

ARTICLE / INVESTIGACIÓN

Identification and Antifungal susceptibility of *Rhodotorula mucilaginosa* isolated from women patients in Erbil City-Iraq Kurdistan

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Abstract: *Rhodotorula* is emerging as an essential cause of nosocomial and opportunistic infections. This is the first study on Cervical-Vulvovaginal yeast infections aimed to find the prevalence of *Rhodotorula mucilaginosa* as the causative agent of Cervical-Vulvovaginal infection in women in Erbil City. For identification methods, API 20C AUX system, CHROMagar and Multiplex PCR amplification of universal primer confirmed by DNA sequencing using the ITS1 and ITS4 universal primer. Sequence data of *Rhodotorula mucilaginosa* provided GenBank accession number OQ568163 for nucleotide sequence. According to Congo red agar (CRA) and molecular method for amplifying the ALS1 and HWP1 genes, the isolates produced biofilms. Furthermore, evaluating the antifungal susceptibilities of *Rhodotorula mucilaginosa* by disc diffusion method revealed that the isolates were sensitive to all tested antifungal discs: Econazole (20mm), Ketoconazole (22mm), Nystatin (24mm), except Miconazole (9mm), which had a little effect on inhibition zone. Agar Well Diffusion Method showed that the isolates were resistant to all tested antifungal drugs: Griseofulvin (4mm), Itraconazole (0mm), Ketoconazole (6mm), and Nystatin (6mm), except Fluconazole (24mm), which affected inhibition zone.

Key words: *Rhodotorula mucilaginosa*, Econazole, Fluconazole, CHROM Agar, biofilm.

Introduction

In regular practice, vulvovaginitis is an issue that is frequently encountered. Vaginal discharge and discomfort are the typical first signs of it. To avoid missing the less prevalent causes, a comprehensive evaluation is necessary¹. *Rhodotorula* are common environmental yeasts once considered non-pathogenic. However, over the past three decades, various species of this yeast have established themselves in people as pathogens, leading to systemic illnesses in the immunocompromised N². Treatment of such novel infections requires knowledge of the *Rhodotorula* strains' susceptibility to commonly used antifungal medications³. The yeasts in the genus *Rhodotorula* are saprophytic. *R. mucilaginosa*, *R. glutinis*, and *R. minuta* are species of the genus, but none have yet been linked to human disease⁴. Recent research has identified the pigmented yeast *Rhodotorula* spp. as an emerging opportunistic pathogen that can colonize and infect immunocompromised patients and cause oral ulcers, aortic homograft endocarditis, meningitis, and peritonitis dermatitis⁵⁻⁷. Blood cultures can become contaminated, and *Rhodotorula mucilaginosa* may be on the skin. Due to the multiresistant profile, fungemia caused by *R. mucilaginosa* is a rare clinical entity that requires risk factors⁸. Regarding the epidemiology, risk factors, and consequences of *Rhodotorula*-related mycoses in people, very little scientific information is available^{9,10}. The majority of these systemic infections are treated using antifungal agents such as amphotericin B and azoles viz. ketoconazole, fluconazole etc¹¹. The objective of this study was to isolate and identify species of *Rhodotorula mucilaginosa* from cervical-vulvovaginal samples of women patients by using the conventional and molecular-based method and its iden-

tification was confirmed by DNA sequencing using universal primers, ITS1 and ITS4 and detection of biofilm virulence gene, ALS1 and HWP1, then determine the efficacy of different antifungal discs and drugs against it.

Materials and methods

Collection and Isolation of samples

Fifty strains were collected from Cervical-Vulvovaginal patients' samples, including signs and symptoms of VVC assessed during the gynecological exam at different hospitals. Prior to the test, the positive isolate samples were cultivated on Sabouraud's dextrose agar medium, with the addition of antibiotics to prevent the growth of bacteria. After 3–5 days of incubation at 37°C, cultures were separated into positive (yeast growth) or negative cultures (no yeast growth). Using this approach, we isolated and determined the number of yeast colonies¹².

