Original Article | Iran J Med Microbiol. 2023; 17(4): 447-456

10.30699/ijmm.17.4.447

Iranian Journal of Medical Microbiology | ISSN:2345-4342

Azole Resistance and *erg11* Gene Expression in Non-*albicans Candida* Strains Isolated from Raw Milk and Human Samples: Cross-sectional Study from 14 Farms and 2 Hospitals, Iran, 2021-2022

Zahra Namvar[1,2], Abbas Akhavan Sepahy[1], Robab Rafiei Tabatabaei[1], Somayeh Sharifynia[3], Sassan Rezaie[4*]

1. *Department of Microbiology, School of Biological Sciences, North Tehran Branch, Islamic Azad University, Tehran, Iran*
2. *Department of Biotechnology, National Animal Breeding Center and Promotion of Animal Products, Karaj, Iran*
3. *Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and long Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran*
4. *Department of Medical Mycology and Parasitology, Division of Molecular Biology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

ABSTRACT

Background and Aim: Nowadays, *non-albicans Candida* are common in human pathogens, and some of these cases were found in milk. Therefore, as well as the lack of accurate estimates of its global prevalence and severity, the present study aims to assess the demographic features of non-albicans Candida (NAC) spp. and determine the species distribution of NAC. It also evaluated the in vitro Azole susceptibility of NAC species and identified the *erg11* gene and *erg11* expression in fluconazole-resistant isolates of NAC spp., in Iran.

Materials and Methods: In the present study, *non-albicans Candida*, including *Candida glabrata, Candia krusei*, *Candida parapsilosis*, and *Candida tropicalis,* were isolated and identified from 14 farms (raw milk) and human patients using culture methods, Real-Time PCR and sequencing. The resistance and susceptibility of the samples to azole were examined and *erg11* expression was evaluated by RT-qPCR. The results were analyzed by REST Software to compare the levels of *erg11* gene expression involved in drug resistance of NAC.

Results: 74 and 52 NAC strains were isolated in 262 collected milk samples and human samples. Based on ITS sequencing, 0.76% were identified as *C. glabrata*, 2.29% as *C. tropicalis*, 4.19% as *C. parapsilosis,* and 19.8% as *C. krusei*. The expression of the *erg11* gene in the NAC was increased in samples isolated from humans compared to samples isolated from livestock ($P>0.05$), while no significant difference was found in the case of *Candida glabrata* isolated from both sources ($P<0.05$). All NAC isolates were sensitive to flucytosine.

Conclusion: non-albicans Candida (NAC) isolates from cows' milk have antifungal resistance genes while they had not taken any antifungal drugs. The resistance gene is transferred from antifungal agents in crop protection medications. Clinical isolates also had increased resistance to antifungal activity. Also, using Azole antibiotics can increase resistance gene level activity. This phenomenon should be considered for treatment program protocols.

Keywords: Azole, *erg11, non-albicans Candida*, Real-time PCR, genes Expression,

Received: 2023/05/07; **Accepted**: 2023/08/03; **Published Online:** 2023/09/27

Corresponding Information: **Sassan Rezaie**, *Department of Medical Mycology and Parasitology, Division of Molecular Biology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran* Email: srezaie@tums.ac.ir

Use a device to scan and read the article online

Namvar Z, Akhavan Sepahy A, Rafiei Tabatabaei R, Sharifynia S, Rezaie S. Azole Resistance and *erg11* Gene Expression in Non-albicans Candida Strains Isolated from Raw Milk and Human Samples: Cross-sectional Study from 14 Farms and 2 Hospitals, Iran, 2021-2022. Iran J Med Microbiol. 2023;17(4):447-56.

Download citation: BibTeX | RIS | EndNote | Medlars | ProCite | Reference Manager | RefWorks

Send citation to: Mendeley Zotero RefWorks

1. Introduction

The prevalence of mycotic infections has steadily increased in recent years **(1)**. Once thought to be nonpathogenic or less virulent, Fungi are now recognized as a leading cause of morbidity and mortality in immunocompromised and critically ill patients **(2)**.

