sequence of *C. kefyr ERG2* gene recorded in NCBI database, table (4-5).

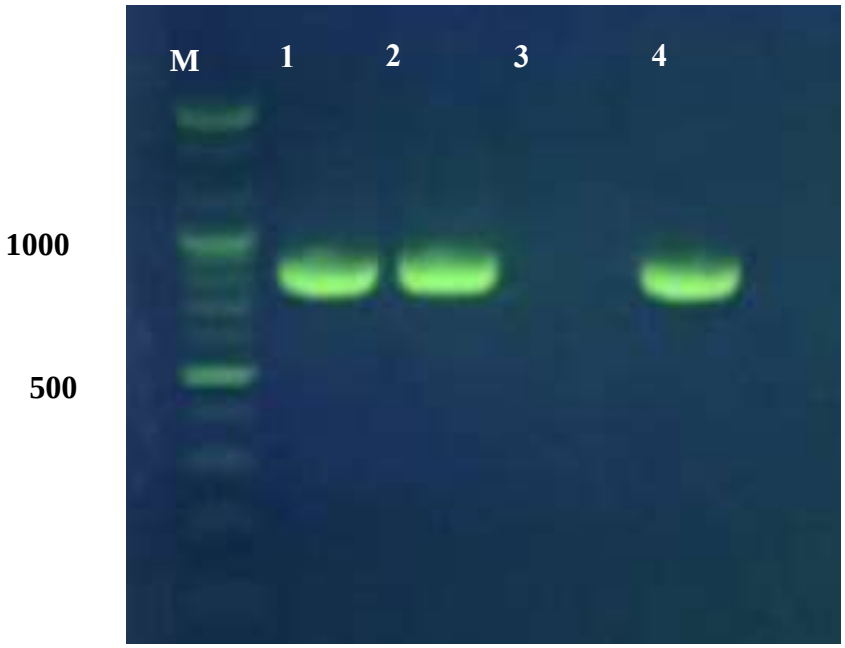

Figure (4-13): PCR amplification of *ERG2* of *C. kefyr* isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm.
Lane (M): DNA ladder marker
Lane (1-4): *C. kefyr* isolates

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Query  177
AATACTGCGCTAGCTGAATATGATTTGGAATCTGAGTCGTTTGACTTGAAAGTAATGTTG  236

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Sbjct  121
AATACTGCGCTAGCTGAATATGATTTGGAATCTGAGTCGTTTGACTTGAAAGTAATGTTG  180


Query  237
CAGGACATCAGGGACAGATTAGCCAATCATTATGGTGAAGAGTACATCAACAAGTACGTT  296

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Sbjct  181
CAGGACATCAGGGACAGATTAGCCAATCATTATGGTGAAGAGTACATCAACAAGTACGTT  240
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A

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Query  8       CTCTTTACACTTGTTAGGAACCTCAAATAAATATAAACCTC  48
               ||||||||||||||||||||||||||||||||||||||||
Sbjct  971128  CTCTTTACACTTGTTAGGAACCTCAAATAAATATAAACCTC  971168
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B

