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The First Isolation and Diagnosis of *Cytobasidium slooffiae* from Women Suffering from Vulvovaginitis in the City of Erbil, Kurdistan Region, Iraq

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ABSTRACT

Vaginitis is still a health concern for women. The vagina is susceptible to trichomoniasis, bacterial vaginosis, and yeast infections due to its squamous epithelium. This is an original research project on the isolation of *Cystobasidium slooffiae*, from patients with cervical vulvovaginal infection in Erbil hospitals. In order to determine the frequency of *C. slooffiae* as the main reason of vaginal-cervical infection in women of reproductive age in Erbil city, this innovative study set out to identify it. By employing the universal primers *ITS1* and *ITS4*, DNA sequencing verified the phenotypic and molecular identification techniques. The study also contains a test for the production of biofilms using Congo red agar (CRA), along with the discovery of the biofilm virulence genes; *ALS1* and *HWP1*. Moreover, using the disc diffusion method to assess *C. slooffiae's* antifungal susceptibilities. The identification of *Cystobasidium* isolates were tested phenotypically. The positive cultures include: non-Cystobasidium species, and *C. slooffiae*. Nucleotide sequence information for *C. slooffiae* is available under GenBank accession number OQ568166. The results of the ANOVA test revealed significantly variations in the level of sensitivity of *C. slooffiae* in opposition to antifungal discs, it was noted to be sensitive to every antifungal disc that was tested: Econazole (24mm) and ketoconazole (20mm), while resistance for each of Miconazole (8mm) and Nystatin (12mm). *C. slooffiae* was identified using a traditional and sequencing strategy, which seems to be a dependable, quick, and economical method. Not creating a biofilm and their sensitivity to different antifungal drugs were observed in *C. slooffiae*.

Keywords: Cervical-vulvovaginal infection, biofilms, *Cystobasidium slooffiae*.

INTRODUCTION

The effects of fungi on agriculture, biodiversity, and world health are significant, and they are essential to biogeochemical cycles and the manufacturing and biomedical research sectors (Sun *et al*., 2020). Pathogenic fungi can enter the body by skin contact, inhalation, translocation over physical barriers, host defects, or unintentional or intentional disruption of epithelial integrity. They can cause a wide range of infections, from bothersome to potentially fatal. Some of these agents are also components of normal human flora or may be present in the absence of clinical symptoms (Calderone and Cihlar, 2002).

Due to serious complications of diseases like cervix erosion and/or ulceration, ectopic pregnancy, HIV transmission, cancer, infertility, perinatal morbidity, related pathologies with obstetric infections, abortions, salpingitis, pelvic inflammatory disease and endometritis, the genital infections are among the diseases that most frequently cause health decline and years of productive life (Carr *et al*., 2005; Fosch *et al*., 2006; Angotti *et al*., 2007).

Vaginitis, sometimes called vulvovaginitis occasionally, is a medical word for vulva and vaginal irritation. Discharge, burning, discomfort, itching, and an unpleasant smell are a few signs. Certain types of vaginitis might cause problems during pregnancy, specifically trichomoniasis, vaginal yeast infection, and bacterial vaginosis. Low estrogen levels during or after menopause or during nursing, as well as chemical sensitivity and intolerance to substances like soaps and spermicides, are additional risks. There may be several reasons present at once. With age, the typical causes alter. Prepubescent females are commonly at risk for getting vulvovaginitis because of low estrogen levels and undeveloped labia minora. Steps in the diagnosing procedure frequently include physical examination, pH monitoring, and discharge culturing. It is important to rule out other potential sources of symptoms, including cervical inflammation, pelvic inflammatory disease, foreign substances, malignancy, and skin diseases (Ferri, 2016; Beyitler and Kavukcu, 2017; Romano, 2020).