Candida spp. are among the most common fungi. They can cause infections in both immunocompetent and immunocompromised hosts, but the incidence of infections is higher in immunocompromised individuals; candidiasis is thus appropriately referred to as the "disease of diseases." **(3)**.

Candida spp., despite being commensal organisms that colonize mucosal surfaces asymptomatically, can become one of the most significant causes of disabling and lethal infection. *Candida* spp. cause a variety of clinical manifestations ranging from mucocutaneous overgrowth to potentially fatal disseminated infections such as candidemia **(3)**.

Even though *Candida albicans* is the most widely distributed species involved in both mucocutaneous and disseminated infections, the frequency and severity of candidiasis associated with non-albicans Candida (NAC) spp. is growing rapidly **(4)**. This change has been linked to several factors, including severe immunosuppression or illness, prematurity, broad-spectrum antibiotics, the empirical use of antimycotic drugs, and diet. Clinical manifestations of infections caused by different NAC spp. are usually indistinguishable, but several NAC spp. are inherently resistant or acquire resistance to commonly used antifungal drugs, or both **(4-6)**.

Human food sources can also be contaminated by *Candida* spp. **(6)**. This pollution causes the spread of diseases and the loss of food. Humans need cow's milk as one of their most important foods. The interest in drinking raw milk is important since it is crucial for human health. It is possible for *Candida* spp. to multiply in cow's milk and cause disease in humans. It can be microbiologically dangerous for consumers. Raw milk is sold directly from farms in some European countries **(7)**.

The epidemiology of *non-albicans candidaes* in cows with mastitis in Yinchuan, Ningxia, China, was examined in 2018, and *Candida krusei* and *Candida parapsilosis* were the most identified cases **(8)**. *Candida glabrata* has been identified as an important pathogen and an important nosocomial infection and is the third leading agent of death after *Candida albicans* and *Candida tropicalis*. *Candida glabrata* is important not only because of its prevalence but also because of its high complications and mortality **(9)**. The pathogenicity of fungi is increasing sharply, and the number of antifungal drugs has nowadays increased **(10)**.

Drugs such as amphotericin B and azoles act on cell membranes or cell walls. Azoles are the most widely used antifungal drugs and are derivatives of imidazole and triazole **(11)**. One of the most common problems in the treatment of *candida* infections is treatment failure, especially due to clinical resistance to antifungal drugs **(12)**.

Candida species have several mechanisms for drug resistance **(13)**. The mechanisms of resistance to azoles are being extensively investigated **(14)**. One of the important mechanisms is the overexpression of the *erg11* gene and its effect on lanosterol 14 - alpha demethylase, which is the target of azoles **(13),** and also, point mutations in the *erg11* gene that reduce the adhesion of azoles to their target **(15)**.

Mutations occur in *erg11* (the gene that produces lanosterol 14 - alpha demethylase) and erg6, which are normally required for membrane function (but are not required for sterol biosynthesis). The development of using azole antifungals increases the treatment of fungal infections and its low toxic effects on the host, increasing its use, and this is not surprising to increase resistance to these drugs, especially fluconazole **(16, 17)**.

Due to the importance of non-albicans *Candida* spp. infection and its pathogenicity, as well as the lack of accurate estimates of its global prevalence and severity, the present study was conducted with the following purposes: a) assessing the demographic features of *non-albicans Candida* (NAC) spp. and determining the species distribution of *non-albicans Candida* (NAC) spp., 2) evaluating the in vitro Azole susceptibility of non-albicans Candida (NAC) species, 3) identifying *erg11* expression in fluconazole-resistant isolates of non-albicans Candida (NAC) spp., in Iran.

2. Materials and Methods

Sample Collection

In this cross-sectional-descriptive study, 262 samples were collected from 14 livestock farms in Tehran Province, Iran, and 48 blood samples were randomly obtained from patients hospitalized in Tehran, Iran with clinically proven or suspected systemic *Candida* infection. The samples were selected through the random sampling method from January 2021 to April 2022.