Phenotypic identification of yeast

Microscopic examination

Each sample was stained with lactophenol cotton blue and inspected under a microscope (40X).

Chromogenic medium

(*CHROM Agar Candida*): After incubation (48hr at 37°C), all positive cultures were cultured on CHROM Agar (BioMérieux, France); yeast identification was performed

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based on colony color.

Biotyping identification

The yeast isolates were biochemically identified using API 20C AUX strips. A single colony from a young yeast culture obtained from an SDA plate was submerged in an API 20C suspension tube. The strips were incubated for (24, 48, and 72) hr at 37°C after being filled with the suspension from the API 20C medium tube in the cupules of the strip. Following incubation, cupule turbidity was measured recorded, and a profile number was created¹³.

Molecular identification via amplification of ITS region

DNA extraction and PCR amplification: The yeast isolates were grown on SDA at 37°C for 48 hours, and then DNA was extracted using a Genomic DNA isolation kit (Fungi/ Yeast Genomic DNA isolation kit. Norgen Biotek/Canada), depending on the manufacturer's instructions. The internal spacer region was amplified by using universal primers¹⁴. A total of 25µl PCR master mix reaction volume was performed containing 3 µl of genomic DNA, 12.5µl of 2X GoTaq-Green Master Mix (Promega/USA), and 1µl was added for each of the forward and reverse primer for both ITS1 and ITS4, forward (ITS1, F'5-TCC GTA GGT GAA CCT GCG G-'3), reverse (ITS4, R-5' TCC TCC GCT TAT TGA TAT GC-'3) primer then the mixture was completed by adding 7.5µl of nuclease-free water. The thermal cycles were programmed as follows: initial denaturation cycles at 95°C for 5 min., followed by 35 cycles at 94°C for 1 min., 55°C for 1min., 72°C for 1min. and a last extension at 72°C for 7 minutes. The 505bp of PCR products were confirmed by using 2% agarose gel electrophoresis in 1XTBE buffer, and PCR product of yeast isolates was sent to (Macrogen/South Korea) for sequencing.

Sanger Sequencing Technique for Fungal Strain Identification

Integrated DNA technologies/USA provided oligonucleotide primers for polymerase chain reaction. The internal transcribed spacer regions ITS1 and ITS4 were amplified using nonspecific primers. All Fungal isolates' targeted DNA was effectively amplified, with PCR amplicon sizes ranging from 500 to 800bp¹⁵.

Genetic analyzer 3500 (Macrogen/South Korea) was used to test the amplified amplicons of ITS isolates. The fasta file sequences were modified, aligned, and submitted to GeneBank to obtain an Accession number, after which they were compared to other reference strains from the different sequences.

Detection of biofilm formation by Congo red method (CRA)

It is an alternative method for biofilm formation described by Freeman *et al.*¹⁶. A positive result was indicated by black colonies with a dry crystalline consistency. A non-biofilm producer usually remains pink. The experiments were performed in triplicate and repeated three times.

Detection genes associated with biofilm formation

Amplification of ALS1 and HWP1 genes: PCR amplification was carried out by using ALS1 primers Forward (F'5-GAC TAG TGA ACC AAC AAA TAC CAG A-'3) and Reverse (R-5'CCT GAA GAA ACA GCA GGT GA-'3) and HWP1 primers Forward (F'5- ATG ACT CCA GCT GGT TC), Reverse (R-5'TAG ATC AAG AAT GCA GC-'3). A total of 25 PCR

master mix reaction volumes was performed containing 3µl of genomic DNA, 12.5 µl of 2X GoTaqGreen, Master Mix (Promega/ USA) and 1µl was added for each of the forward and reverse primer for both genes and volume completed with 5.5µl of DNase, RNase free water. The PCR was conducted as follows: 1 cycle of 94°C for 4min., followed by 35 cycles of 94°C for 30s, 52°C for 1min., and 72°C for 2min. A final extension cycle was performed at 72°C for 5 minutes. The PCR products were separated on 2% agarose. The 318bp and 572bp mean the amplification was successfully done for ALS1 and HWP1 genes¹⁷.

