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Detection and Identification of *Candida auris* and *Candida kefyr* from Different Clinical Samples by Conventional and Molecular Methods

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Abstract

Background Candidiasis is an opportunistic fungal disease. Some Candida species (*Candida spp.*) exhibit greater resistance to antifungals, leading to difficulty in treatment.

Objective To search for pathogenic *C. auris* and *C. kefyr* isolates from specimens taken up from patients admitted to Kirkuk hospitals, to test their susceptibilities to various antifungals, and the genetic background for their antifungal resistance pattern.

Methods The study comprised the collection of 1047 samples. All isolated *Candida spp.* were identified morphologically by direct and indirect methods, and then subjected to antifungal susceptibility testing. Polymerase chain reaction (PCR) was used for identification of *Candida spp.* and detection of resistance genes.

Results Among the total samples, 215 were positive for *Candida spp. C. auris* has not been detected, and four isolates of *C. kefyr* were found: two isolates from urine samples and the others from wound swabs. All of *C. kefyr* isolates displayed amphotericin resistance. Regarding azoles, all isolates were sensitive, excluding one isolate that was found to be voriconazole and 5-flucytosine resistant. Ergosterol3 (ERG3), ERG6, and ERG11 genes were expressed in all isolates. The ERG2 was not found in isolate number 3, contrary to the uracil phosphoribosyl transferase (FUR1) gene, which was noted only in this isolate.

Conclusion The extreme resistance to amphotericin B occurred by the increased expression of ERG3, ERG6, and ERG11genes. The expression of the FUR1 gene was responsible for resistance against the 5-flucytosine.

Keywords *Candida auris*, *Candida kefyr*, antifungals, amphoterecin B.

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List of abbreviations: AGE = Agar gel electrophoresis, API = Analytic profile index, AST = Antifungal susceptibility test, CLSI = Clinical Laboratory Standards Institute, ERG = Ergosterol, EUCAST = European Committee on Antimicrobial Susceptibility Testing, FUR1 = Uracil phosphoribosyl transferase, GD = Genomic DNA. I = Intermediate, MDR = Multi-drug resistant, MIC = Minimum inhibitory concentration, NAC = Non-albicans Candida, PDR = Potato dextrose agar, R = resistant, S = Sensitive, SDA = Sabouraud dextrose agar, SDB = Sabouraud dextrose broth, YEPD = Yeast extract peptone dextrose

Introduction

Candida species (Candida spp.) 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 spp.' resistance to antifungal medications is becoming a growing concern, as there are only a limited number of antifungal classes available, and Candida spp. 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 spp. 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 spp. 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].

This study aimed for isolation and identification of pathogenic *C. auris* and *C. kefyr* from clinical samples by phenotypic and molecular methods, studying the antifungal susceptibilities of the isolates using disk diffusion method, Etest, and VITEK 2 compact system and for determination of the ergosterol (ERG(2, ERG3, ERG6, ERG11, and uracil phosphoribosyl transferase (FUR1) genes responsible for the antifungal-resistant profile of the mentioned isolates by polymerase chain reaction (PCR).

Methods

Patients

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

The Institutional Review Board of the College of Medicine, Al-Nahrain University (No. 20211162 in 30/12/2021) and the formal letter issued by the Kirkuk Health Office (No. 142 in 28/2/2022) 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.

This study involved the collection of 1047 samples from inpatients admitted to medical, surgery, medical emergency, surgery emergency, and intensive care unit 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.

Conventional Methods

Isolation and Identification

The impregnated cotton end of each swab was spread onto Sabouraud dextrose agar (SDA) (Himedia, India), 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 (Bio Research, United States) [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 (Himedia, India) at 37°C for 1-4 days to

differentiate Candida isolates by their color and morphology of colonies [2].

The incubation of CHROMagar Candida took 48 hr, and the colonies of each isolate appeared in a specific colour based on the Candida spp [10]. The suspected *C. kefyr* and *C. auris* isolates were preserved at −70°C in the SDA slants with 10 % glycerol (Thomas Baker, India) until they were further identified by biochemical profile and PCR [11].

All the preserved isolates were subcultured on yeast extract peptone dextrose (YEPD) broth (Himedia, India) at 44 °C for 48 hours. Then, a loop full from broths was transferred to YEPD agar and incubated at 44°C for 48 hr to obtain yeast colonies [12]. For precise identification by carbohydrate assimilation reactions of analytic profile index (API) system represented by KB006 HiCandida Test kit (Himedia, India). The numerical code of API was 2366027 of *C. kefyr* [13].

Finally, all isolates were subjected to species confirmation using the VITEK 2 Compact System Version 07.01 (Biomérieux, France) following the manufacturer's instructions [14]. At the end of biochemical reactions, Sabouraud dextrose broth (SDB) (Oxoid, United Kingdom) 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 (Himedia, India) 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]. ETest 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), 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 C. auris and C. kefyr with their antifungal resistance genes

For identification of *C. auris*, isolates were taken from the −20°C frozen SDA slants and revived a YEPD broth for 48 hours at 37°C. For each isolate, a loop full of yeast suspension was transferred from YEPD broth to fresh YEPD agar plates and incubated at 37°C for 48 hr. For identification of *C. kefyr*, the yeast cells were retrieved and subcultured onto potato dextrose agar (PDA) (Oxoid, United Kingdom) 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], which was done through an application of Presto™ Mini gDNA Yeast (Geneaid, Taiwan) and Quick-DNATM Fungal/Bacterial Miniprep (Zymo Research, United States) 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 (iNtRON, South Korea). The PCR amplification of *C. auris* by using ITS1 and ITS2 universal primers for amplification of the ITS1 and ITS2 which were of great importance in distinguishing fungal species by PCR analysis. To ensure the accuracy of *C. kefyr* identification, the PCR was performed on the ITS1 and ITS4 regions in the rDNA [24], on the 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 (Bioneer, South Korea).

PCR was carried out in total volume of 25 µl which based on the information of Moody [28].