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Query  6
CTCTTTAAACTTGTTAGGAACCTCAAATAAATATAAACCCTCTTCCAATTACTATGAAA  64
               |||||||
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Sbjct  971128
CTCTTTACACTTGTTAGGAACCTCAAATAAATATAAACCCTCTTCCAATTACTATGAAA  971184
```

C

Figure (4-14): Sequence alignment of *ERG2* gene of *C. kefyr* isolates. Sequences from the isolate were deposited in GenBank

A: CK-1

B: CK-2

C: CK-4

Table (4-5): Mutations of *ERG2* gene of *C. kefyr* clinical isolates

Isolate Symbol	Nucleotide	Type of Mutation	Location
CK-1	–	–	–
CK-2	–	–	–
CK-4	A/C	Base Pair Substitution	971135

***Ergosterol 3* (*ERG3*)**

The results of gel electrophoresis for the *ERG3* gene in *C. kefyr* isolates are shown in figure (4-15). The conventional PCR for the identification of the *ERG3* gene showed that the amplified product was ~796 bp that was detected in all of isolates (CK-1, CK-2, CK-3 and CK-4). To determine any mutation in *ERG3* in each isolate of *C. kefyr* CK-1, CK-2 and CK-4, the amplified gene products were sequenced and aligned with *ERG3*reference sequence. In this study, 10 mutations were detected rather than a complete sequence identity between *ERG3* of *C. kefyr* CK-1, CK-2 and CK-4 and the reference sequence. Figure (4-16) (A) clarified that isolate CK-1 contained two mutations (one transition A→G; one transversion T→A), while isolate CK-2 exhibited three transition mutations (A→G, T→C and G→A), figure (4-16) (B). On the other hand, five mutations (three transitions G→A, T→C and G→A; two transversions C→A and T→G) were found in isolate CK-4 as appeared in figure (4-16) (C). The resultant nucleotide substitutions are listed in table (4-6), with complete identity with the reference sequence of *C. kefyr ERG3* gene recorded in NCBI database.

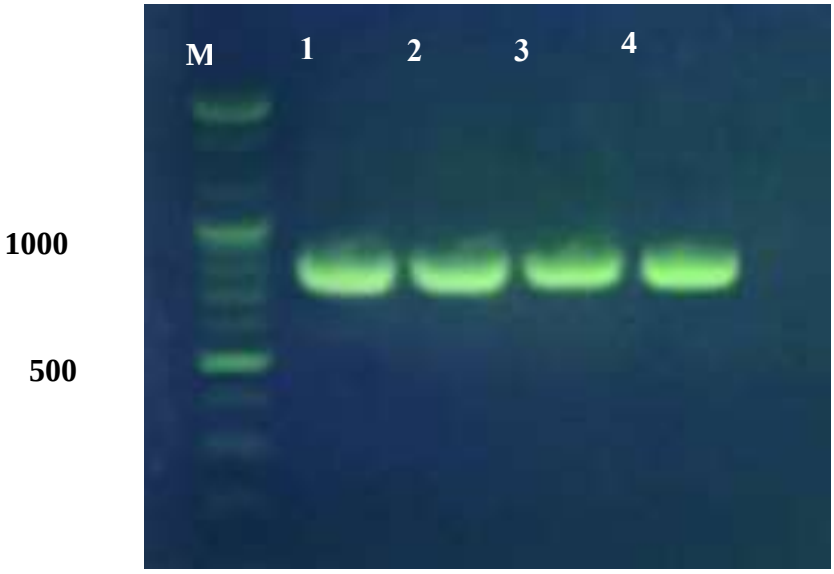

Figure (4-15): PCR amplification of *ERG3* of *C. kefyr* isolates after electrophoresis on 1.5%

agarose for 1hour at 50 V/cm.

Lane (M): DNA ladder marker

Lane (1-4): *C. kefyr* isolates

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Query  121
AATTGCCAGGTTTCAACAGTTCGGTGAAGCCTCCGGGCGATGTGTATGGGTACGAGCCTT  180

               ||||||||||||||||||||||||||||||||||
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Sbjct  651515
AATTGCCAGGTTTCAACAGTTCGGTGAAGCCTCCAGGCGATGTGTATGGGTACGAGCCTT
651456


Query  181
TCCTATTGGATTTCACCGAATACACTTTCCAATCGCTTTTCCCTCGTCACAATTTGTTTA  240

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Sbjct  651455
TCCTATTGGATTTCACCGAATACACTTTCCAATCGCTTTTCCCTCGTCACAATTTGTTTA
651396


Query  241
GACAAACACTTACAATGTTCGATTTGATGACCATATTCGGTTGGTTGTTGTACTTCAGCG  300

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Sbjct  651395
GACAAACACTTACAATGTTCGTTTTGATGACCATATTCGGTTGGTTGTTGTACTTCAGCG
651336
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A

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Query  121
TGCCAGGTTTCAACAGTTCGGTGAAGCCTCCGGGCGATGTGTACGGGTACGAGCCTTTCC  180

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|||||||||||||||
Sbjct  651512
TGCCAGGTTTCAACAGTTCGGTGAAGCCTCCAGGCGATGTGTATGGGTACGAGCCTTTCC
651453


Query  181
TATTGGATTTCACCGAATACACtTTCCAATCGCTTTTCCCTCGTCACAATTTGTTTAGAC  240

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Sbjct  651452
TATTGGATTTCACCGAATACACTTTCCAATCGCTTTTCCCTCGTCACAATTTGTTTAGAC
651393


Query  241
AAACACTTACAATGTTCGTTTTGATGACCATATTCGGTTGGTTGTTGTACTTCAGCGTTG  300

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Sbjct  651392
AAACACTTACAATGTTCGTTTTGATGACCATATTCGGTTGGTTGTTGTACTTCAGCGTTG
651333


Query  301
CTACTGTGTCGTACCTATTTGTGTTTGACAAGGCAGTGTTTAACCATCCACGTTATTTGA  360

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Sbjct  651332
CTACTGTGTCGTACCTATTTGTGTTTGACAAGGCAGTGTTTAACCATCCACGTTATTTGA
651273


Query  361
AAAACCAAATGTCTTTGGAGATGAAGCAAGCCTTCTCTGCCATCCCAACCATGGTTCTGT  420

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Sbjct  651272
AAAACCAAATGTCTTTGGAGATGAAGCAGGCCTTCTCTGCCATCCCAACCATGGTTCTGT
651213
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B

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Query  1
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TTTGGTTTGGACCATTTGTACGCCAAGCTTTTCCCAGCAAACTTGCTTCCCGCTGACATC  60

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Sbjct  651606
TTTGGTTTGGACCATTTGTACGCCAAGCTTTTCCCAGCAAACTTGCTTCCCGCTGACGTT
651547


Query  61
CCAAAAAATGTTATACAGCTGATAGACTTGCAATTGCCAGGGTTCAACAGTTCGG  115
                |||||  ||||||||||||||  ||||||||||||||||||
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Sbjct  651546
CCAAAGAATGTTATACAGCTGCTAGACTTGCAATTGCCAGGTTTCAACAGTTCGG  651492
```