Milk and Clinical Samples

Briefly, 10 mL milk and blood samples were extracted from milk storage tanks that had been filled less than 24 hours ago. At 4°C, the samples were sent to the diagnostic laboratory. After one week of incubation at 37°C, they were cultured on Sabouraud dextrose agar (SDA) containing penicillin and streptomycin and incubated at 37°C for 72h.

Identification of Samples

The yeast colonies were then examined using candida Chrom-agar culture media (CHROMagar Candida, France), and their colors were used to differentiate them. To ensure complete identification of NAC, a portion of the rDNA in the Internal transcribed spacer (ITS) region was amplified and sequenced in all isolates. In brief, high molecular weight DNA was extracted from the samples using the previously described glass bead and phenol-chloroform method **(18)**. A portion of the 28S rDNA was amplified in each isolate by PCR using specific nucleotide primers for the ITS region; ITS1-S:3'TCC GTA GGT GAA CCT GCG G5' and ITS4-AS:3' TCC TCC GCT TGA TAT GC5' **(18)**. The obtained amplicons were sequenced nucleotides, and the results were compared in gene data banks (NCBI, NIH) to confirm identity.

Candida albicans strain ATCC 24433 was cultured on Sabouraud 4% Dextrose Agar (MilliporeSigma, USA-Canada) for 72 h as a positive control group.

Determination of Antifungal Susceptibility Tests (MIC)

Antifungal susceptibility testing is a way to determine the level of fungal resistance, the best treatment for a particular fungus, and the epidemiology of global resistance to antifungal agents. Microdilution is the reference and standard gold technique. Non-*albicans Candida Candida*tes, isolated from raw cow's milk samples and patients' samples, were examined against amphotericin B, itraconazole, fluconazole, and flucytosine using clinical and laboratory standard protocol (CLSI, M27-A3).

To collect new colonies from isolates, samples were cultured on Sabouraud 4% Dextrose Agar (MilliporeSigma, USA-Canada), and after 24 hours of incubation, a massive culture was prepared from the grown colonies. Then, the microdilution method was used to identify the susceptibility pattern of isolated samples against amphotericin B, itraconazole, fluconazole, and flucytosine according to CLSI protocol (CLSI, M27-A3). *C. parapsilosis* ATCC 22019 was used for quality control in this study.

Real-Time Quantitative PCR (RT-qPCR) Reaction

RNA Extraction and cDNA Synthesis

NAC pellets were used and collected in A600=20. The MICs of antibiotics were determined before RNA was extracted. The samples were cultured in the medium containing the antibiotic MIC concentration from the previous study. Once the samples had been collected, RNA was extracted using the RNAx kit's (EX6101-RNX Plus Solution, Sina Colone, Iran) RNA extraction protocol. The concentration and purity of the isolated RNA were measured using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Finally, cDNA was prepared from the extracted RNA.

For this purpose, Buffer Mixm, Enzyme (Reverse Transcriptase), Depc Water, and RNA were used. This protocol obtained the final volume of 20 µL and all samples were transferred to the sterilized PCR microtubes under sterile conditions and placed in a thermal cycler (ABI Quant Studio 12 Flex, USA) according to the gene amplification program: 10 Min in 25°C, 60 Min in 47°C and 5 Min in 85°C.

RT-PCR Test and Sequencing

RT-PCR was performed to ensure cDNA synthesis. Therefore, using PCR Kit (SinaClon BioScience, Iran) 7.5 µL H_2O, 12.5 µL Master Mix, 1.5 µL P_S (ITS1 primer), and 1.5 µL P_{AS} (ITS4 primer), and 2 µL cDNA were mixed according to the kit protocol. Based on the kit manual, the final volume was 25 µL, and the following program was used to amplify the products. Ten minutes at 25°C, 60 minutes at 47°C, and 5 minutes at 85°C.