Go taq master mix thawed at room temperature, then mixed by vortex, and volume of the reaction mixture completed to 25 µl using nuclease free water, according to Najafov and Hoxhaj [29].

No statistical analysis was needed in this study.

Results

Isolation and identification

Out of the 1047 samples incubated on Sabouraud dextrose agar, 215 yielded Candida spp., which were directly inoculated on CHROMagar Candida medium. The number of pink colored Candida isolates observed on CHROMagar Candida after 48 hours of incubation were 15 out of 215.

Candida isolates that displayed pink colored colonies on CHROMagar Candida medium were subjected to 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 (1).

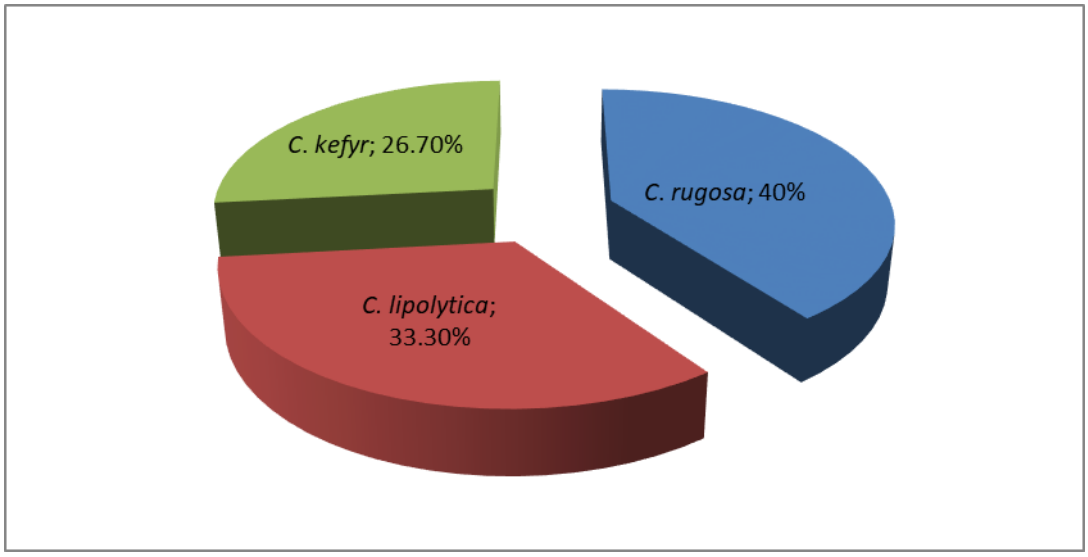

Figure 1. Proportions of the pink-colored Candida species identified by VITEK 2 system

In the current study, the API system correctly diagnosed all of *C. kefyr* with excellent identification, as displayed in figure (2).

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

Figure 2. API Candida system results obtained from all isolates of *C. kefyr*

Table 1. Clinical characteristics of candidiasis caused by *C. kefyr* isolates

Isolate Number	Sample	Sex	Age	Signs and symptoms	Risk factors
1	Wound Swab	Male	44 year	Chest pain, back pain and chronic illness	Total parenteral nutrition
2	Wound Swab	Male	67 year	Sweeting, loss of appetite and cough	Immunosuppression due to neutropenia
3	Urine	Female	72 year	Sudden loss of consciousness	Hypertension and diabetes mellitus
4	Urine	Female	35 year	Shortness of breath	Pregnancy and diabetes mellitus

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 2).

Table 2. Susceptibility of *C. kefyr* isolates to different antifungals using disc diffusion method

Drug	Isolate number	Interpretation
AP	1	R
	2	R
	3	R
	4	R
CC	1	S
	2	S
	3	S
	4	S
FLC	1	S
	2	S
	3	S
	4	S
IT	1	S
	2	S
	3	S
	4	S
KT	1	S
	2	S
	3	S
	4	S
MIC	1	S
	2	S
	3	S
	4	S
POS	1	S
	2	S
	3	S
	4	S
VO	1	S
	2	S
	3	R
	4	S

AP = Amphoterecin B, CLO = Clotrimazole, FLC = Fluconazole, ITR = Itraconazole, KET = Ketoconazole, MIC = Miconazole, POS = Posaconazole, VRC = Voriconazole, R = Resistant, S = Sensitive

Figure (3) A and B showed the susceptibility of *C. kefyr* against antifungals: Amphoterecin, clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, and posaconazole

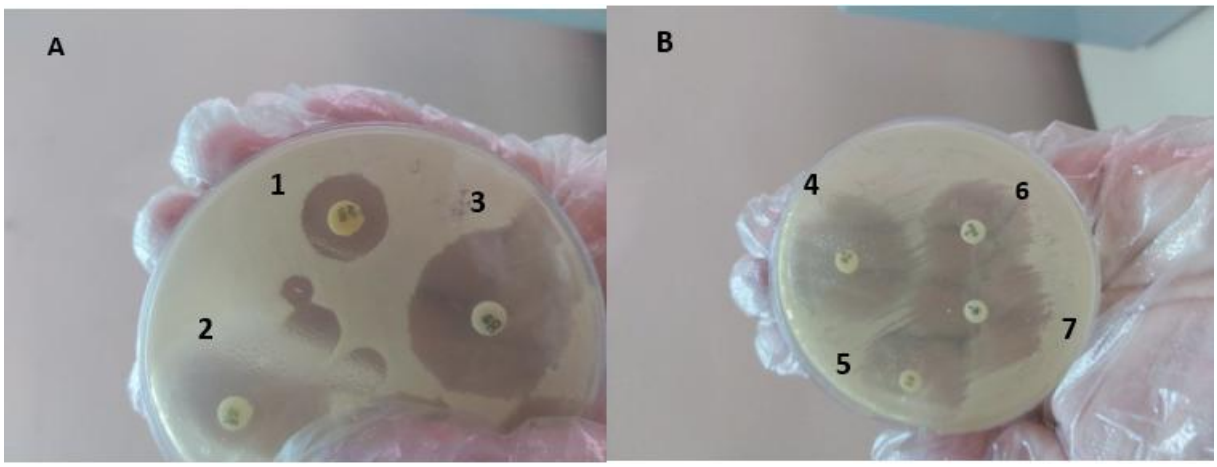

Figure 3. Susceptibility of *C. kefyr* against different antifungals by disc diffusion method
A: Agents: 1) Amphoterecin B 2) Clotrimazole 3) Fluconazole
B: Agents: 4) Itraconazole 5) Ketoconazole 6) Miconazole 7) Posaconazole

Using the CLSI or Eucast guideline, the minimum inhibitory concentration (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 3). The MIC pattern of fluconazole and amphotericin B Etest strips displayed in figure (4).