C

Figures (4-16): Sequence alignment of *ERG3* gene of *C. kefyr* isolates. Sequences from the isolate were deposited in GenBank

A: CK-1

B: CK-2

C: CK-4

Table (4-6): Mutations of *ERG3* gene of *C. kefyr* clinical isolates

Isolate Symbol	Nucleotide	Type of Mutation	Location
CK-1	G/A	Base Pair Substitution	651508
	A/T	Base Pair Substitution	651374
CK-2	G/A	Base Pair Substitution	651481
	C/T	Base Pair Substitution	651469
	A/G	Base Pair Substitution	651244
CK-4	A/G	Base Pair Substitution	651549
	C/T	Base Pair Substitution	651547
	A/G	Base Pair Substitution	651541
	A/C	Base Pair Substitution	651525
	G/T	Base Pair Substitution	651505

***Ergosterol 6* (*ERG6*)**

PCR results of the *ERG6* gene from the four isolates (CK-1, CK-2, CK-3 and CK-4) showed that the amplification product corresponded to the expected DNA fragment length (860 bp). *ERG6* gene increased expression is presented in figure (4-17). To determine any mutation in *ERG6* in each isolate of *C. kefyr* CK-1, CK-2 and CK-4, the amplified gene products were sequenced and aligned with *ERG6* reference sequence. Results of alignment showed a complete sequence identity between *ERG6* of *C. kefyr* CK-4 and the reference sequence except two sites of substitution mutation (transition mutations A→G and G→A), figure (4-18) (C), while the nucleotide sequence of *ERG2* of the isolates CK-1 and CK-2 was free of genetic mutations as appeared in figures (4-18) (A) and (B). Table (4-7) displays the occurrence of mutation in the *ERG6* gene with complete identity with the reference

sequence of *C. kefyr ERG6* gene recorded in NCBI database.

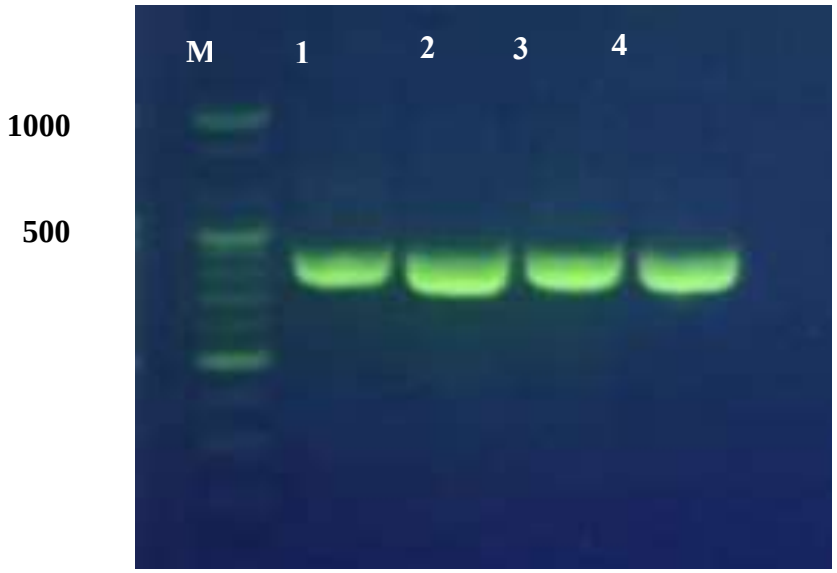

Figure (4-17): PCR amplification of *ERG6* of *C. kefyr* isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm.
Lane (M): DNA ladder marker
Lane (1-4): *C. kefyr* isolates

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Query  134
CGACGTCACCAAGAGCGGTATGTCTGCTTTACTCTCCAAGAACAAGAGCGCACAAAGCGA  193

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Sbjct  63
CGACGTCACCAAGAGCGGTATGTCTGCTTTACTCTCCAAGAACAAGAGCGCACAAAGCGA  122


Query  194
GGCTGTTGCCAAGTACTTGAAGCATTGGGACGGTAAGACAGACGAGGATGCAGAACGCAG  253

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Sbjct  123
GGCTGTTGCCAAGTACTTGAAGCATTGGGACGGTAAGACAGACGAGGATGCAGAACGCAG  182
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A