By preparing 1% agarose gel, the samples were analyzed. The cDNA concentration was examined by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Since the values of RNA concentration from the cells are different, and mRNA volume is 1 to 2% of the total RNA, internal standards or control were selected to compare the expression levels of a particular gene in different samples (Housekeeping genes). Therefore, this study designed a pair of primers to express the β-actin gene. In the case of selected sequences, their homology was examined using Primer Blast.

The obtained amplicons were sequenced nucleotides, and the results were compared in gene data banks (NCBI, NIH) for identity confirmation.

Comparison of Gene Expression Levels

The mRNA expression levels of the studied genes were determined by qRT-PCR using SYBER Green (DNA-binding agents) and LightCycler® 96 Instrument

(Roche Molecular Systems, Inc., Dubai). The results of CT analysis obtained from real-time PCR reaction were analyzed using REST software **(19)** in *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida glabrata* with animal and human origins and according to the expression level of the β-actin gene. For this purpose, the High ROX kit of (Biofact, Korea) was used, and for each of the studied genes, H_2O: 6 µL Master Syber: 10 µL P_S: 1 µL, and P_{AS}: 1 µL and cDNA: 2 µL protocol was used.

Based on this protocol, the final volume was 20 µL, and the amplification program was 25°C for 20 Sec, 58°C for 30 Sec, and 70°C for 30 Sec in 40 cycles. It should be noted that the reactions were triplicate, and its melting temperature (Melt Curve) was set at 72-95°C. In the studied samples, the amplification of the beta-actin gene in all samples started from approximately cycle 25, indicating that the expression of the housekeeping gene remained constant in all samples and was not affected by the resistance and origin of the species. In all species, it has shown the same expression, indicating the stability of this gene. For this study, the following sequences were used as *erg11* primer; (*erg11* Forward) 5'-ACTCATGGGGTTGCCAATGT-3' and (*erg11* Reverse): 5'-AGCAGCATCACGTCTCCAAT-3'. Primers were designed using the PRIMER 3 web-based software (http://bioinfo.ut.ee/primer3-0.4.0) **(20)**.

Statistical Analysis

Statistical analysis was performed using the SPSS 24.0 (SPSS Inc., Chicago, IL., USA). Data analysis (binomial, unpaired) was performed using a t-test. Three biological replicates were used to calculate the mean and standard errors.

3. Results

74 and 52 non-albicans candida strains were isolated in 262 collected milk samples and human samples. Based on ITS sequencing results, 2 cases (0.76%) were identified as *C. glabrata*, 6 cases (2.29%) as *C. tropicalis*, 11 cases (4.19%) as *C. parapsilosis* and 50 cases (19.8%) as *C. krusei*.

Except for *C. krusei*, flucytosine with MIC_{50} had a more favorable effect on the specimens under study. In addition to flucytosine, itraconazole with MIC_{50} = 1.125 was determined to be the preferred drug for the three strains of *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*.

Flucytosine with MIC_{50} = 1 was more effective against *C. krusei* strains than itraconazole with MIC_{50} = 0.5. Figure 1 shows the results of in vitro antimicrobial susceptibility testing of non-albicans Candida species isolated from livestock.

Flucytosine was sensitive to 100% of *C. glabrata* and *C. tropicalis* isolates and 94 percent of *C. krusei* isolates in the study. Fluconazole was also effective against 100, 100, 72.72, and 80% of *C. glabrata, C. tropicalis, C. parapsilosis*, and *C. krusei* strains, respectively. This discovery demonstrated the potency of this antimicrobial medication against non-albicans Candida spp. Figure 1 shows the frequency of sensitive and resistant strains isolated from livestock based on the results.