Table 3. Antifungal MIC values for the four *C. kefyr* isolates included in this study

Isolate	Antifungal MIC (µg/ml)									
No.	FLU	AP	CLO	FLC	ISA	ITR	KET	MIC	POS	VRC
1	-	24	0.016	0.38	0.047	0.064	0.125	0.032	0.047	0.064
2	-	16	0.012	0.125	0.064	0.064	0.125	0.047	0.032	0.032
3	-	16	0.047	0.032	0.032	0.047	0.016	0.016	0.064	0.25
4	-	12	0.016	0.094	0.032	0.047	0.125	0.125	0.012	0.016

FLU = 5-Flucytosine, AP = Amphoterecin B, CLO = Clotrimazole, FLC = Fluconazole, ISA = Isavuconazole, ITR = Itraconazole, KET = Ketoconazole, MIC = Miconazole, POS = Posaconazole, VRC = Voriconazole

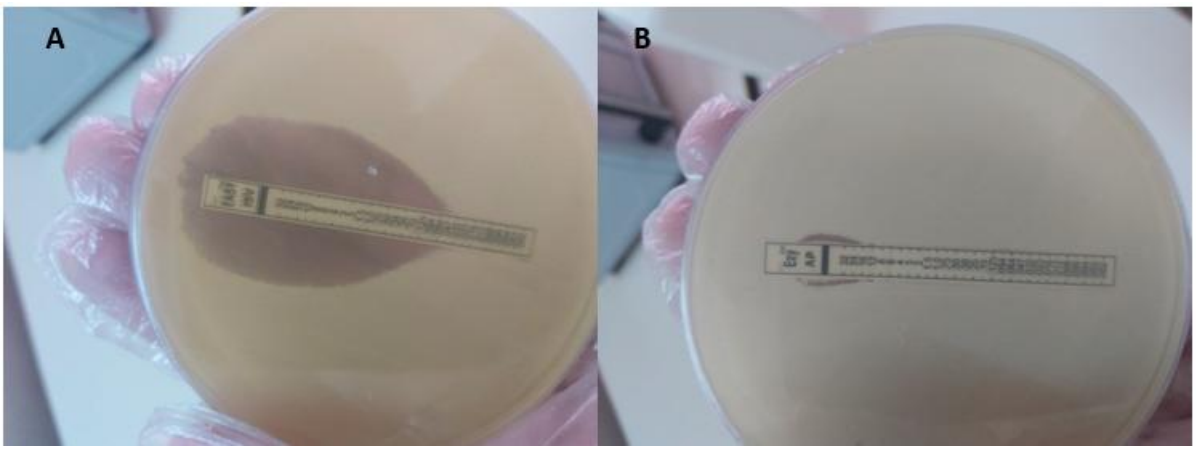

Figure 4. Antifungal susceptibility of C. kefyr represented by Etest strips
A: Fluconazole, B: Amphoterecin B

Table (4) illustrates the MIC ranges and interpretation of different antifungals against *C. kefyr* isolates employing VITEK 2 system. Drug resistance patterns varied between the classes of test drugs. For 5-Flucytosine, the high MIC (≥64) in isolate number 3 was encountered, sensitive pattern (≤1) was observed in both number 1 and 2 isolates. On the 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. Results of antifungal susceptibility testing by VITEK 2 method

Antifungal	Isolate Number	Results	
		MIC	Interpretation
5-Flucytosine	1	≤1	S
	2	≤1	S
	3	≥64	R
	4	8	I
Amphoterecin B	1	1	S
	2	2	I
	3	≥16	R
	4	≥16	R
Voriconazole	1	≤0.12	S
	2	≤0.12	S
	3	≥8	R
	4	≤0.12	S

FLU = 5-Flucytosine, AP = Amphoterecin B, VOR = Voriconazole, S = Sensitive, R = Resistant, I = Intermediate

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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 spp. 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 (5) A and B showed clear and sharp DNA bands visualized under ultraviolet ray).

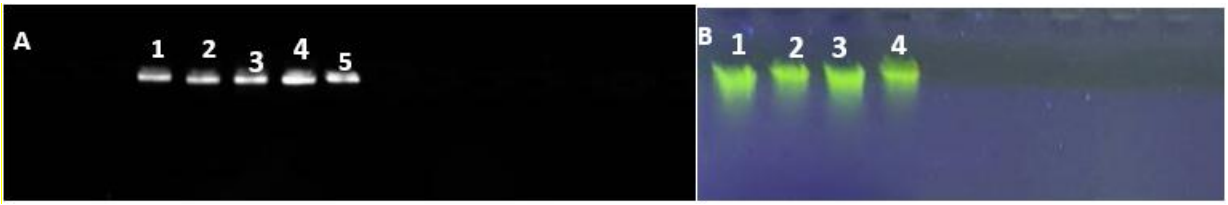

Figure 5. Genomic DNA bands for suspected *C. auris*, and *C. kefyr* after electrophoresis on 1% agar at 5 V/cm for 60 minutes. A: Lane (1-5): Isolates suspected to be *C. auris*. B: Lane (1-4): *C. kefyr* isolates