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Query  121
TACGGCTGGGGTTCCTCGTTCCACTTCTCAAGATTCTACAAAGGCGAGTCCTTTGCTGCT  180

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Sbjct  247
TACGGCTGGGGTTCCTCGTTCCACTTCTCAAGATTCTACAAAGGCGAGTCCTTTGCTGCT  306
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Query  181
AGTGTCGCCAGACACGAACACTATTTGGCGTACAAGGCCGGCATCAAGGAGAACGACCTT  240

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Sbjct  307
AGTGTCGCCAGACACGAACACTATTTGGCGTACAAGGCCGGCATCAAGGAGAACGACCTT  366


Query  241
GTGCTCGATGTCGGTTGCGGTGTTGGTGGTCCAGCGCGTGCTATCGCCCGTTTCACCGGC  300

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Sbjct  367
GTGCTCGATGTCGGTTGCGGTGTTGGTGGTCCAGCGCGTGCTATCGCCCGTTTCACCGGC  426
```

B

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Query  241
TGGGGTTCCTCGTTCCACTTCTCGAGATTCTACAAAGGCGAGTCCTTTGCTGCTAGTGTC  300

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Sbjct  253
TGGGGTTCCTCGTTCCACTTCTCAAGATTCTACAAAGGCGAGTCCTTTGCTGCTAGTGTC  312


Query  301
GCCAGACACGAACACTATTTGGCGTACAAGGCCGGCATCAAAGAGAACGACCTTGTGCTC  360

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||||||||||||||||||

Sbjct  313
GCCAGACACGAACACTATTTGGCGTACAAGGCCGGCATCAAGGAGAACGACCTTGTGCTC  372
```

C

Figures (4-18): Sequence alignment of *ERG6* gene of *C. kefyr* isolates. Sequences from the isolate were deposited in GenBank

A: CK-1

B: CK-2

C: CK-4

Table (4-7): Mutations of *ERG6* gene of *C. kefyr* clinical isolates

Isolate Symbol	Nucleotide	Type of Mutation	Location
CK-1	–	–	–
CK-2	–	–	–
CK-4	G/A	Base Pair Substitution	276
	A/G	Base Pair Substitution	254

***Ergosterol 11* (*ERG11*)**

In the present study, the *Erg11* gene was amplified with ERG11 primer yielding fragments of ~997 bp. The overexpression of the *ERG11* gene in *C. kefyr* was detected in all four isolates (CK-1, CK-2, CK-3 and CK-4), figure (4-19). To determine any mutation in *ERG11* in each isolate of *C. kefyr* CK-1, CK-2 and CK-4, the amplified gene products were sequenced and aligned with *ERG11* reference sequence. Three substitution mutations were revealed as listed in table (4-8), with complete identity with the reference sequence of *C. kefyr ERG11* gene recorded in NCBI database. Two mutations were transversion substitution (C→A and T→G) observed in isolates CK-1 and CK-2 separately, while a transition mutation (C→T) identified in isolate CK-1, figures (4-20) A and B. The nucleotide sequence of *ERG11*of the isolate CK-4 was free of genetic mutations, figure (4-20) C.

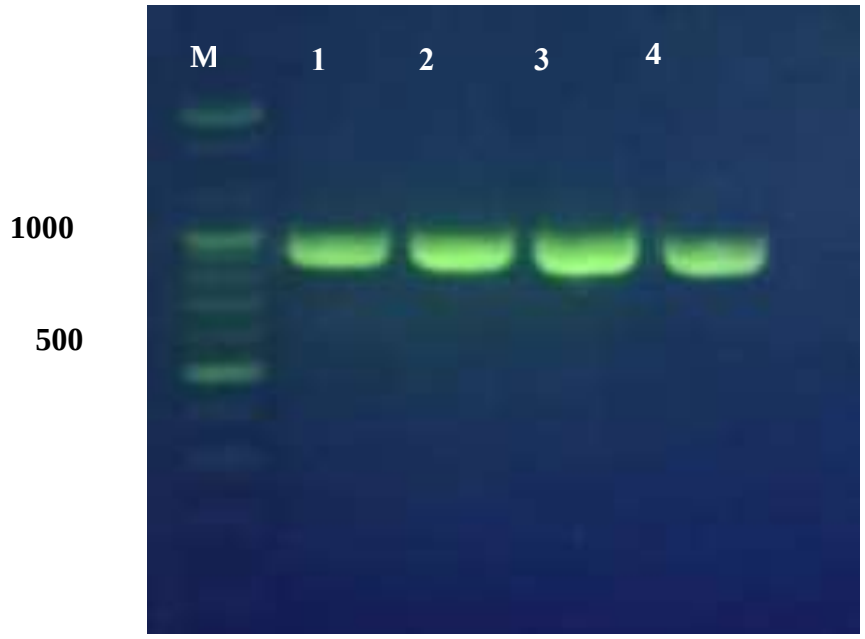

Figure (4-19): PCR amplification of *ERG11* of *C. kefyr* isolates after electrophoresis on 1.5%

agarose for 1 hour at 50 V/cm

Lane (M): DNA ladder marker

Lane (1-4): *C. kefyr* isolates

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Query  7
GCTAGTGTGTGCGTGTGTGTACGACAAAAAGATACAAAAGAAAAAGACCACGATAATAG  65

