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Pages: 29-41

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Molecular Identification and Sequences Analysis with Phylogenetic Tree for *Nakaseomyces glabratus* Isolated from Vulvovaginitis in Mares in Baghdad, Iraq

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Abstract:
Nakaseomyces glabratus, a non-dimorphic yeast formerly known as *Candida glabrata*, has been recognized as an opportunistic pathogen associated with vulvovaginal infections. Its relevance has been increasing due to its adaptability and resistance, posing challenges in both human and veterinary medicine. This study represents the first molecular and phylogenetic investigation of *Nakaseomyces glabratus* in equine vulvovaginitis cases in Iraq. It underscores the significance of incorporating molecular diagnostic techniques for accurate identification and improved management of fungal infections in veterinary practice.

Background:

Nakaseomyces glabratus has emerged as a significant opportunistic fungal pathogen, particularly in cases of vulvovaginal infections. Formerly known as *Candida glabrata*, this yeast is non-dimorphic and exhibits considerable resistance to antifungal agents, raising concern in both human and veterinary health. Despite its growing importance, limited data exist regarding its role in animal infections, especially in equine species in Iraq.

Aims:

This study aimed to molecularly characterize and assess the genetic relationships of *Nakaseomyces glabratus* isolates obtained from mares clinically diagnosed with vulvovaginitis in Baghdad City, Iraq.

R**esults**:

Between August 2024 and January 2025, 100 vaginal swabs were collected from mares showing clinical signs of vulvovaginitis in various locations around Baghdad City. Phenotypic identification included macroscopic growth on Sabouraud dextrose agar and CHROM agar, microscopic examination, and trehalose assimilation testing. Molecular identification was performed via

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)
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conventional PCR targeting the NL (D1/D2) region, followed by sequencing and phylogenetic analysis using MEGA 11 and BioEdit.

Out of the 100 samples, 20 (20%) were identified as Nakaseomyces glabratus. Four of the most virulent isolates were confirmed via PCR and sequencing. The NL gene was detected in all PCR-positive samples, producing a characteristic 620 bp band. Sequence analysis demonstrated 99–100% similarity to reference strains from Belgium, Kuwait, South Africa, and the Netherlands, indicating strong genetic conservation.

Conclusions:

The findings highlight the presence and genetic relatedness of Nakaseomyces glabratus strains in equine vulvovaginitis in Iraq, marking the first such report in the country. Molecular tools proved essential for accurate identification, and their use should be encouraged in veterinary diagnostics to enhance fungal infection management strategies.

Keyword: *Nakaseomyces glabratus*, vulvovaginitis, mares, molecular identification, PCR, sequencing, phylogenetic analysis, Baghdad, Iraq, non-*albicans Candida*

Introduction:

Nakaseomyces glabratus, formerly identified as *Candida glabrata*, is a yeast like fungus that exhibits non-dimorphic characteristics and has emerged as an opportunistic pathogen associated with mucosal infections in both humans and animals **(Kalia *et al.*, 2021; Beardsley *et al.*, 2024)**. This organism is classified within the *Nakaseomyces* genus of the *Saccharomycetaceae* family and is typically located in the gastrointestinal tract, oral cavity, and genital region **(Beardsley *et al.*, 2024)**. The morphological and growth properties of *N. glabratus* are characterized by asexual reproduction through budding, resulting in small, round to oval cells observable under a microscope. Notably, it does not produce true hyphae or pseudohyphae, which restricts its morphological variability. The fungus thrives on standard fungal media, yielding smooth, cream-colored colonies on Sabouraud Dextrose Agar **(Al-Tameemi *et al.*,2013).**

Vulvovaginitis, a common inflammatory condition affecting the vulva and vagina, is observed in various species, including mares and women. Among the pathogens associated with this condition, **(Al-Kubaisy *et al.*,2023)** *Nakaseomyces glabratus* has gained increased prominence. Although *Candida albicans* remains the leading cause of fungal vulvovaginitis *N. glabratus* ranks as the second most prevalent cause of vulvovaginal candidiasis **(VVC)** in women, and its isolation from reproductive tract infections in mares has become more frequent **(Hadi et al., 20250).** The organism is often linked to recurrent infections due to its ability to form biofilms and exhibit antifungal