Cystobasidium (Langerheim) Neuhoff (1924) was described as a genus of mycoparasites of *Coprophilous ascomycetes*. The genus includes simple pored, auricularioid, and dimorphic basidiomycetous yeasts that are members of the family Cystobasidiaceae, order Cystobasidiales, class Cystobasidiomycetes, subphylum Pucciniomycotina and phylum Basidiomycota. A sexual stage is found on the fungal host growing on animal dung (Sampaio and Oberwinkler, 2011). When this yeast is cultured on mycological agars, it produces smooth, soft, light orange to pinkish colonies. There are now 21 species of *Cystobasidium* described, including: *C. sloofae*, *C. minutum*, *C. fmetarium*, *C. pinicola*, *C. calyptogenae*, *C. benthicum*, *C. laryngis*, *C. lysinophilum*, *C. pallidum*, *C. alpinum*, *C. portillonensis*, *C. psychroaquaticum*, *C. oligophagum*, *C. ongulense*, *C. rietchieii*, *C. tubakii*, *C. halotolerans*, *C. keelungensis*, *C. terricola C. rafnophilum* and *C. iriomotense*. *Cystobasidium* yeasts have been found in a variety of habitats and ecologies, including supraglacial deposits, aquatic settings, soil, and phylloplanes (Yurkov *et al*., 2015; Turchetti *et al.*, 2018; Chang *et al.*, 2019). The existence of this strain raises questions about the cleanliness and sanitary maintenance of industrial facilities, particularly in hard-to-reach regions, and possible hygiene violations by staff members. Additionally, it implies heavily contaminated raw materials (Bazhenova *et al*., 2021).

This research aimed to isolate *C. slooffiae* from cervical-vulvovaginal infection patients in Erbil hospitals, using conventional and molecular-based methods and DNA sequencing was used to confirm its identity. Additionally, to detect biofilm virulence genes then assess *C. slooffiae*'s antifungal susceptibility profiles. It is the first study of *C. slooffiae* in Cervical-vulvovaginal samples in Erbil-Iraqi Kurdistan.

MATERIAL AND METHODS

Collection and isolation of samples

This study was accepted by the Ethics Board of Salahaddin University-College of Science-Biology Department. The study covered the period from March to July 2022. One hundred samples

from cervical-vulvovaginal infection patients (married women), between the ages of 19 to 68 years were taken. As described by obstetrics-gynecology specialists working at various hospitals in Erbil, Iraqi Kurdistan, including: Balsam hospital, CMC hospital, Hawler hospital Maternity Teaching hospital, Shar hospital and Zhin hospital. The patient including those who had leucorrhea, pruritus, vulvovaginal erythema and edema, as well as those who had whitish plaques in the mucosa. By completing the clarified informed consent form, the women gave their permission to engage in the procedure, which included using a sterile swab to obtain a sample of cervical-vaginal mucosa during the gynecological exam. In order to stop bacteria from growing, antibiotics were added to Sabouraud dextrose agar (SDA) medium where the isolate samples were cultivated. The cultures were divided into positive (yeast growth) and negative (no yeast growth) groups after 48 hours of incubation at 37°C (Babic and Hukic, 2010).

Identification of yeast

Phenotypic identification

Microscopic examination:

Before being viewed at a 40X magnification, each sample was stained with lactophenol cotton blue.

Germ tube test:

A small amount of a pure yeast colony was added to sterile test tubes together with 0.5 mL of human serum, and the yeast was then injected. To perform the germ tube test, the samples were incubated for three hours at 37°C (Kim *et al*., 2010).

Chromogenic medium:

After 48 hours of incubation at 37°C, all positive cultures were grown on CHROM Agar (BioMérieux, France). The colour of the colonies must be determined for the purpose of distinguishing between the species being studied (Baradkar *et al.*, 2010).

ITS region amplification for molecular identification:

DNA extraction and PCR amplification: Following the manufacturer's instructions, the yeast isolates were cultivated on SDA at 37ºC for 48 hours, and DNA was then extracted using a genomic DNA isolation kit (Fungi/Yeast Genomic DNA isolation kit. Norgen Biotek /Canada). Universal primers were used to amplify the internal spacer region. The total of 25 µl PCR master mix reaction volume was performed containing 3 µl of genomic DNA, 12.5 µl of 2 X GoTaq Green Master Mix (Promega/USA), and 1 µl 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 7.5 µl of nuclease free water.