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Sbjct  29
GCTAGTGTGTGCGTGCGTGTACGACAAAAAGATACAAAAGAAAAAGCCCACGATAATAG  87
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A

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Query  61
TTTATTCGATGAGAAAAGACCGTGTTCCATTGGTGTTTTACTGGATTCCATGGGTTGGTT  120

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Sbjct  233
TTTATTCTATGAGAAAAGACCGTGTTCCATTGGTGTTTTACTGGATTCCATGGGTTGGTT  292
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B

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Query  423
TGTTTCTGCTGAAGCAGCATACACCCATTTGACCACACCAGTGTTTGGTGAAGGTGTCAT  482
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
|||||
Sbjct  447
TGTTTCTGCTGAAGCAGCATACACCCATTTGACCACACCAGTGTTTGGTGAAGGTGTCAT  506


Query  483
CTATGACTGTTCTAACAGTCGTCTAATGGACCAAAAGAAGTTCGTTAAGGGTGCTTTGAC  542

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Sbjct  507
CTATGACTGTTCTAACAGTCGTCTAATGGACCAAAAGAAGTTCGTTAAGGGTGCTTTGAC  566
```

C

Figures (4-20): Sequence alignment of *ERG11* gene of *C. kefyr* isolates. Sequences from the isolate were deposited in GenBank

A: CK-1

B: CK-2

C: CK-4.

Table (4-8): Mutations of *ERG11* gene of *C. kefyr* clinical isolates

Isolate Symbol	Nucleotide	Type of Mutation	Location
CK-1	T/C	Base Pair Substitution	44
	A/C	Base Pair Substitution	75
CK-2	G/T	Base Pair Substitution	240
CK-4	–	–	–

***Fluorouidine 1* (*FUR1*)**

Figure (4-21) demonstrates the expressions of the *FUR1* gene in C. *kefyr* isolates. Only isolate CK-3 possessed the *FUR1*gene, as the agarose gels of conventional PCR products confirmed the amplification of the target with their respective expected molecular size (957 bp) of identifiable band.

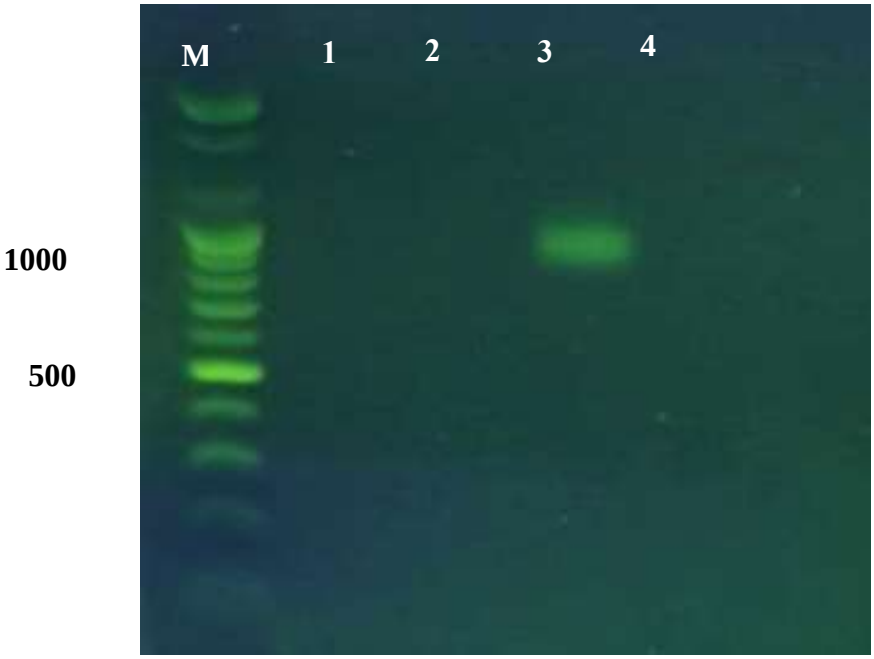

Figure (4-21): PCR amplification of *FUR1*Gene of *C. kefyr* isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm
Lane (M): DNA ladder marker
Lane (1-4): *C. kefyr* isolates

Discussion

Identification of Candida Species

Identification of Candida auris

In this study, the infection with *C. auris* tried to be investigated in the samples collected from patients and by applying all the successive steps in the conventional methods, the most important of which is the Vitek 2 on the isolates that presented pink colonies in CHROMagar Candida medium, considering that pink is the distinctive color of this fungus, as well as the molecular diagnosis by PCR amplifying and sequencing of the *ITS1*, *ITS2* and *28S rDNA* genes, which are considered universal genes in the diagnosis of this fungus; *C. auris* was not found and the result is in agreement with what was displayed in Lahore city of Pakistan by (Huma *et al*, 2023) when they did not identify *C. auris* isolate using the Vitek 2 compact identification system or real-time PCR-based molecular identification (32), but when various specimens were examined, an outbreak of *C. auris* was documented in a tertiary care hospital of Karachi, another city of Pakistan (33). Therefore, the first interpretation of the current results is that the lack of prevalence of *C. auris* in Kirkuk hospitals does not negate the presence of patients infected with this fungus in other provinces of Iraq.

After *C. auris* was isolated from a sandy beach and a salt marsh in a tropical coastal environment, it was determined that the fungus could survive in harsh wetlands and that it may therefore be associated with the marine ecosystem as a niche for itself apart from its human host. For this reason, *C. auris* is thought to be an environmental microorganism (34). The global warming emergence theory requires that *C. auris* was present in the environment prior to its clinical identification and developed into a pathogen that is harmful to people as a result of thermal adaptation in response to climate change (35).

It's possible that migratory birds brought thermotolerant *C. auris* to rural areas where people and birds coexist regularly (36). Recently (Glushakova and Kachalkin, 2024) determined that a number of Candida species were present in the feces of partially synanthropic birds, indicating that these birds