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)
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resistance. In immunocompromised individuals, *N. glabratus* can lead to more severe infections, including candidemia **(Pappas *et al.*, 2018)**. In women, it accounts for approximately 10-20% of non-albicans vulvovaginal cases, particularly among those with predisposing factors such as diabetes, immunosuppression, or prolonged antibiotic use **(White *et al.*, 2020)**. The characterization and management of N. *glabratus* infections have emerged as a significant clinical challenge, primarily due to the organism's diminished susceptibility to standard first-line antifungal therapies. Although the implications of N. *glabratus* for human health are extensively documented, there exists a paucity of data concerning its role in vulvovaginitis within equine species, particularly among mares. Research on fungal infections in mares has identified various species of *Candida*, yet the clinical relevance of N. *glabratus* in equine vulvovaginitis remains inadequately investigated **(Fan *et al.*, 2022)**. This yeast exhibits distinctive virulence factors that enhance its pathogenicity; N. *glabratus* adheres to epithelial cells of the host through adhesins, facilitating colonization of mucosal surfaces. Additionally, it can form biofilms on these surfaces and medical devices **(Dhamraa *et al.*,2014),** thereby augmenting its resistance to host immune responses and antifungal therapies. The immune evasion strategies employed by *N. glabrata* include mechanisms that allow it to circumvent the host's immune system, such as exhibiting a reduced inflammatory response in comparison to C. *albicans*. This organism can exist in the host asymptomatically until it triggers an infection under conditions of immune compromise **(Fidel et al.,1999; de Oliveira *et al.*, 2015; Al-Zubidi *et al.*, 2023)**. Genetic analysis of N. *glabrata* isolates from mares has revealed similarities to human strains, indicating potential zoonotic or environmental connections **(Hadi et al., 2025).** The yeast's capacity to thrive in various environments, including the reproductive tracts of mammals, along with its antifungal resistance, raises concerns in both veterinary and human medical contexts. In equine practice, addressing fungal vulvovaginitis in mares poses its own set of challenges, primarily due to the absence of standardized antifungal treatment protocols in veterinary medicine. While empirical treatment with antifungal agents such as Amphotericin B or Nystatin may be considered, the resistance profile of N. *glabrata* in animals is not as thoroughly characterized as it is in humans**.**These characteristics make conventional identification methods less reliable, necessitating molecular techniques for accurate species differentiation **(Abdul-Rahman *et al.*, 2016).** PCR amplification and sequencing of the ITS region and D1/D2 domains are considered the gold standard for fungal identification **(Al-Waily *et al.*, 2020).**

This study aimed to molecularly confirm the presence of *N. glabratus* in mares with vulvovaginitis in Baghdad and analyze its evolutionary relationships using phylogenetic analysis.

Materials and Methods:

Sample Collection and Culture

A total of 100 Vaginal swab were collected from mares in different ages between **(3.5-20 years old)** with clinical vaginitis from different places in Baghdad province like Al- Fdhelia, Doura, Equestrian club in Abu-Ghraib, and Equestrian club in Al-Jadrea during the period from August 2024 to January 2025. by passing each swab several times across vaginal surface then kept it in ice

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)
https://doi.org/10.71375/djvs.2025.03203

box, all samples were transported as soon as possible under complete aseptic condition (**de Oliveira *et al.*, 2015**), to the mycology laboratory in the College of Veterinary Medicine / University of Baghdad for processing, isolation and identification of fungi specially *N. glabratus*

Phenotypic Identification

Morphological characteristics were assessed, including:
Its depending on the Colony appearance, *N. glabratus* colonies appeared as smooth, cream-colored formations on CHROMagar, also the microscopic analysis by using lactophenol cotton blue staining to observe yeast morphology, and Trehalose assimilation test to differentiate *N. glabratus* from other *Candida* species **((Freydiere *et al.*, 2003; Khairallah *et al.*,2020; Kareem *et al.*, 2022).**

Molecular Identification

Template Preparation by boiling method

DNA template was prepared by a boiling method as described by **(Looke *et al.*, 2011; Ahmed *et al.*, 2024).** Briefly, 5 isolated colonies of overnight growth yeast were suspended thoroughly in 2 ml distilled water and boiled in a water bath, for 10 min. After centrifugation, the supernatant was used as template DNA for the PCR.