Antifungal susceptibility test

Antifungal disc by Disc Diffusion Method (DDM)

This is the current reference method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). The Kirby-Bauer disk diffusion method evaluated yeast isolates in vitro for antifungal drug susceptibility. The disks used in the testing were Biorex diagnostics microbiology sensitivity discs manufactured in the U.K., mainly Econazole50mg, Ketoconazole50mg, Miconazole50mg and Nystatin100mg. It was performed on SDA agar supplemented with 8% glucose.

Antifungal drugs by Agar Well Diffusion Method (AWDM)

The antifungal drugs, including Fluconazole, Griseofulvin, Itraconazole, Ketoconazole, and Nystatin, were tested against *Rhodotorula mucilaginosa*. The stock solution was prepared for each drug, except for Itraconazole, which was dissolved using DMSO; other medicines' stock solutions were made by dissolving them in SDW. The inoculum of *Rhodotorula mucilaginosa* was prepared using 48hr fresh yeast cultures grown on SDB, and they were adjusted to (1x10⁶/ml) with a bright line hemocytometer (Hausser Scientific, Horsham, Pa)¹⁸. Briefly, 0.1mL (100µL) suspension of each isolate was spread over SDA; a sterile cork borer was used to punch 6mm diameter wells in the culture media. Then, 100µL of each drug was poured into each well until complete. The treated plate was incubated at 37°C for 24 hours. The diameters of inhibition zones were measured in millimeters using a ruler for each antifungal drug^{19,20}.

Statistical Analysis

The distribution of the data was analyzed according to the Shapiro-Wilk and Kolmogorov-Smirnov normality test. Then, the data were analyzed according to a one-way analysis of variance (ANOVA) followed by multiple comparisons of Tukey's test. For other obtained data, an unpaired t-test was applied using GraphPad Prism software (version 9). P < 0.05 is considered statistically significant.

Results

Phenotypic identification

The identification of the samples isolated from cervical-vulvovaginal patients is phenotypically summarized in (Table 1). Nine non-duplicated *Rhodotorula mucilaginosa* isolates from Vulvovaginal candidiasis (VVC) patients in Erbil City-Iraqi Kurdistan. Identification was performed on SDA; the colony color glistening, smooth, mucoid, orange-pink colonies of *Rhodotorula mucilaginosa*. These iso-

lates failed to produce germ tube in human serum after 3 hours of incubation at 37°C. Identification was performed on CHROM Agar after incubation 48hr at 37°C based on colony color. By using this method, the color of *Rhodotorula mucilaginosa* has not changed.

performed on SDA agar; the ANOVA test showed significant differences in the sensitivity of *Rhodotorula mucilaginosa* against antifungal discs. It was reported as sensitive for each of the tested antifungal's discs: Econazole50mg (20mm), ketoconazole50mg (22mm) and Nystatin100mg (24mm),

Phenotypic method	
Microscopic examination	Positive (yeast)
Biotyping identification	<i>Rhodotorula</i>
CHROMagar	no change in color of colony

Table 1. Phenotypic characteristics of *Rhodotorula mucilaginosa* isolates.

Molecular identification (PCR and sequences): The result of the molecular identification, by using ITS1 and ITS4 gene Amplification, was consistent with the phenotypic analysis (Figure 1). Isolated *Rhodotorula* shared the identical ITS sequence, which differs from others in one position within the 35 bp ITS2 signature sequence. The amplification products were submitted to sequencing analysis and showed 99% to 100% sequence identity with the *Rhodotorula* strains. Sequence data of *Rhodotorula* provided GenBank accession number OQ568163 for nucleotide sequence.

while resistance for Miconazole50mg (9mm), which had a little effect on inhibition zone. The zone of inhibition showing the interpretive categories by the disk diffusion technique is presented in (Table 3, Figures 3 and 4). For Antifungal drugs, the ANOVA test showed significant differences in the sensitivity of *Rhodotorula mucilaginosa*, including Fluconazole, Griseofulvin, Itraconazole, Ketoconazole, and Nystatin. It revealed that all isolates were resistant to Griseofulvin (4mm), Itraconazole (0mm), Ketoconazole (6mm), and Nystatin (6mm), which did not affect inhibition; nevertheless, tested isolates were sensitive to Fluconazole (24mm) which affected inhibition zone (Table4, Figure5 and 6).