may act as carriers of highly pathogenic strains of opportunistic Candida species from urban settings to natural biotopes (37). There are now data on animals that house*C. auris*; the fungus was discovered in a dog's oral mycobiome in Kansas, but there have been no reports of human infection or colonization to far (38), Additionally, the fungus was identified molecularly in the cloaca and blood of a single Egyptian cobra (*Naja haje legionis*) after being isolated from its cloacal swab (39); thus these documents suggest that the mentioned animals could play as potential reservoirs of this emerging pathogen raise the possibility the role of these animals for the zoonotic transmission of this pathogenic fungus. Therefore, the second explanation for such phenomena is the possibility that the people in all Iraqi provinces are not infected with *C. auris* due to the drying up of the southern Iraqi marshes, which have become unsuitable environments for migratory birds carrying this fungus and spreading them to different areas of Iraq.

Identification of *Candida kefyr*

In this study, regarding to the prevalence of *C. kefyr* among clinical samples, Vitek 2 system distinguished four pink-colored isolates as *C. kefyr* which was followed with the confirmation of species identification utilizing the PCR amplification and sequencing of the *ITS1* and *ITS4* region of the four isolates' DNA. The final result of the *C. kefyr* diagnosis rate was 1.9% (4/215 Candida isolates); a very similar result was obtained in Iran by (Badiee *et al*, 2022) when they reported eleven (1.8%) positive C. *kefyr* from 598 clinical Candida isolates (40). On the contrary, a higher ratio of this species found as 22/813 (2.7%) by (Turan and Aksaray, 2022) (7). Other investigators have got lower rates in Kuwait [69/8257 (0.83%)] by (Ahmad *et al*, 2020) (26)

Based on the information provided in the above references, the epidemiology of *C. kefyr* differs from one country to another; geographical variation of *C. kefyr* distribution may be influenced by the type, the number of searched specimens and the application of molecular approaches to yeast identification in recent years; (Zhang *et al*, 2015) documented geographical variations in the prevalence of Candida species, particularly NAC, in different geographical areas (41).

In the current study, the urine sample was the source of two *C. kefyr* isolates; A study of (Ahmad *et al*, 2020) clarified that most of *C. kefyr* [31/69 (44.9%)] was commonly detected in urine samples over seven years in Kuwait (26). Patients with risk factors should have surveillance urine cultures for fungal infections as one of the main causal agents in these patients is fungus. Given that a significant portion of urinary fungal germs are NAC species, species identification is especially crucial (42).

Each of the other two *C. kefyr* isolates was obtained from patients number 1 and 2 separately by culturing wound swabs; the result is consistent with a case report that illustrates kerion-like scalp mycosis caused by *C. kefyr* occurred in a child exposed to a local trauma (43).

Antifungal Susceptibility of *Candida kefyr* Isolates

Conventional Methods

In the current study, concerning with amphotericin B, all *C. kefyr* isolates seemed to be resistant by conducting the disc diffusion method, the Etest revealed MIC range (12-24 µg/ml), while Vitek 2 system showed that only isolates number 3 and 4 were resistant with ≥ 16 µg/ml of both isolates; these data concur with previous investigation of (Mishra *et al*, 2014), where all *C. kefyr* were uniformly resistant to amphotericin B in India (42). By applying the MIC breakpoints, 4/5 (80%) of *C. kefyr* isolates were resistant to amphotericin B, which is considered the most likely *Candida* spp. resistant to amphotericin B (8). (Asadzadeh *et al*, 2023) identified eight isolates from 8 patients with reduced susceptibility to amphotericin B by Etest in Kuwait (25). It had become clear the decreased susceptibility of two, one and four *C. kefyr* isolates to amphotericin B at 1, 4 and 32 µg/ml MIC individually and 0.002-32 µg/ml MIC range of all 63 isolates against amphotericin B was observed by (Ahmad *et al*, 2020) in Kuwait (26).

Regarding the azole antifungals tested against *C. kefyr* by disc diffusion method in this study, no resistance was detected excluding isolate number 3 which was resistant to voriconazole and also appeared as the same in the analysis of the Vitek 2 system, the MIC ranges against fluconazole and voriconazole were 0.032-0.38 µg/ml and 0.016-0.25 µg/ml separately; the result was strongly supported by findings of researches when the resistance of only one isolate of *C. kefyr* against voriconazole was stated by (Wang *et al*, 2015) in United States (8) and (Ahmad *et al*, 2020) in Kuwait (26). The good activity of fluconazole against all isolates in the present work was similar to that reported in studies of (Seyoum *et al*, 2020) in Ethiopia (44). The data of the papers carried out by (Dagi *et al*, 2016) in Turkey, (Badiee *et al*, 2011) in Iran and (Mishra *et al*, 2014) in India were in parallel with current results regarding to 100% susceptibility of isolates to posaconazole (45), ketoconazole (46) and itraconazole (42) respectively.