Molecular identification of C. auris

Results of amplification illustrated in figure (6) showed an amplified product of 290 bp represents ITS1 and ITS2 domain. The amplified product was sequenced according to Sanger method, and then sequence was aligned with the reference sequence recorded in National Center for Biotechnology Information online using Bioedit program. Results of alignment illustrated in figure (7) showed up to 99% sequence similarity with corresponding sequence recorded for Pichia kudriavzevii by comparing the observed nucleotide sequence of the investigated isolates with retrieved reference sequence recorded in Genbank with an accession number of CP039612.1**.**

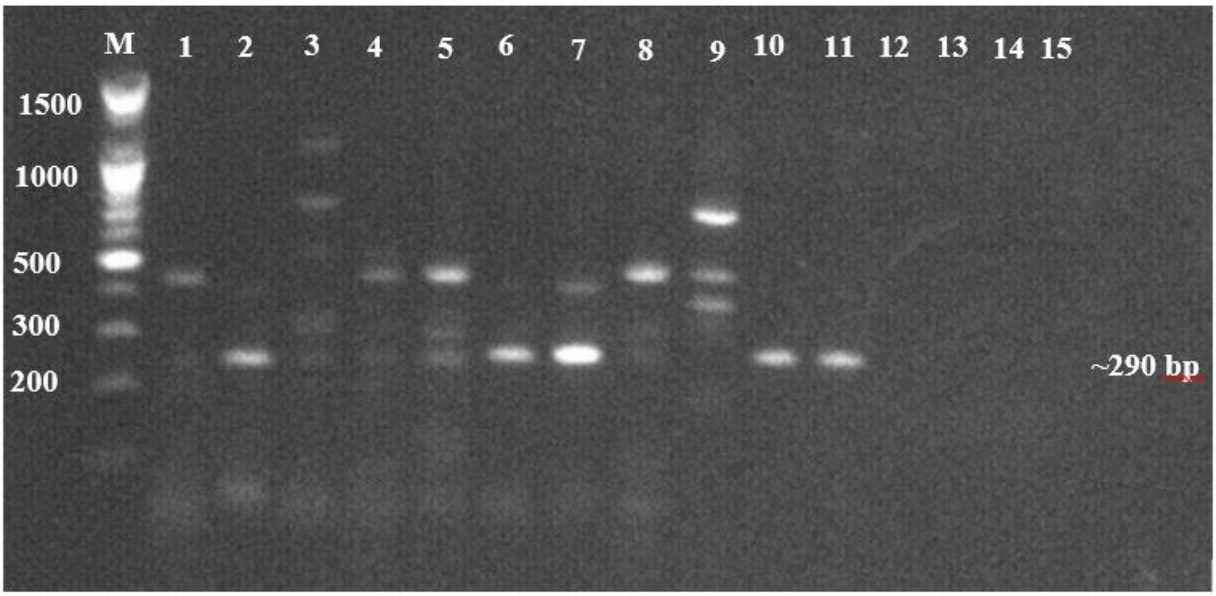

Figure 6. The amplified products of ITS1 and ITS2 regions after electrophoresis on 1.5% agarose gel for 60 minutes at 5 V/cm. Lane (M): DNA ladder marker; Lane (1-15): Fifty pink colored Candida species on VITEK 2 system; Lane (2, 6, 7, 10 and 11): Suspected *C. auris* isolates

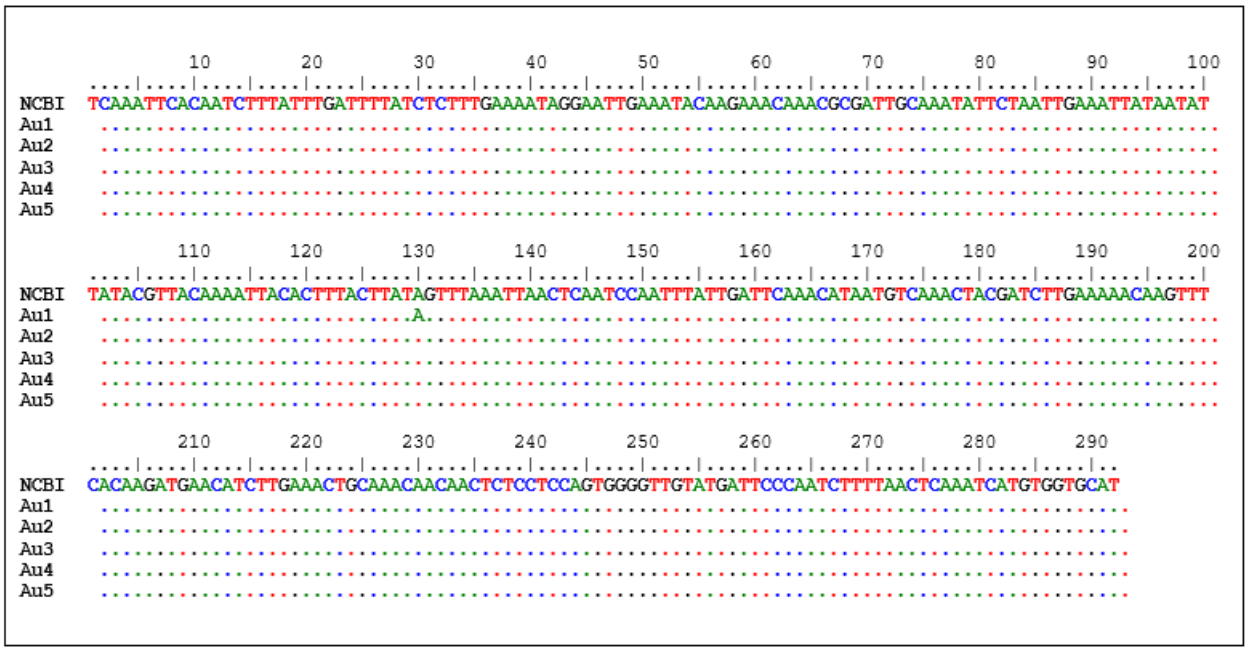

Figure 7. Nucleotide sequence alignment of five *C. auris* suspected isolates with their corresponding reference sequences of the amplified 290 bp amplicons. The letters "Au", followed by a number refer to the sample number

The 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 (8) 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 (9).