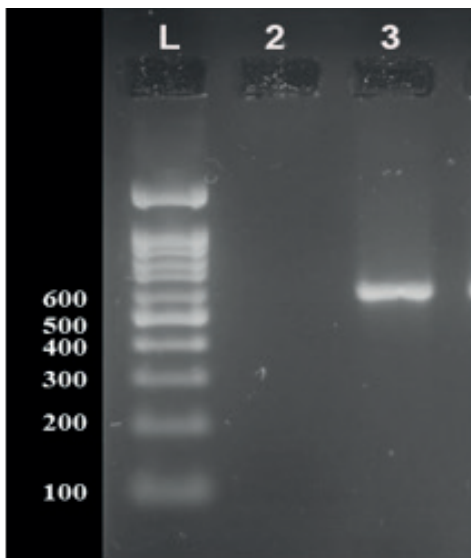


Figure 1. PCR amplification of Internal Transcribed spacer region. Lane L: DNA ladder 1kb, Lane 2: negative control 3: Correspond to Fungal ITS region amplification.

Biofilm formation

In this study, Congo red Agar was used for the detection of the ability of *Rhodotorula mucilaginosa* to form biofilm. It was revealed that all *Rhodotorula mucilaginosa*, isolated from women patients with cervical-vulvovaginitis were biofilm producers.

Detection of ALS1 and HWP1 genes associated with biofilm formation: The prevalence of the studied virulence genes in *Rhodotorula mucilaginosa* isolates was HWP1 and ALS1 using PCR. The gene HWP1 was found, but ALS1 was not found in *Rhodotorula mucilaginosa*. In the present study, biofilm formation was evaluated to see the reflection of gene presence on phenotype (Table 2, Figure 2).

Susceptibility testing of antifungal drugs

In this study, all the isolates of *Rhodotorula mucilaginosa* were studied against antifungal disc and drugs based on the zone of inhibition of growth. Disk diffusion test was

Discussion

An understanding of the role of yeasts in the environment has been uncertain because population size and diversity estimates have often been based on species iden-

Phenotype test	
CRA	+
Molecular test	
ALS1 gene	-
HWP1 gene	+

Table 2. Phenotypic and molecular test of *Rhodotorula mucilaginosa* for biofilm formation.

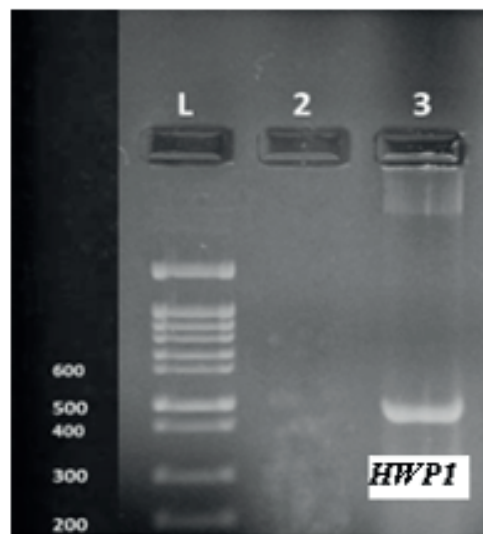


Figure 2. Agarose gel electrophoresis of ALS1 and HWP1 gene amplification (PCR products) for biofilm formation *Rhodotorula mucilaginosa*.