Conversely to the described in the current study; studies of (Dagi *et al*, 2016) in Turkey (45) revealed that voriconazole susceptibility appeared against all *C. kefyr* isolates.

By applying the MIC breakpoints, out of 63 isolates, four (6.3%) showed reduced susceptibility to fluconazole at MIC =1 µg/ml and one (1.6%) showed resistance to fluconazole at MIC > 64 µg/ml (26). Among five isolates, 2 (60%) were resistant to fluconazole (8).

The Vitek 2 system showed that isolate number 3 was resistant to 5-flucytosine at MIC of ≥ 64; this finding is in line with a trial of (Gopinathan *et al*, 2013) when they studied the antifungal susceptibility profile of 72 Candida isolates and found that 30 (41.66%) of the isolates among them *C. kefyr* were resistant to 5-flucytosine in India (27).

In the current study, isolate number 3 was MDR to amphotericin B, voriconazole and 5-flucytosine as appeared in Vitek 2 results; previous research works identified MDR *C. kefyr* against amphotericin B, fluconazole and voriconazole (Asadzadeh *et al*, 2023) in Kuwait (25)

According to the references mentioned above, the AST profile of *C. kefyr* differs from region to region which is in agreement with the findings of report performed by (Zhang *et al*, 2015) in China, when they revealed that the frequency of antifungal resistance in isolates of Candida varies by different areas (41).

Molecular Methods

Regarding the current results, PCR amplification revealed a mutation [single nucleotide polymorphism (SNP)] in *ERG2* gene in isolate number 4, and one substitution mutation was detected in the isolate after sequencing of the *ERG2* gene; the result is relatively agree with the findings of (Hull *et al*, 2012b) report in Wales, whereas two isolates of *C. glabrata* harboring mutations in *ERG2* and exhibiting reduced sensitivity to amphotericin B (MIC 8 µg/ml) (47).

According to the findings of PCR products, the variation of four isolates' *ERG3* gene was obvious, with 10 mutations presence as a sum of all the mutations of the isolates after application of sequencing; the result was in the line with (Carolus *et al*, 2021) experimental trial in Belgium, whilst nonsense mutations in *ERG3* had a role to decrease amphotericin B susceptibility against *C. auris* (48).

The amplified gene products also represented mutations in *RRG6* gene in four isolates, and the sequencing of *ERG6* displayed two substitution mutations in isolate number 4; the result matches with the work achieved by (Rybak *et al*, 2022) in United States, when they found a mutation in the *C. auris ERG6* gene to be associated with amphotericin B resistance, and this mutation alone conferred a > 32-fold increase in such resistance (49).

In support of the flow in results, out of eight amphotericin B resistant *C. glabrata* strains isolated in Kuwait, six isolates contained a nonsense or nonsynonymous mutation in *ERG6*, while two isolates contained a nonsynonymous mutation in *ERG2*. The data showed that *ERG6* and *ERG2* are major targets conferring resistance to amphotericin B in clinical *C. glabrata* isolates (50).

In the present study, it is clear that there are mutations in *ERG11* gene in the four isolates after carrying out PCR. The isolates number 2 and 1 were exposed to the one and two mutations separately, that appeared by subjecting the PCR product to genetic sequencing; These findings are in accession with study of (Sanglard *et al*, 2003) that revealed a mutant *ERG11* in *C. albicans* strain could be responsible for amphotericin B resistant mechanisms during antifungal therapy in Switzerland. This strain demonstrated an increasing in the level of amphotericin B MIC (51).