PCR Amplification

The PCR amplification procedure for detected *N. glabratus local* isolates included:

Final volume for PCR mixture was 25 µl **(12.5 of Master Mix 2x, 5 µl template DNA, 1 µl primers for each forward and reverse primer, finally, 5.5 µl nuclease free water)** in uniplex PCR Eppendorf tubes but amount changed in multiplex PCR, mixed briefly via vortex then been placed in thermocycler polymerase chain reaction. Annealing gradients were set, where appropriate, increments from 52°C to 62°C, the program used for each PCR mixture were illustrated in the table. **(2-1)**.

Table (2-1) Primers Oligonucleotide Sequences Used in This Study.

No.	Gene	Primer	Sequences (5'_3')	Product size/bp
1	ITS	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Approximately 600
2	NL	NL1 NL4	GCATATCAATAAGCGGAGGA TTGGTCCGTGTTTCAAGACG	620

Table (2-2): Amplification program of primers

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)
https://doi.org/10.71375/djvs.2025.03203

No.	Amplified gene	Initial denaturation	No. of cycle	Denaturation	Annealing	Elongation	Final extension
1	**ITS**	**95°C/ 5min**	**35**	**94°C/ 45 sec**	**52.2°C/45 sec**	**72°C/1min**	**72°C/5min**
2	**NL**	**95°C/ 5min**	**35**	**94°C/ 45 sec**	**52.2°C/45 sec**	**72°C/1min**	**72°C/5min**

Primer dilution

The oligonucleotide primers, initially in a lyophilized state, were first reconstituted and diluted in nuclease-free double distilled water **(D.D.W.)** in accordance with the manufacturer's specifications to achieve a concentration of 100 picomol/µl. Subsequently, this stock solution was further diluted in nuclease-free D.D.W. to reach an approximate concentration of 10 picomol/µl. This dilution method was consistently applied to all primers utilized in this study.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was employed to detect the amplified PCR products, which were visualized using Ethidium bromide stain and documented with a UV transilluminator system. Specifically, one gram of agarose was dissolved in 100 ml of 1x TBE buffer. Additional TBE buffer was added to submerge the gel, after which the tank was sealed, and electrophoresis was conducted for one hour at a voltage of 5 volts/cm² across the gel for both DNA extracts and PCR products. Following electrophoresis, the agarose gel was removed from the tank and visualized using the UV transilluminator system, with subsequent photographs taken using a digital camera. Prior to electrophoresis, 3.5 µl of the extracted DNA was combined with 1.5 µl of loading dye **(Bromophenol blue)** and loaded into the wells of the gel **(Sambrook *et al.*, 2001).** In contrast, each well designated for PCR products received 5 µl of the monoplex PCR products **(White *et al.*, 1990)**. DNA ladders were concurrently run with each electrophoretic session to determine the size of the PCR products. Visualization of the DNA bands was accomplished via the UV transilluminator documentation system.

Sequencing of PCR Amplicons

The successfully amplified PCR amplicons were subjected to sequencing using the ABI3730XL automated DNA sequencing platform provided by Macrogen Corporation, South Korea. The same forward primer was consistently utilized throughout the sequencing process. The resultant ABI sequence files were analyzed with BioEdit and MEGA 11 software, during which any extraneous nucleotide sequences were discarded. To verify the identity of the obtained nucleotide sequences, the BLAST tool available on the NCBI website was employed **(Sievers *et al.*, 2018).**The oligonucleotide primers which in lyophilization status were dissolved and diluted first in free nuclease D.S.D.W **(amount according to recommended of manufactured company)** to obtains 100 picomol/µl, then

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)
https://doi.org/10.71375/djvs.2025.03203

this stock was diluted in free nuclease D.D.W to obtain nearly 10 picomol/µl. This technique accorded on all primers in this study.