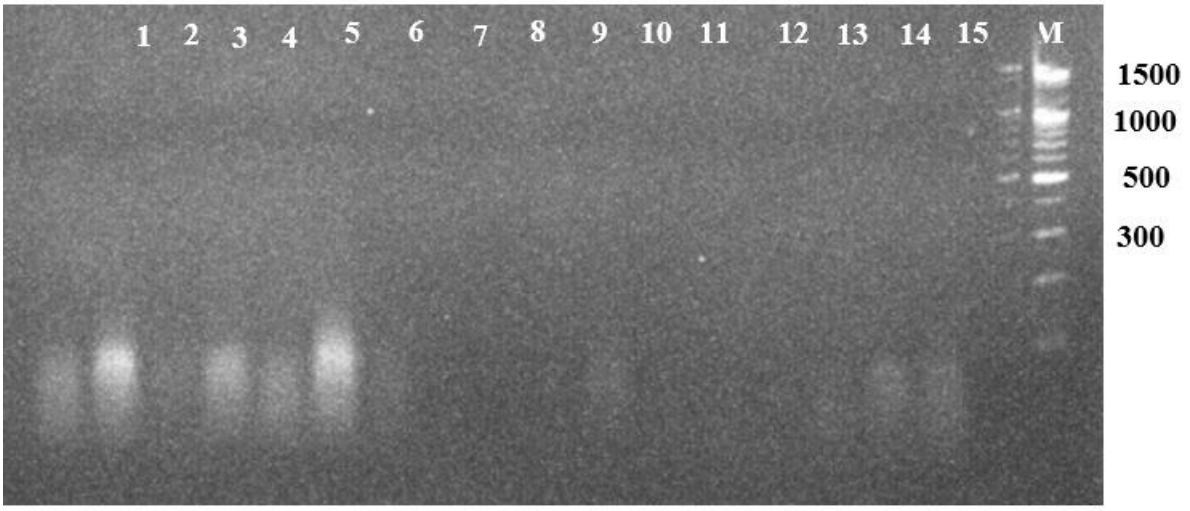

Figure 8. The amplified products of ITS2 and 28S rDNA regions after electrophoresis on 1.5% agarose gel for 60 minutes at 5 V/cm. Lane (M): DNA ladder marker; Lane (1-15): Fifty pink colored Candida species on VITEK 2 system

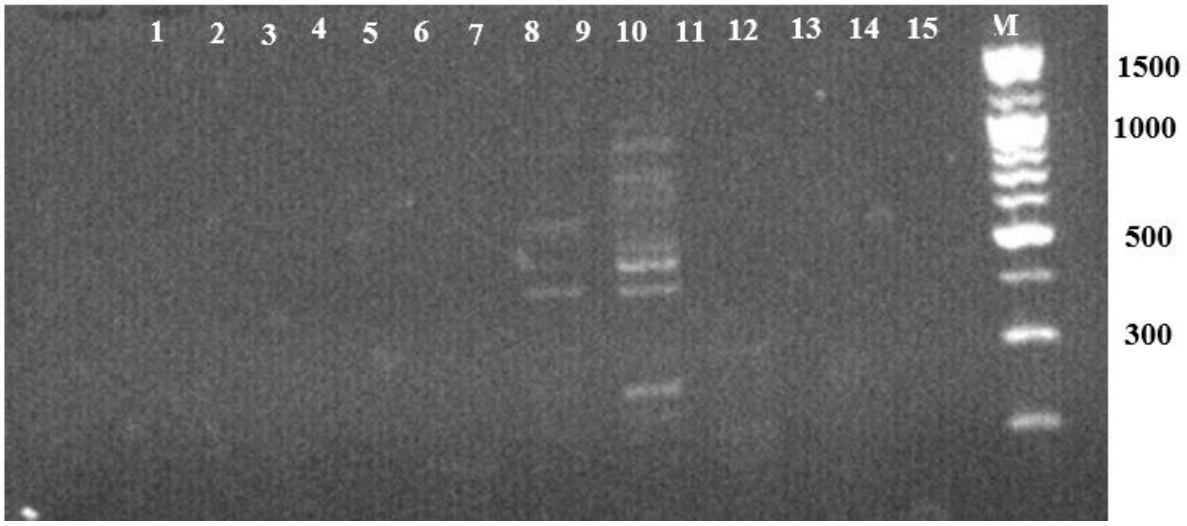

Figure 9. PCR amplification of D1 and D2 regions of 28S rDNA after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm. Lane (M): DNA ladder marker; Lane (1-15): Fifty pink colored Candida species on VITEK 2 system

Molecular identification of C. kefyr

Universal oligonucleotide primers were used for amplification of ITS1 and ITS4 domains for identification of *C. kefyr*. Results of amplification illustrated in figure (10) showed an amplified product of 650 bp appeared after electrophoresis on a 1.5% agarose gel.