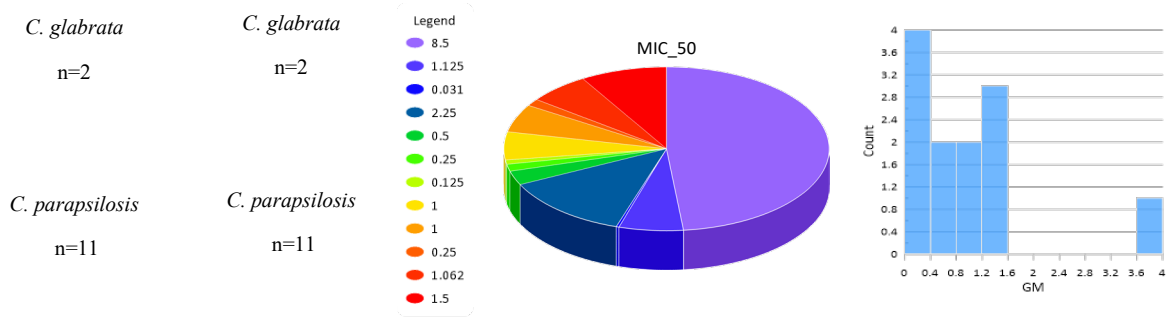

Figure 1. The results of in vitro antimicrobial susceptibility testing of NAC isolated from livestock.

The susceptibility testing results of cow milk-isolated and clinically isolated NAC strains were compared in this study. Clinical NAC isolates were also found to be flucytosine sensitive in all samples. Amphotericin B had the greatest effect on this group of fungi after flucytosine. Figures 2 and 3 show the MICs of antifungal medications as well as the frequency of resistant and sensitive clinical non-albicans Candida isolates used in this study.

MIC ranges for fluconazole, itraconazole, amphotericin B, and flucytosine against NAC isolated from livestock in this study were 0-64, 0.015-16, 0.0125-16, and 0.031-16 µg/mL, respectively, while MIC ranges for fluconazole, itraconazole, amphotericin B, and flucytosine against clinically NAC were 0.125-64, 0.015-16, 0.06-4, and 0.031-1 µg/mL, respectively (Figure 3 & 4).

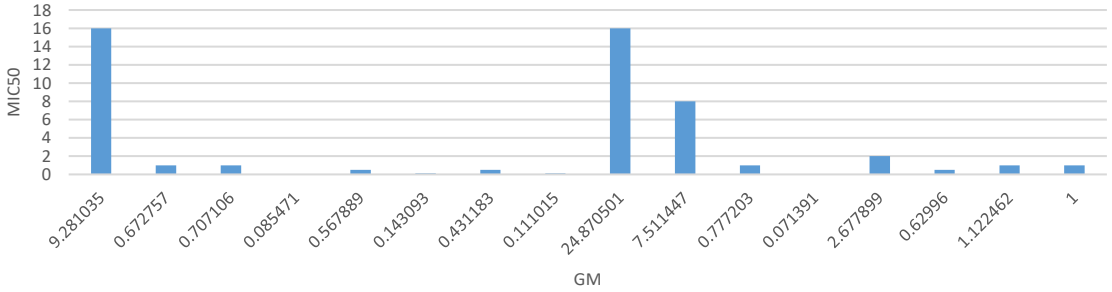

Figure 2. Interpretation of the sensitivity results of NAC from humans according to CLSI-M27-A3