The following was the programming for the thermal cycles: An initial denaturation cycle 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72 °C for 1 min and a last extension at 72°C for 7 min. The 505 bp of polymerase chain reaction (PCR) products were confirmed by using agarose gel (2%) electrophoresis in 1XTBE buffer and for sequencing, PCR product of yeast isolates was sent to (Macrogen/ South Korea) (Jafari *et al*., 2017).

Sanger sequencing technique for fungal strain identification:

The PCR oligonucleotide primers were supplied by Integrated DNA technologies/USA. The internal transcribed spacer regions *ITS1* and *ITS4* were amplified using non-specific primers. Targeted DNA from fungal isolate was successfully amplified using PCR, with amplicon sizes ranging from 500 to 800 bp. Tests on the amplified amplicons of the *ITS* isolates were performed using the genetic analyzer 3500 (Macrogen/ South Korea). After being altered, aligned and submitted to obtain an accession number from GenBank, the fasta file sequences were then contrasted with other reference strains from different sequencing (White *et al.*, 1999).

Phylogenetic analysis should be implemented. *ITS* sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) following BLASTN searches with sequences (http://www.ncbi.nlm.nih.gov/Blast.cgi). In the phylogenetic tree, only sequences having 100%

similarity with query sequences were included. A test was performed by MEGA 11 to determine the proper model for the phylogenetic analysis and the model with the lowest BIC score, Tamura 3 parameter. MEGA 11 was used to run a test to identify which model should be used for the phylogenetic study, and the model with the lowest BIC score, tamura 3 parameter, was chosen.

Based on linked *ITS* rDNA sequences, a phylogenetic tree of *Cystobasidium slooffiae* was generated using the maximum likelihood technique. The *ITS1* and *ITS4* genes were sequenced to describe fungal cells isolated from vulvovaginal swab in this study. These sequences were compared to those from other countries.

Detection of biofilm development by Congo red method (CRA):

It is an alternative approach for biofilm development given by Freeman and his colleagues. A dry, crystalline black colony was an indication of success. A non-biofilm producer typically doesn't change color. Three times the experiments were conducted (Freeman *et al*., 1989).

Detection genes linked to the biofilm development:

***ALS1* and *HWP1* genes amplification:**

Use of *ALS1* primers was made during the PCR amplification process, forward (F´5-GAC TAG TGA ACC AAC AAA TAC CAG A -´3) and Reverse (R-5´CCA 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). 12.5 µl of 2 X GoTaq green, master mix (Promega/ USA), 3 µl of genomic DNA, and 1µl of each forward and reverse primer for both genes, and 5.5 µl of DNase and RNase free water made up the entire 25 µl of the PCR master mix reaction volume. The procedure for the PCR was as follows: 1cycle of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 52°C for 1 min, and 72°C for 2 min. A last round of extension was carried out at 72°C for 5min on 2% agarose, the PCR products were separated. The amplification of the *ALS1* and *HWP1*genes was successful, as indicated by the 318 bp and 572 bp results (Inci *et al*., 2013).

Test for antifungal susceptibility

Up to the present time, the national committee for clinical laboratory standards (NCCLS) recommends using Kirby-Bauer Disc diffusion method as the current technique. The susceptibility of yeast isolates to antifungal medications were assessed *in vitro* using this method. Biorex diagnostics microbiology sensitivity discs, mostly produced in the UK, were the disks used in the testing and included the following: Ketoconazole 50 mg, Econazole 50 mg, Nystatin100 mg and Miconazole 50 mg. It was carried out using SDA agar that had eight percent glucose added to it (Othman *et al*., 2018).