Antifungal disc			
Diameter of inhibition zone(mm)			
Econazole	Ketoconazole	Miconazole	Nystatin
20mm	22mm	9mm	24mm

Table 3. Susceptibility testing of *Rhodotorula mucilaginosa* isolates against the antifungal disc.

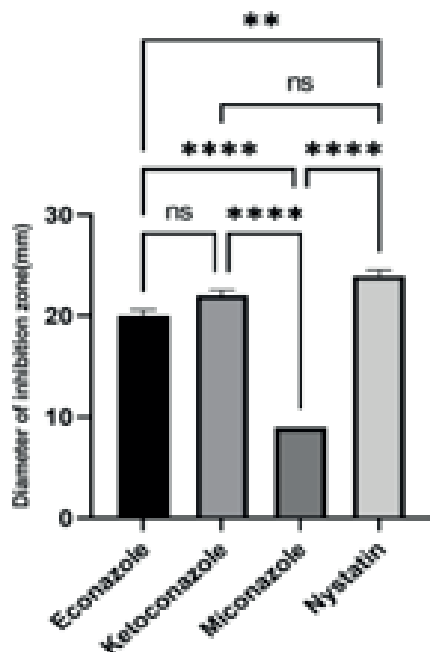


Figure 3. Quantitative measurement of inhibition zone for *Rhodotorula mucilaginosa* to antifungal discs by DDM. Data are presented as mean \pm SE of three biological replicas. All data were significant at $P < 0.0001$ (***)



Figure 4. Disc diffusion method with antifungal azoles: Econazole, Ketoconazole, Miconazole and Nystatin.

Antifungal disc				
Diameter of inhibition zone(mm)				
Fluconazole	Griseofulvin	Itraconazole	Ketoconazole	Nystatin
24mm	4mm	0mm	6mm	6mm

Table 4. Susceptibility testing of *Rhodotorula mucilaginosa* isolates against antifungal disc.

tifications that were determined from a limited number of phenotypic characteristics. The widespread use of DNA-based species identification now enables a precise evaluation of species in various settings. However, because specific taxa are polyphyletic, there are still classification issues. As a result, it is still challenging to identify yeasts, estimate their genus-level diversity, and assign genera to higher taxonomic orders²¹.

Historically, *Rhodotorula* species were regarded as contaminants, but during the past two decades, they have gradually come to be understood as human infections. Although less virulent than *Trichosporon* or *Candida*, reports have revealed that *Rhodotorula* can produce severe, sometimes deadly, invasive infections²².

Almeida *et al.*, described 25 cases of *Rhodotorula* spp. Isolated from blood cultures at a Brazilian tertiary teaching hospital and investigated the *in vitro* activity of four antifungal drugs using a standardized method. All strains were identified as *R. mucilaginosa* by conventional methods. Misidentification of the species was observed with the Vitek

Yeast Biochemical Card and API 20C AUX systems; Fluconazole demonstrated good activity high for all strains²³.

Rhodotorula mucilaginosa yeasts can be challenging to distinguish phenotypically²⁴. In order to accurately identify these opportunistic fungal infections for surveillance, molecular screening is essential. It is imperative to conduct more research to understand the mechanisms of *R. mucilaginosa* infection, mycoparasitism in the human oral cavity, and the numerous underlying factors that may raise the risk of angular cheilitis²⁵.

Rhodotorula infections are uncommon, but given the expected evolution of the virulence factors, it is likely that *Rhodotorula* infections will become more common in the future, as shown by the increasing number of case reports in the microbiological literature^{26,27}.