Interpretation of Sequencing Data

The sequencing outcomes obtained from the PCR products were compared with their respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 **(DNASTAR, Madison, WI, USA**). The acquired sequences were aligned and analyzed to identify any discrepancies or similarities. Each recognized nucleic acid was assigned a distinct identifier that corresponded to its position within both the PCR amplicons and the reference genome. The NCBI-BLAST program was utilized to extract specific target regions, aiding in the identification of sequence matches for each sample. This analysis facilitated the identification and characterization of the sequences obtained from the PCR products.

Comprehensive Phylogenetic Tree Construction

The cladogram construction method was employed to enable a thorough examination of the relationships and similarities between the identified sequences and their respective reference sequences. To create a specific and detailed phylogenetic tree, the Clustal Omega tool was applied for multiple sequence alignments on the retrieved nucleic acid sequences. Following this, the neighbor-joining method was used to develop a comprehensive phylogenetic tree, which was visualized with the assistance of BioEdit and MEGA 11. The variations detected, along with their corresponding reference sequences, were incorporated into the complete cladogram

Results:

Cultural and Morphological Identification

Among the 100 vaginal samples analyzed, 20 isolates **(20%)** were suspected to be *N. glabratus* based on CHROMagar morphology Figure **(3-1)**, trehalose assimilation Figure **(3-2),** and microscopic examination on sabuorod dextrose agar **(Alsaidy *et al.*,2014; Mahmoud *et al.*, 2018)** as shown in Figure **(3-3)**

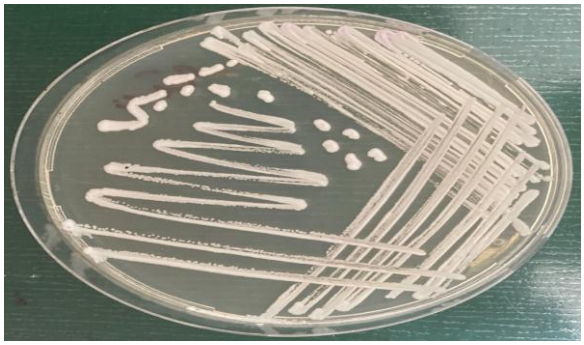

Figure (3-1): *Nakaseomyces glabratus* on CHROMagar shows cream to pinkish white smooth colored colonies after inculation at 37 ºC for 24-48hrs.

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)

https://doi.org/10.71375/djvs.2025.03203

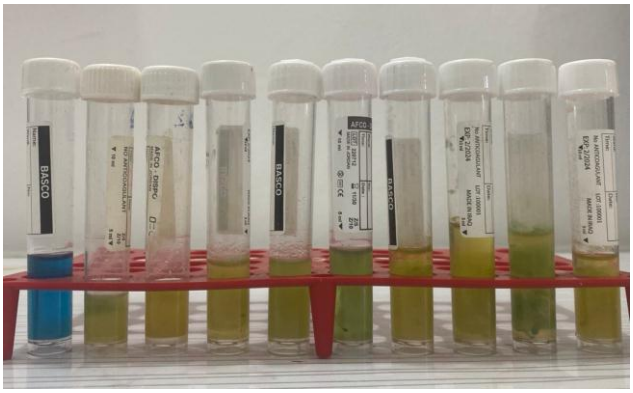

Figure (3-2): Trehalose Assimilation Test of *N. glabratus* after 3 hours at 42 ºC