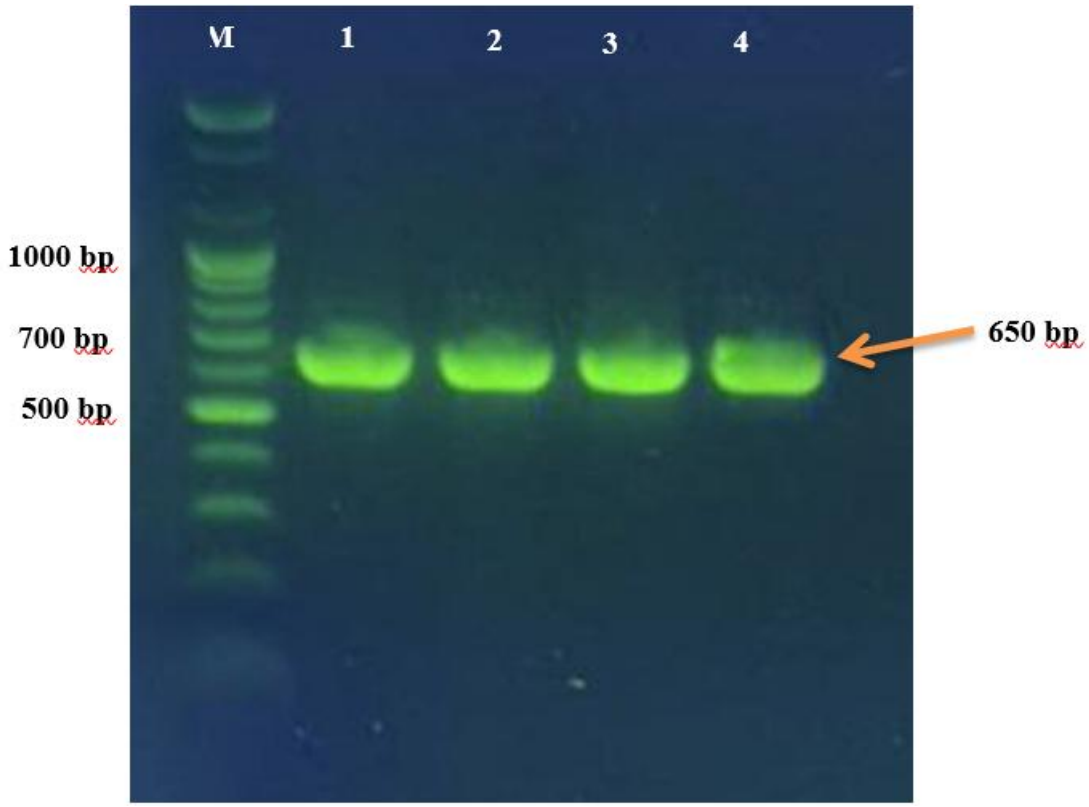

Figure 10. PCR amplification of ITS1and ITS4 regions of C. kefyr isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm. Lane (M): DNA ladder marker; Lane (1-4): C. kefyr isolates

Molecular characterization of C. kefyr antifungal resistant genes

Ergosterol 2 (ERG2)

PCR amplification of the ERG2 gene resulted in a ~846 bp band in three *C. kefyr* isolates (numbers 1, 2, and 4), while ERG2 was not detected in isolate number 3, as shown in figure (11).

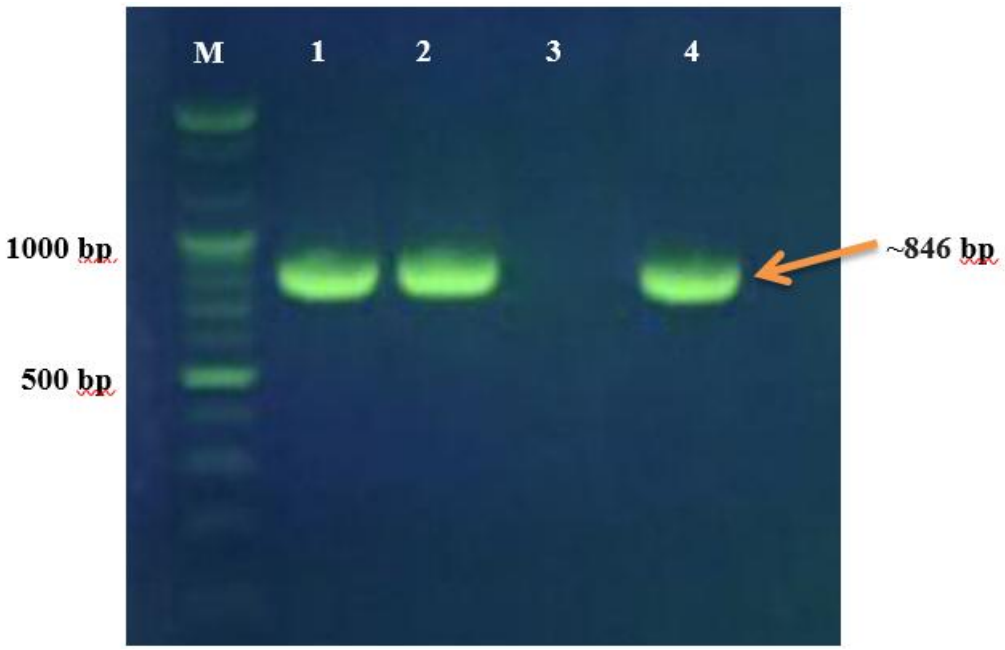

Figure 11. PCR amplification of ERG2 of C. kefyr isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm. Lane (M): DNA ladder marker; Lane (1-4): *C. kefyr* isolates

Ergosterol 3 (ERG3)

The results of gel electrophoresis for the ERG3 gene in C. kefyr isolates are shown in figure (12). 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.

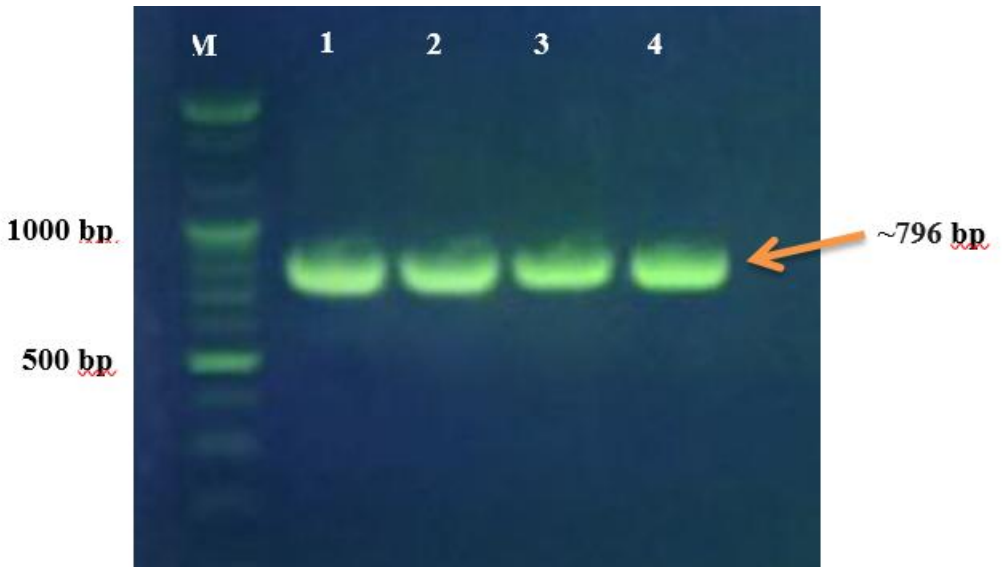

Figure 12. PCR amplification of ERG3 of *C. kefyr* isolates after electrophoresis on 1.5% agarose for 1hour at 50 V/cm. Lane (M): DNA ladder marker; Lane (1-4): *C. kefyr* isolates