Ergosterol content is involved in amphotericin B fungicidal effects (52). The ergosterol biosynthesis is disrupted by the mutations in the *ERG2*, *ERG3*, *ERG6*, and *ERG11* genes, leading to a significant change in the sterol profiles of cells or ergosterol depletion. This adaptive process is required for yeasts to develop resistance to amphotericin B (48, 49, 50, 51). As noted in the present study, each of the three isolates (number 1, 2 and 3) had mutations in more than one *ERG* genes, which may have led to a significant loss of ergosterol and thus the phenotype of high amphotericin B resistance at MIC range of 12-24 µg/ml; Similarly, (Asadzadeh *eta al*, 2023) detect a nonsynonymous mutation in *ERG2* in the majority of amphotericin B resistance isolates, whereas one or more nonsynonymous mutations were detected in *ERG3*, *ERG11*, and *ERG6* respectively in a small number of isolates with the amphotericin B resistance. These findings suggest that *ERG2* is a major target conferring amphotericin B resistance in clinical *C. kefyr* isolates. The majority of amphotericin B resistance isolates did not contain ergosterol, and the overall cell sterol profiles correlated with the loss of *ERG*2 function in multiple isolates and the loss of *ERG3* activity in a single isolate (25).

In the current study, isolate number 3 was cross-resistant to voriconazole and amphotericin B, and had a high expression of *ERG11* gene in the PCR product, it is may be possess a mutation in *ERG11* gene; (Hull *et al*, 2012a) revealed a clinical isolate of *C. glabrata* was showing signs of cross-resistance to fluconazole, voriconazole, and amphotericin B in Wales. Due to a single missense mutation in the *ERG11* gene, sterol absorption was impacted. This resulted in a lack of cellular ergosterol and minimal or no affinity for azoles with high-level resistance to amphotericin B (53). (Couzigou *et al*, 2014) found that two missense mutations in the *ERG11* allele, which result in the amino acid changes E123Q and K151E, were present in a clinical isolate of *C. kefyr* in France. The high degree of cross-resistance to fluconazole and voriconazole phenotypic of *C. kefyr* may be explained by the combination of mutations (54).

In this study, the PCR technique demonstrated the band of *FUR1* gene product was seen only in isolate number 3; previous study performed by (Gopinathan *et al*, 2013) have illustrated that the primary resistance of Candida species including *C. kefyr* to 5-flucytosine was possibly mediated by the mutant *FUR1* gene. Through application of PCR, the amplified product of the mutant *FUR1* gene from Candida isolates was detected in all 5-flucytosine-resistant isolates (27). *In vitro*, (Vandeputte *et al*, 2011) indicated that mutations in *FUR1* gene represent the most common cause of resistance to 5-flucytosine among Candida species in France (55).

It was shown through the present results of the genetic sequencing of ITS1-ITS4 and antifungal resistant genes (*ERG2*, *ERG3*, *RRG6* and *ERG11*) of the four isolates, each one of them carried different mutations from the other, and this is an indication of the multiple sources of infection of the isolates because genotypic diversity of them; the result is supported by findings clarified in (Ahmad *et al*, 2020) research, as genotypically heterogeneous invasive and amphotericin B-resistant isolates ruled out the possibility of a dominant and invasive *C. kefyr* strain spreading throughout Kuwait (26).

The summative conclusions are *C. auris* did not detected from any leftover clinical samples collected from different hospitals and a private laboratory in Kirkuk City by conventional nor by molecular methods. *C. kefyr* was isolated and identified from the collected samples in a percentage of (1.9%). *Candida kefyr* isolates from different clinical specimens were resistant to amphotericin B, while they were sensitive to clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, posaconazole, and voriconazole, except one isolate that presented voriconazole resistance, in addition to 5-flucytosine

resistance that was characterized by *FUR1* gene expression. *ERG2*, *ERG3*, *ERG6*, *ERG11*, and *FUR1* antifungal genes were detected in *C. kefyr* isolates from different clinical specimens. Single nucleotide polymorphism (base pair substitution) was detected in *ERG2* gene in only one isolate of *C. kefyr*, while the other isolates were free. Other SNPs were also detected in *ERG3*, *ERG6*, and *ERG11* genes in different isolates of *C. kefyr*.

It is suggested that further studies are recommended to detecting and identifying *C. auris* in other provinces in Iraq. Searching for *C. kefyr* resistance against other classes of antifungals, for example, echinocandin, as well as the study of the genes responsible for such resistance. Studies should be focused on other rare species of NAC and determining their antifungal resistance profile conventionally and molecularly. Whole genome sequencing for locally isolated *C. kefyr* isolates comparing with reference isolates

4. Conclusion and future scope

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