Statistical analysis

According to the Shapiro-Wilk and Kolmogorov- Smirnov normality tests, the data distribution was analyzed, one-way analysis of variance (ANOVA) was used to analyze the data, and graphpad prism software (version9) was used for this purpose. P-value < 0.05 considered statistically significant, ANOVA was used for multi-group comparisons in the experiment related to the impact of antifungal discs (Ghoodjani, 2019).

RESULTS

Phenotypic identification:

In the current study, the identification of *Cystobasidium* isolates among 100 Cervical-vulvovaginal patients (CVV) patients (married women) in Erbil city, Iraqi Kurdistan were tested phenotypically as summarized in (Table 1). The negative cultures were 59(59%), while the positive cultures were 41(41%), includes: Non-*Cystobasidium* species were 37(37%), and *Cystobasidium slooffiae* were 4(4%). On SDA, identification was done, the colony color of *C. slooffiae* was light orange to pinkish colonies. After three hours at 37°C, in human serum, these isolates were unable to produce germ tubes. Based on colony color, identification was carried out on CHROM Agar at 37°C after 48 hours. The colony color of *Cystobasidium* is not changed by this procedure.

Table 1: Phenotypic identification of the isolates

Age	Isolates	No.	%	ME	GTT	CHROM agar
19-55	No growth	59	59	-------	-------	-------
20-43	Non-*Cystobasidium* spp.	37	37	+(yeast)	-and+	Change in colony color
23-68	*C. slooffiae*	4	4	+(yeast)	-	No change in colony color

No.: Number, ME: Microscopic examination, GTT: Germ Tube Test.

Molecular identification (PCR and sequences):

Cystobasidium isolates were submitted to multiplex-PCR gene amplification with universal primers for molecular identification. Accurate characterization and identification of isolates were made possible by molecular data of many specimens using *ITS* nucleotide sequences Fig. (1).

There was agreement between the results of the molecular identification using *ITS1* and *ITS4* gene amplification and the phenotypic research. *Cystobasidium* that was isolated had the same *ITS* sequence. After being subjected to sequencing analysis, the amplification products revealed 99% to 100% sequence identity with the strains of *C. slooffiae*. Nucleotide sequence information for *C. slooffiae* is available through GenBank accession number OQ568166.

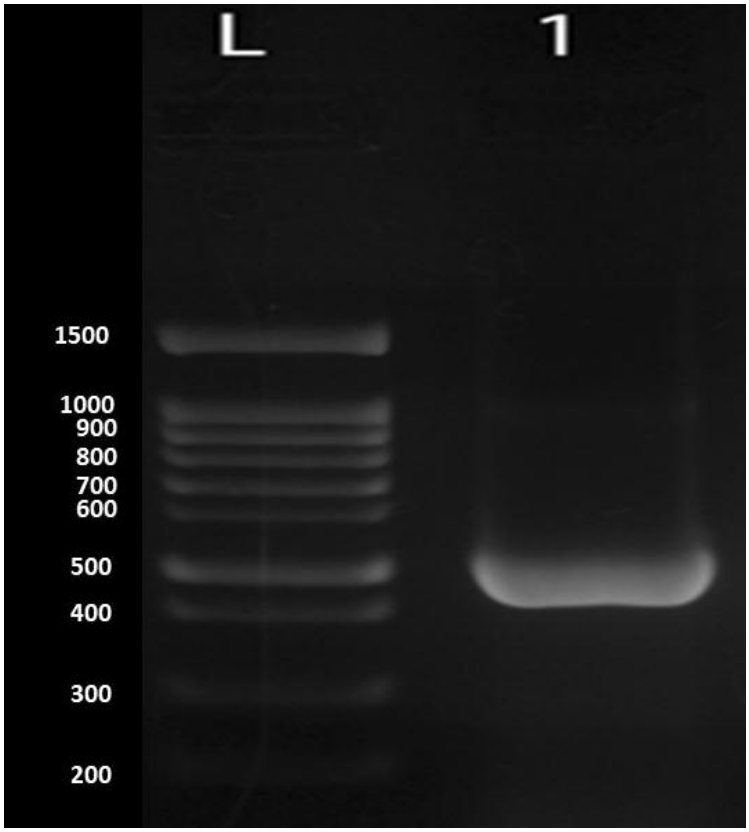

Fig. 1: PCR amplification of adjacent segments of the nuclear small subunit rDNA gene. Lane L: DNA ladder 100 bp, Lane 1: Correspond to Fungal ITS region amplification (500bp).