Using the disk diffusion technique, the antifungal drugs ketoconazole, nystatin, and Fluconazole were evaluated in *Rhodotorula*, which showed sensitivity to ketoconazole and nystatin. No antifungal sensitivity to Fluconazole has been reported (2). In homemade fermented rice water, *Rhodo-*

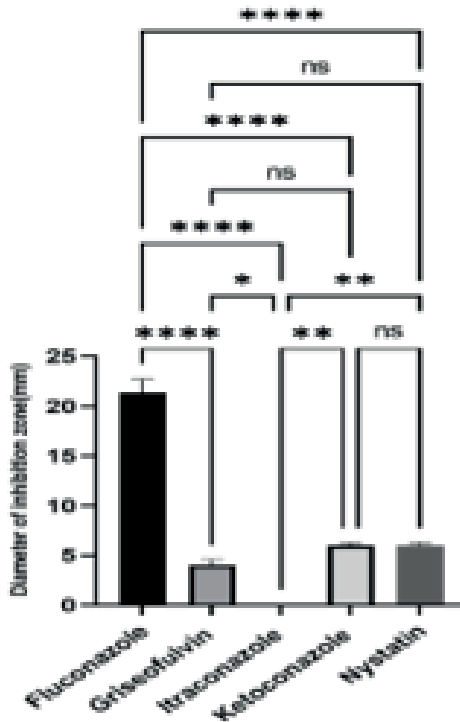


Figure 5. Quantitative measurement of inhibition zone for *Rhodotorula mucilaginosa* to antifungal drugs by AWD. Data are presented as mean \pm SE of three biological replicates. All data were significant at $P < 0.0001$ (****).

torula sp. was discovered utilizing phenotypic techniques, such as biochemical traits using API ID 32C and molecular techniques²⁸.

Turchetti *et al.* isolated 23 yeast strains that could not be assigned to any known fungal taxa during ecological studies of yeast communities carried out in cold ecosystems in the Italian Alps, Svalbard (Norway, Arctic region), and Portugal; in particular, two of them were first identified as *Rhodotorula* sp.²⁹.

Conclusions

This is the first identification of *Rhodotorula mucilaginosa* from cervical-vulvovaginal candidiasis of women patients in Erbil city, Iraqi Kurdistan. A conventional sequencing technique was used to identify the *Rhodotorula mucilaginosa*, which was a reliable, rapid, and cost-effective technique. Biofilm production and antifungal drug resistance were observed in *Rhodotorula mucilaginosa*. Biofilm production could act as one factor in reducing the antifungal agent's penetrability. The biofilm virulence gene ALS1 was undetected, while HWP1 was detected in isolated strains. Funding: We relied on ourselves in all aspects, as no research funding sources were reported. Informed Consent Statement: The patient's consent was made in writing through the term of informed consent after the human ethics committee of the University of Salahaddin-Erbil approved this study. Inclusion criteria were vaginal swabs from women with cervical-vulvovaginal candidiasis who tended to participate in the study. All procedures performed in this study involving women participants followed the rules on patient sampling and fully complied with the ethics committee.

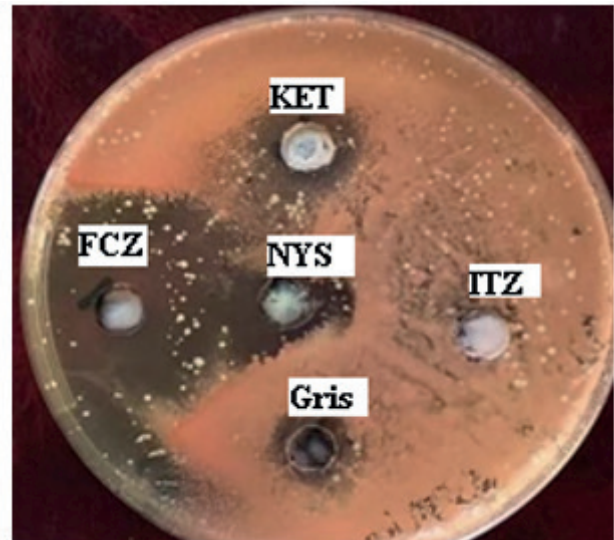


Figure 6. Agar diffusion method with antifungal azoles: FCZ, Ketoconazole KET, and Nystatin NYS Gris, ITZ.

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Conflicts of Interest

The authors declare no conflict of interest.

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