Ergosterol 6 (ERG6)

PCR results of the ERG6 gene from the four isolates showed that the amplification product corresponded to the expected DNA fragment length (860 bp). The expression of the ERG6 gene is presented in figure (13).

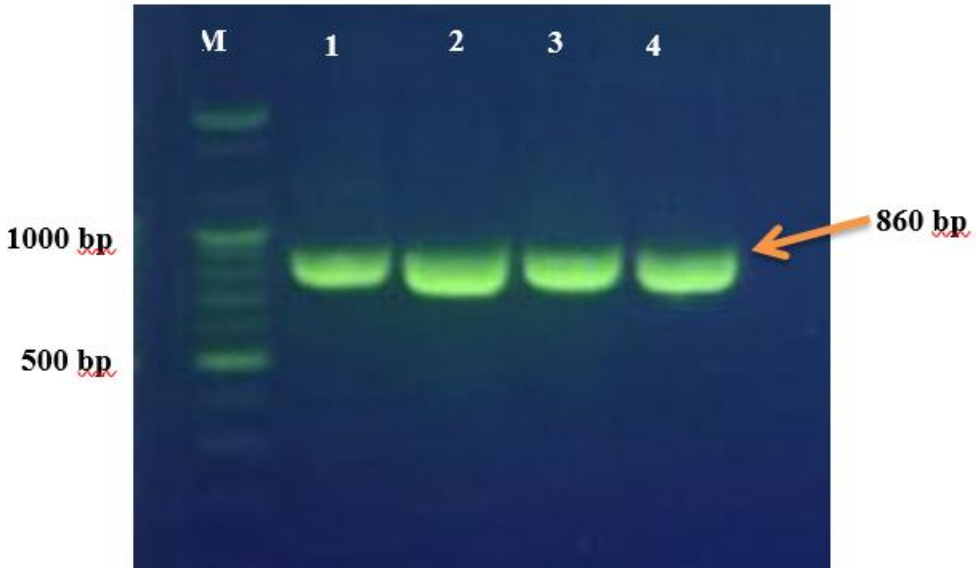

Figure 13. PCR amplification of ERG6 of C. kefyr isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm. Lane (M): DNA ladder marker; Lane (1-4): *C. kefyr* isolates

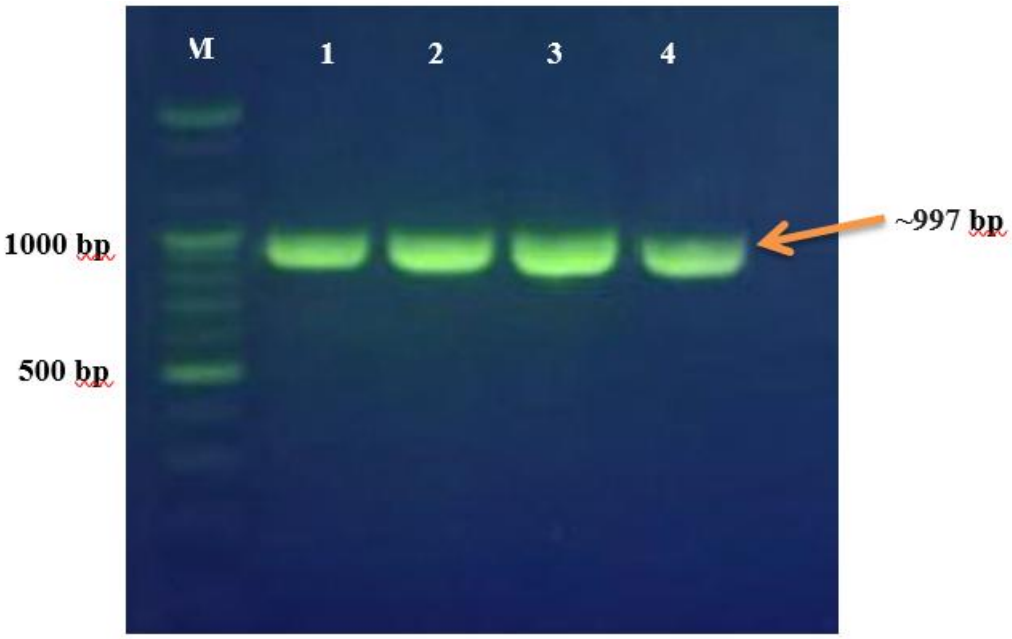

Figure 14. PCR amplification of ERG11 of C. kefyr isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm. Lane (M): DNA ladder marker; Lane (1-4): *C. kefyr* isolates

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Uracil phosphoribosyl transferase (FUR1)

Figure (15) demonstrates the expressions of the FUR1 gene in C. kefyr isolates. Only isolate number 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.