The phylogenetic tree revealed that the isolate consists of one group and one out group. The majority of the species belong to one group. Based on the alignment of current and global reference sequences the accession number of *C. slooffiae* OQ568166, was closely related to another *C. slooffiae* found in China: MN075256, MK386939, USA: MK267900, MK990671, AF444589, Canada: MW895734, MW895055, MW895735, Italy: MN848423, New Zealand: KT819329 and South Africa: JQ993376, while it was no closely relationship with the out-group fungal sequence like *C. Africana*: MZ770755 that found in Iraq Fig. (2).

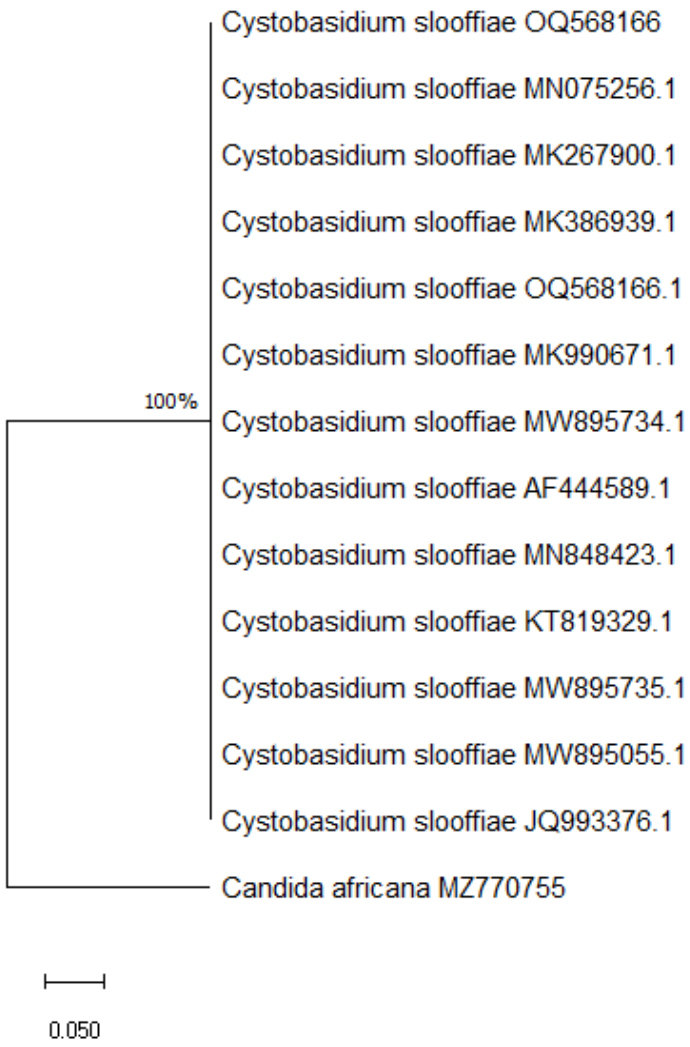

Fig. 2: Phyloge using primers (*ITS1*, *ITS4*) showing relationship between representative strains along with related sequences retrieved from GenBank. The numbers at the nods indicate the level of bootstrap support (%) base on maximum likelihood analysis of 100 re-sample datasets. The scale bar indicates the phylogenetic distance corresponding to 0.050 changes per 100 bases.