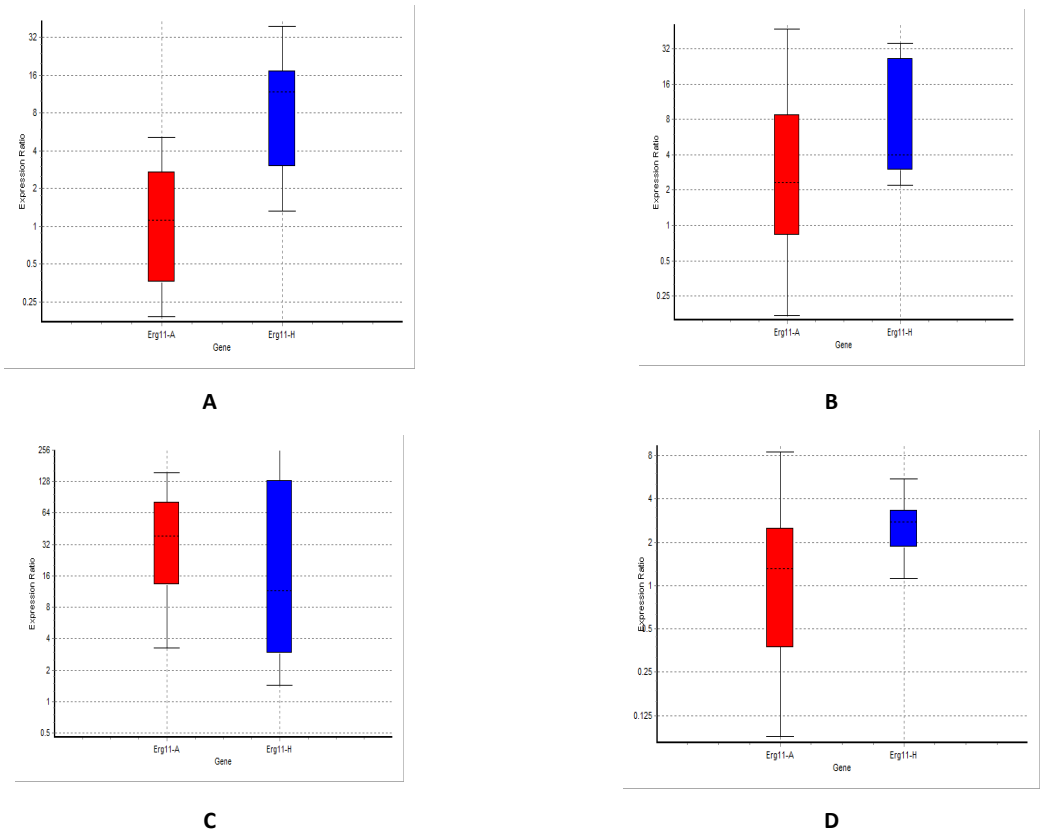

Figure 3. A. *Candida glabrata*, B. *Candida krusei*, C. *Candida parapsilosis*, D. *Candida tropicalis*

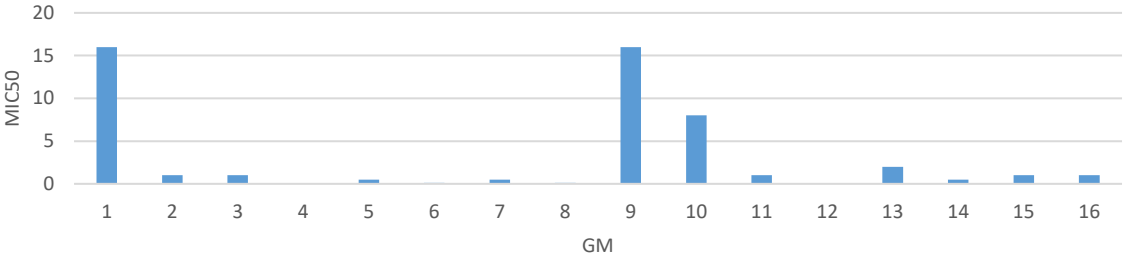

Figure 4. Susceptibility of NAC from humans tested against antifungal medications.

Figure 3 shows the *erg11* expression in *Candida glabrata*, *Candida krusei*, *Candida* parapsilosis, and *Candida tropicalis.*

Therefore, the present study evaluated the *erg11* gene expression in *Candida glabrata, Candida krusei, Candida parapsilosis*, and *Candida tropicalis* from animal and human origins. As results in Table 1, all *Candida* samples of human origin had an increased gene expression. Also, in *Candida glabrata* samples, there were no changes in gene expression levels.