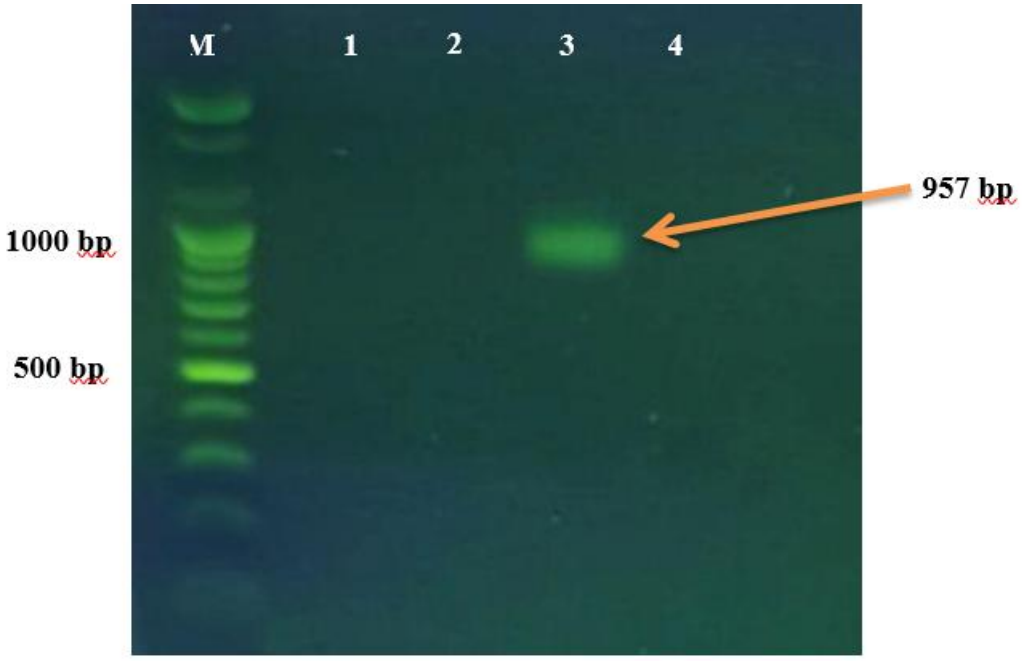

Figure 15. PCR amplification of FUR1Gene of *C. kefyr* isolates after electrophoresis on 1.5% agarose for 1 hour at 50 V/cm; Lane (M): DNA ladder marker; Lane (1-4): *C. kefyr* isolates

Discussion

Identification of Candida Species

Identification of C. auris

In this study, the infection with *C. auris* was 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 [30], but when various specimens were examined, an outbreak of *C. auris* was documented in a tertiary care hospital of Karachi, another city of Pakistan [31]. 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 [32]. 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 [33].

Identification of C. kefyr

In this study, regarding 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 [34]. 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 non-albicans Candida (NAC), in different geographical areas [35].

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 [36].

Each of the other two *C. kefyr* isolates was obtained from patient's 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 [37].

Antifungal susceptibility of *C. kefyr* isolates

Conventional methods

In the current study, concerning 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 the 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 investigations of Mishra et al. (2014), where all *C. kefyr* were uniformly resistant to amphotericin B in India [36]. 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; the result 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 [38]. 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 [39], ketoconazole [40], and itraconazole [36], respectively.

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Conversely to the described in the current study, studies of Dagi et al. (2016) in Turkey [39] 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 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 [35].

Molecular methods

Regarding the current results, PCR amplification revealed an increased expression in ERG2 gene in isolate number 4; the result is relatively in agreement with the findings of Hull et al. (2012a) report in Wales, whereas two isolates of *C. glabrata* revealed overexpression in ERG2 and displayed reduced sensitivity to amphotericin B (MIC 8 µg/ml) [41].

According to the findings of PCR products, the variation of four isolates' ERG3 gene was obvious; the result was in line with Carolus et al. (2021) experimental trial in Belgium, whilst increased expression in ERG3 had a role to decrease amphotericin B susceptibility against *C. auris* [42].

The amplified gene products also represented expression in RRG6 gene in four isolates; the result matches with the work achieved by Rybak et al. (2022) in United States, when they found an expression in the *C. auris* ERG6 gene to be associated with amphotericin B resistance, and this expression alone conferred a >32-fold increase in such resistance [43].

In support of the flow in results, the data of paper showed that ERG6 and ERG2 are major targets conferring resistance to amphotericin B in clinical *C. glabrata* isolates [44].

In the present study, it is clear that there are expressions in ERG11 gene in the four isolates after carrying out PCR. These findings are in accession with study of Sanglard et al. (2003) that revealed an ERG11 expression in *C. albicans* strain could be responsible for amphotericin B resistant mechanisms during antifungal therapy in Switzerland. This strain demonstrated increasing in the level of amphotericin B MIC [45].

Ergosterol content is involved in amphotericin B fungicidal effects [46]. 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 [42-45]. As noted in the present study, each of the three isolates (number 1, 2 and 3) had expressions 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 et al. (2023) found that ERG2, ERG3, ERG11, and ERG6 respectively are targets 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 ERG2 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; Hull et al. (2012b) revealed a clinical isolate of *C. glabrata*

was showing signs of cross-resistance to fluconazole, voriconazole, and amphotericin B in Wales [47].

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 spp. in France [48].

The summative conclusions are *C. auris* not detected but *C. kefyr* isolated and identified from the collected samples in a percentage of (1.9%). *C. kefyr* isolates were resistant to amphotericin B, while they were sensitive to clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, posaconazole, and voriconazole, except for one isolate that presented voriconazole resistance, in addition to 5-flucytosine resistance that was characterized by FUR1 gene expression. In other hand, ERG2, ERG3, ERG6, and ERG11, genes were detected in *C. kefyr* isolates.

In conclusion, *C. auris* was not detected but *C. kefyr* was isolated and identified from the collected samples in a percentage of (1.9%). *C. kefyr* isolates were resistant to amphotericin B, while they were sensitive to clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, posaconazole, and voriconazole, except for one isolate that presented voriconazole resistance, in addition to 5-flucytosine resistance that was characterized by FUR1 gene expression. In other hand, ERG2, ERG3, ERG6, and ERG11, genes were detected in *C. kefyr* isolates.

It is suggested that further studies are recommended to identify *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.

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Author contribution

Arjan: PhD student, collection of samples, application of conventional methods and writing the manuscript draft. Dr. Al-Attraqchi and Dr. Jasim supervised the work, edit and finalize the writing of the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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