Biofilm formation:

Each Congo red agar plate was used in this study to test *Cystobasidium*'s capacity to build biofilms. It was discovered that none of the *Cystobasidium* isolated from cervical-vulvovaginitis patients in women produced biofilm.

Identification of the genes *HWP1* and *ALS1* linked to the production of biofilms

HWP1 and *ALS1* were the most often found virulence genes in *Cystobasidium* isolates, according to PCR analysis. These genes were not found in *Cystobasidium*, it was verified that Congo red agar (CRA) did not reveal the formation of biofilms. To see how the absence of a gene reflected in phenotypic, biofilm formation was not assessed in this study.

Susceptibility testing of antifungal drugs

Based on the zone of growth inhibition, all *Cystobasidium* isolates were tested in this investigation against antifungal medications. Disk diffusion test on SDA agar with 8% glucose addition: The ANOVA test revealed a substantial variation in *Cystobasidium slooffiae* susceptibility

to antifungal discs. It was found to be sensitive to all tested antifungal discs, including ketoconazole 50 mg (20 mm) and econazole 50 mg (24 mm), while resistance for each of miconazole 50 mg (8 mm) and nystatin100mg (12 mm). According to the disk diffusion approach, the zone of inhibition displaying the interpretative categories is shown in (Fig. 3 and 4).

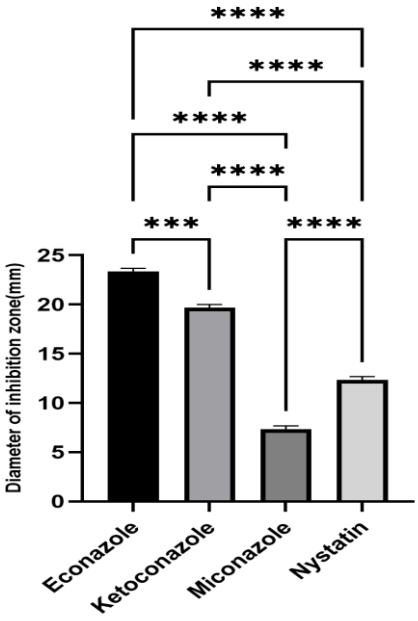

Fig. 3: Quantitative measurement of inhibition zone for *Cystobasidium slooffiae* to antifungal discs by DDM. Data are presented as mean ±SE of three biological replicas. All data were significant at P-value < 0.0001(***).

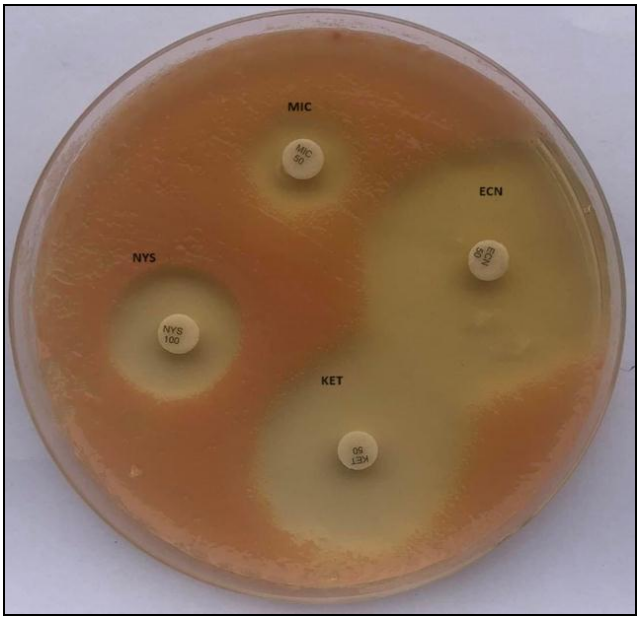

Fig. 4: Disc diffusion method with antifungal azoles: ECN: Econazole, KET: Ketoconazole, MIC: Miconazole and NYS: Nystatin.