Table 1. The *erg11* gene expression in four studied *Candida*tes

Candida	Source	Type	Reaction Efficiency	Expression	Std. Error	95% C. I.	P(H1)	Result
Candida glabrata	A*	TRG	1. 0	1. 030	0. 315 - 3. 962	0. 212 - 4. 988	0. 929	unchanged
Candida glabrata	H*	TRG	1. 0	8. 515	2. 474 - 26. 649	1. 519 - 37. 530	0. 053	unchanged
Candida krusei	A	TRG	1. 0	2. 573	0. 545 - 10. 498	0. 226 - 39. 697	0. 475	unchanged
Candida krusei	H	TRG	1. 0	6. 805	2. 395 - 27. 096	2. 204 - 34. 022	0. 000	UP
Candida parapsilosis	A	TRG	1. 0	30. 344	8. 605 - 98. 186	3. 992 - 144. 268	0. 100	unchanged
Candida parapsilosis	H	TRG	1. 0	16. 412	1. 899 - 139. 438	1. 455 - 240. 427	0. 000	UP
Candida tropicalis	A	TRG	1. 0	1. 000	0. 180 - 3. 920	0. 093 - 7. 700	0. 900	unchanged
Candida tropicalis	H	TRG	1. 0	2. 567	1. 644 - 4. 408	1. 210 - 5. 360	0. 000	UP
	β *actin*	REF	1. 0	1. 000				

A: Animal; H: Human.

4. Discussion

The expression of *erg11* genes in *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida glabrata* were examined using Rest software **(19)**. Fungi are not microorganisms transmitted from milk to humans, but their secondary metabolites, such as mycotoxins, are pathogenic to humans. Cows supply almost 85% of human milk. In many countries, using unheated raw milk due to reduced allergies has increased the importance of milk hygiene and led to accuracy in production quality because its consumption is crucial for children, the elderly, and patients with immunodeficiency.

Yeasts and molds are essential in fermented dairy products and yogurts **(21)**. None of today's problems is food-borne pathogens and new pathogens emerging. However, the effect of fungi has been greatly underestimated **(22)**. Nowadays, drug resistance is a problem that most societies face and seek to solve. The present study compared this issue in animal and clinical samples to find possible resistant samples isolated from the raw milk of cows that had not taken any antifungal drugs and had not faced these factors and determined the sensitivity and resistance of these samples. A significant increase in the use of antifungal agents such as azoles for the treatment of invasive candidiasis and aspergillosis and as fungicides in crop protection have led to the emergence of clinical strains resistant to antifungals, especially triazoles so that fluconazole resistance is increasing in *Candida parapsilosis* and *Candida tropicalis*. Thus, conducting studies and monitoring antifungal resistance is essential **(23)**.

Non-*albicans* yeasts have been shown to cause therapeutic problems in patients with immunodeficiency such as HIV-positive, cancer, or transplant recipients as an opportunistic pathogen **(24)**. In the twentieth century, nosocomial infections and *Candida* infections in patients with blood infections increased. More than 90% of the reports are related to *Candida albicans*, *Candida glabrata*, *Candida* parapsilosis, *Candida krusei,* and *Candida tropicalis* **(12, 24-26)**.

Candida glabrata is an opportunistic fungus, the world's second most common cause of candidiasis, and a major public health concern. The second most common nosocomial infection is *Candida albicans*, although it is historically considered a nonpathogenic flora. In hospital infections, it is often isolated and causes mucosal and systemic infections in patients with immunodeficiency or diabetics. Its treatment is difficult because it is resistant to many azoles and less sensitive to amphotericin B **(27, 28)**. Several resistance mechanisms, such as P-450-dependent ergosterol synthesis and an energy-dependent pump (in the case of fluconazole), have been observed **(29, 30)**. *Candida krusei* is significant since it is communally transmitted in humans and is often isolated from mucosal surfaces **(31)**. Compounds such as *Candida krusei* cell wall are different from other *Candida*tes. *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis,* and *Candida glabrata* are sensitive to lysozyme **(32)**.