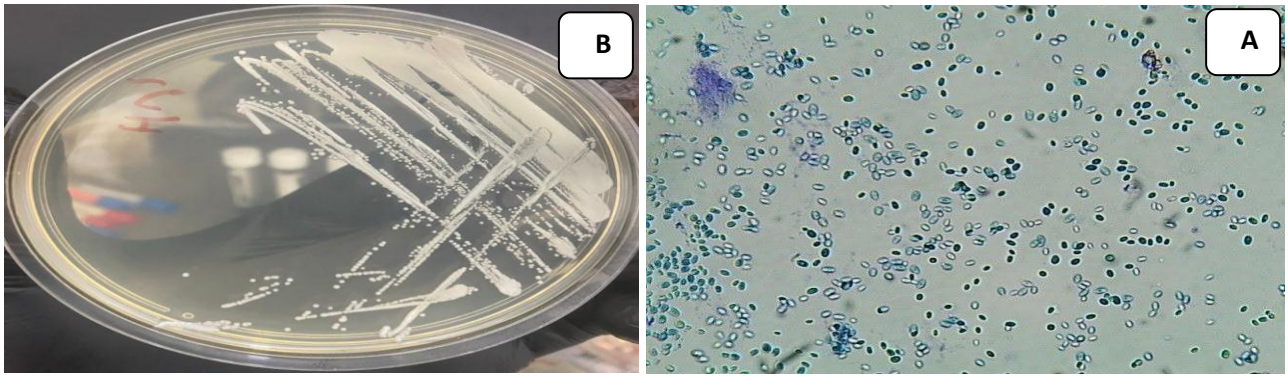

Figure (3-3): (A) Microscopic appearance of *N. glabratus* stained by lacto phenol cotton blue stain (40X) shown Macroscopic appearance of *Nakaseomyces glabratus* on Sabouraud Dextrose round to oval budding cells, (B) agar after 24-48 hours at 37°C shown cream colored, smooth, glossy colonies.

Molecular Confirmation

Out of 20 *N. glabratus* from mares, four isolates from mares were chosen for diagnosis by convential PCR according to virulence factors, these isolates exploring the presence of the NL gene which amplified of D1/D2 region of large subunit ribosomal RNA of PCR technique showed that the NL gene appeared **(100%)** detection rate in all these isolates.

The PCR technique for *N. glabratus* isolated from mares revealed NL gene with 620bp product size Figure **(3-4),** this study demonstrate that NL gene-based PCR has specific and high degree of sensitivity for the detection of *N. glabratus* **(Hameed *et al.*,2019; Abdulla *et al.*, 2023).**

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)

https://doi.org/10.71375/djvs.2025.03203

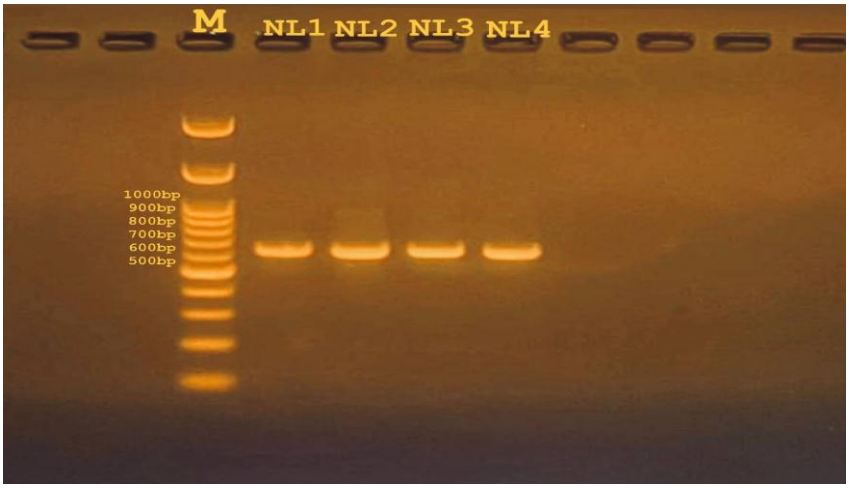

Figure (3-4): Agarose gel electrophoresis 1.5% agarose,7v/cm2 for 60 min was carried out, show PCR amplification of the NL gene in four isolates of *Nakaseomyces glabratus* in mares yields a ~620 bp fragment for specific species identification

Sequences Analysis

The sequence analysis confirmed the identification four isolates of *N. glabratus* (four isolated from each mares) which reported in GenBank NCBI under the following accession number PV138441 for local isolate of mare, and all the phenotypic characterization with molecular identification are listed in Table (3-1).