DISCUSSION

In the present work, *Cystobasidium* isolates were identified by SDA, the colonies' colors ranged from light orange to pink. Generally, torularhodin, a carotenoid pigment, is produced when

Cystobasidium spp. colonies are grown on SDA, resulting in pink to orange colonies (Deepa *et al*., 2014; Chreptowicz *et al*., 2021). Phenotypic methods were performed, such as identification by microscopic examination and cultivation the positive cultures of isolates on CHROM agar (AL Zubaidy and Khidhr, 2014; Faqeabdulla and Ismael, 2022).

In our investigation, multiplex-PCR with universal primer gene amplification was used to identify isolated *Cystobasidium* molecularly. Accurate characterization and identification of isolates were made possible by molecular data from many specimens from *ITS* nucleotide sequences. Because species identifications based on a limited set of morphological features have often been used to estimate population size and diversity, it has been difficult to grasp the significance of yeasts in the environment. A precise assessment of species in different environments is now possible thanks to the extensive use of DNA-based species identification. Nevertheless, classification problems persist because certain taxa are polyphyletic. Consequently, the identification of yeasts, the estimation of their genus-level diversity, and the assignment of genera to higher taxonomic orders remain difficult (Takashima *et al.*, 2012). Since *Cystobasidium* shares many characteristics with the newly developing opportunistic pathogen *Rhodotorula*, surveillance by molecular identification in clinical settings is crucial for accurately diagnosing and treating rare yeast infections (Karajacob *et al*., 2022).

Cystobasidium's capacity to proliferate at 37°C shows that it has the pathogenicity to infect human hosts. Due to the difficulty in phenotypically differentiating *Cystobasidium* yeasts, molecular screening is required to accurately identify these opportunistic fungal infections. Further research is necessary to comprehend the mechanisms underlying mycoparasitism and *Cystobasidium* infection in the human oral cavity, as well as the various underlying conditions that may increase the risk of angular cheilitis (Libkind and Sampaio, 2010; Federico *et al*., 2023). Several authors were isolated *C. slooffiae* from the Arabian Gulf surrounding Qatar. The strains have identical sequences in the D1/D2 domains of the large subunit and the internal transcribed spacer regions of the rRNA gene (Fotedar *et al*., 2019). Other researchers, showed that an accurate identification of *C. slooffiae* was performed based on the analysis of the ribosomal gene sequence obtained by sequencing the DNA region encoding the ITS-D1/D2 rDNA region. The detection of this strain indicates violations of the hygiene rules by personnel and indicates the high contamination of raw materials (Bazhenova *et al*., 2021). Authors, isolated and identified *C. slooffiae* by molecular method from the tubeworm *Lamellibrachia* sp. and the giant white clam *Calyptogena* sp. collected from the deep-sea floor of the Pacific Ocean off Japan (Nagahama *et al*., 2003). While other researchers diagnosed two novel species from natural environments described as *C. Rietchiei* and *C. Psychroaquaticum*. The new species were registered in Myco Bank under MB 809337 and MB 809336, respectively (Yurkov *et al.*, 2015).

C. Calyptogenae was isolated from human oral samples by some researchers, the fact that *C. calyptogenae* is the sole yeast species isolated from the patient's oral samples and that it can grow at 37°C shows that it has pathogenic potential and may be connected to angular cheilitis. It is thought that immunocompromised persons are more likely to get opportunistic infections due to the *Cystobasidium* yeast's widespread distribution (Karajacob *et al*., 2022). Others, identified four strains of the genus *Cystobasidium* from soil samples obtained on east ongul island, East Antarctica, based on sequence similarity of the ITS region and the LSU D1/D2 domain (Tsuji *et al*., 2017). Others identified yeast species present in homemade fermented rice water uses a combination of phenotypic and molecular methods. The large subunit rDNA gene's D1/D2 domain and the yeast genomic DNA's ITS1/ITS2 sections were then amplified using PCR. The yeast species were identified in the fermented rice water, including *Cystobasidium calyptogenae, Candida* spp., *Meyerozyma caribbica* and *Rhodotorula toruloides* (Wongwigkarn *et al*., 2020). Another isolated various yeast strains that could not be assigned to any known fungal taxa during ecological studies of yeast communities conducted in cold ecosystems in the Italian Alps, Svalbard and Portugal, two of them were initially identified as *Rhodotorula* sp. and *Cystobasidium larynges* (Turchetti *et al.*,

2018). In our study, *C. slooffiae* displayed a variety of resistance to the tested antifungals, this result is comparable to other studies (FaqeAbdulla, 2023; FaqeAbdulla and Ismael, 2023).

CONCLUSIONS

Cervical vulvovaginal yeast infection is prevalent among women in Erbil province. For recognizing isolated *Cystobasidium*, phenotypic methods are available, including morphological culture tests and CHROMagar medium. Recent molecular techniques, which are mostly used for epidemiological research, are added to these methods. This is novel study conducted in in Erbil/Kurdistan and in Iraq for identification of *C. slooffiae* as a causative agent of Cervical-vulvovaginal infection among women patients. The *ALS1* and *HWP1* biofilm virulence gene were not detected in isolated strains, which may explain the antifungal drugs sensitivity was commonly seen with different antifungal agent.

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العزل والتشخيص الاولى لخميرة *Cystobasidium slooffiae* من النساء المصابات بالتهاب الفرج والمهبل في مدينة أربيل، إقليم كردستان، العراق

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الملخص

لا يزال التهاب المهبل يشكل مصدر قلق صحي للنساء. المهبل عرضة للإصابة بمرض المشعرات والتهاب المهبل البكتيري وعدوى الخميرة بسبب ظهارته الحرشفية. هذا مشروع بحثي أصلي حول عزل *Cystobasidium slooffiae* من المرضى الذين يعانون من التهاب فرجي مهبلي في عنق الرحم في مستشفيات أربيل. من أجل تحديد *C. slooffiae* باعتباره السبب الرئيسي للعدوى المهبلية وعنق الرحم لدى النساء في سن الإنجاب في مدينة أربيل، شرعت هذه الدراسة المبتكرة في التعرف عليه من خلال استخدام البادئات العالمية *ITS1* و*ITS4*، تم التحقق من تسلسل الحمض النووي من تقنيات تحديد النمط الظاهري والجزيئي. كما تحتوي الدراسة على اختبار لإنتاج الأغشية الحيوية باستخدام أجار الكونغو الأحمر (CRA)، إلى جانب اكتشاف جينات فوعة الأغشية الحيوية؛ *ALS1* و*HWP1*. علاوة على ذلك، باستخدام طريقة انتشار القرص لتقييم حساسية *C. slooffiae* المضادة للفطريات. تم اختبار تحديد عزلات *Cystobasidium* ظاهريًا. وشملت النتائج الايجابية للزرع: الأنواع non–*Cystobasidium* و *C. slooffiae*. ثبتت معلومات تسلسل النوكليوتيدات لـ *C. slooffiae* تحت رقم وصول GenBank OQ568166. كشفت نتائج اختبار ANOVA عن اختلافات كبيرة في مستوى حساسية *C. slooffiae* لمضادات الحيوية تجاه الفطريات، وقد لوحظ أنها حساسة لكل قرص مضاد للفطريات تم اختباره: الإيكونازول (24 ملم) والكيتوكونازول (20 ملم)، في حين أن المقاومة لكل من: ميكونازول (8 ملم) ونيستاتين (12 ملم). تم التعرف على *C. slooffiae* باستخدام استراتيجية تقليدية وتسلسلية، والتي يبدو أنها طريقة يمكن الاعتماد عليها وسريعة واقتصادية. عدم تكوين الغشاء الحيوي وحساسيتها للأدوية المضادة للفطريات المختلفة ل *C. slooffiae* تم اختبارهما.

الكلمات الدالة: عدوى عنق الرحم والفرج المهبلي، الأغشية الحيوية، *Cystobasidium slooffiae*.