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)
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Table (3-1): Phenotypic characterization with molecular identification

No. of isolates	Phenotypic characterization of *N. glabratus*	Macroscopically on CHROMagar	Macroscopically on SDA	Microscopically 40X	Trehalose assimilation test
20	Yeast-like fungi Its existing as a small to oval, budding yeast cells and does not form hyphae or pseudohyphae not forming germ tube	cream to pinkish white smooth colored colonies	cream colored, smooth, glossy colonies	Small, oval budding teast cell	Yellow color
	Molecular characteristic of *N. glabratus*	**Name of gene**	**Accession number**	**Product base pair**	**Identity**
4	Have a haploid genome. with 12.3 Mb in size, its Contains 13 chromosomes, and its relatively compact genome	NL gene	PV138441	**620 bp**	**100%**

Phylogenetic Analysis

Sequencing results showed 99-100% identity with *N. glabratus* strains from Belgium, South Africa,Kuwait, and the Netherlands. The phylogenetic tree confirmed the genetic stability of Iraqi isolates within a globally conserved lineage **(Othman *et al.*, 2018; Al-Khafaji *et al.*, 2019).**

Discussion

The 20% prevalence of *N. glabratus* in vulvovaginitis cases aligns with global reports of increasing non-albicans *Candida* infections **(Gharban *et al.*, 2020).** This trend is clinically significant due to *N. glabratus* inherent resistance to azole antifungals, complicating treatment options (Mohammed *et al.*, 2018).

Molecular identification via PCR and sequencing proved more accurate than traditional methods, which often misidentify non-albicans *Candida* species (**Abdul-Rahman *et al.*, 2016).** A recent investigation conducted by Imran and Ali **(2015)** explored the molecular identification of *Candida* species through NL-PCR, followed by gel electrophoresis and sequencing. In their study, the authors

P-ISSN: 2410-8863
E-ISSN:2958-6178
https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)
https://doi.org/10.71375/djvs.2025.03203

successfully amplified the D1/D2 regions from various *Candida* isolates, yielding distinct, clear PCR bands **(ranging from 510 to 870 bp, depending on the species)** on agarose gels, which were subsequently sequenced to authenticate species identity. Their results reaffirmed the efficacy of NL-PCR as a rapid and sensitive technique for *Candida* speciation, akin to the clear band visualization noted for the NL targets in the current study. Additionally, Waikhom *et al.* **(2020)** illustrated the swift identification of *Candida* species using a multiplex PCR strategy that targeted various genomic regions, including the ITS and D1/D2 domains, followed by Agarose gel electrophoresis 1.5% agarose,7v/cm2 for 60 min **(Saeed *et al.*, 2020).**The study reported distinct bands for each *Candida* species and underscored the importance of combining PCR with sequencing to increase identification accuracies The phylogenetic analysis demonstrated that Iraqi isolates share a stable genetic relationship with international strains, suggesting that environmental persistence and potential healthcare-associated transmission contribute to its spread **(Kadhim *et al.*, 2021).**

Conclusions:

In conclusions *Nakaseomyces glabratus* rate is one of main causes increasing the incidence of vulvovaginitis infection in mares, and this study is considered the firstly for isolation and identification of this yeast from mares in Iraq as well as molecular assay, sequencing and phylogenetic tree have been important tools in the identification and profiling of this species by using NL gene.Future work needed to find the best biocontrol method.

Recommendations:

- this study showed isolation of non-albicans *Candida* **(*N. glabratus* previously *C. glabrata)** as scientific research needs to investigate the others non-albicans *Candida* as causative agent of vulvovaginitis infection in Human or other animals.
- Larger molecular research is required to determine the epidemiology clades of *N. glabratus* in human and different animals in Iraq.
- Further molecular study for the detection of virulence factor genes among local isolation of *N. glabratus* from different hosts.
- Study the immunological parameters and histopathological effects of *N. glabratus in vivo*
- Implementation of Hygiene and Biosecurity Protocols: Effective sanitation, improved management of stables, and stringent hygiene practices in equestrian environments may contribute to the reduction of fungal transmission and colonization among mares,

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Conflict of interest
There is no conflict of interest

Author contributions
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https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
Vol. 3 No.2 June (2025)

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https://djvs.uodiyala.edu.iq
Pages: 29-41

Diyala Journal for Veterinary Sciences
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Pages: 29-41

Diyala Journal for Veterinary Sciences
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