The prevalence of *Candida parapsilosis* has increased in the last two decades, especially in hospitals **(33, 34)**. The *Candida parapsilosis* family has emerged as a pathological and large hospital disease

(35). *Candida tropicalis* is one of the most common causes of the disease in patients admitted to the urinary tract and blood infections wards. Accordingly, based on some studies, *Candida tropicalis* has become resistant to amphotericin B due to long-term treatment **(36)**. In recent years, *Candida tropicalis* has become increasingly isolated from candidiasis and rapidly become resistant to fluconazole **(23)**. Nowadays, fungal genera and species resistant or less sensitive to antifungal agents are a major cause of invasive infections in patients with immunodeficiency. Therefore, they are more important **(37)**. An increase in fungal infections, changes in the epidemiology of their types and drug resistance, and the development of new toxins have led to the identification of various antifungal drugs **(38)**. *Candida glabrata* is less sensitive to azole. Resistance to treatment and Fluconazole prophylaxis has been examined in them. A high resistance rate has caused significant concern for patients exposed to antifungal drugs **(39)**.

Lupetti et al. (2002) showed that mutations in the *erg11* gene also occur in *Candida* species **(40)**. *erg11* is the main target of azoles, and its mutation is associated with drug resistance **(26)**. The association between increased *erg11* expression and resistance of azoles in *Candida dubliniensis*, *Candida tropicalis*, *Candida* parapsilosis, *Candida krusei*, and *Candida glabrata* has been reported **(23)**. These findings are the result of a study on human samples. They are consistent with our findings in comparing the cases of *Candida krusei*, *Candida parapsilosis,* and *Candida tropicalis*, and we showed an increase in expression in human samples in all three cases. However, in the case of *Candida glabrata*, we did not observe any change in the expression of the *erg11* gene. Also, in the samples of *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida glabrata* isolated from animal origin, we did not observe any change in gene expression. However, in research carried out by Du Jun et al. (2018) in China on cows with mastitis, the presence of the *erg11* gene in *Candida* parapsilosis and *Candida krusei* was shown, but their study did not examine the level of expression of the gene, and it examined merely the presence of the gene **(8)**.

5. Conclusion

NAC isolates from cows' milk have antifungal resistance genes while they had not taken any antifungal drugs. The resistance gene is transferred from antifungal agents in crop protection medications. Also, it is noteworthy that the *erg11* genes were expressed in the fluconazole-resistant isolates, especially since they were not found in the susceptible dose-dependent isolates. Clinical isolates also had increased resistance to antifungal activity. Therefore, using Azole antibiotics can increase resistance gene level activity. This phenomenon should be considered for treatment program protocols.

Ethics Approval

Not applicable.

Availability of Data and Materials

All sequence data generated in this are available in GenBank.

Authors' Contribution

Zahra Namvar: carried out the experiment, wrote the manuscript & and analyzed the data. Abbas Akhavan Sepahy: helped supervise the project. Robab Rafiei Tabatabaei: helped supervise the project. Somayeh Sharifynia: analyzed the data. Sassan Rezaie: supervised the project.

Acknowledgment

We thank our colleagues at the Medical Mycology Laboratory, School of Public Health, Tehran University of Medical Sciences.

Conflict of Interest

The authors declared no conflict of interest.

Reference

1. Rayens E, Norris KA. Prevalence and Healthcare Burden of Fungal Infections in the United States, 2018. Open Forum Infect Dis. 2022;9(1): ofab593. [DOI:10.1093/ofid/ofab593] [PMID] [PMCID]

2. Khodadadi H, Zomorodian K, Nouraei H, Zareshahrabadi Z, Barzegar S, Zare MR, et al. Prevalence of superficial-cutaneous fungal infections in Shiraz, Iran: A five-year retrospective study (2015-2019). J Clin Lab Anal. 2021;35(7): e23850. [DOI:10.1002/jcla.23850][PMID][PMCID]

3. Colombo AL, de Almeida Júnior JN, Slavin MA, Chen SCA, Sorrell TC. Candida and invasive mould diseases in non-neutropenic critically ill patients and patients with haematological cancer. Lancet Infect Dis. 2017;17(11):e344-e56. [DOI:10.1016/S1473-3099(17)30304-3] [PMID]

4. Mendonça A, Santos H, Franco-Duarte R, Sampaio P. Fungal infections diagnosis - Past, present and future. Res Microbiol. 2022;173(3):103915.

[DOI:10.1016/j.resmic.2021.103915] [PMID] [PMCID]

5. Agnelli C, Valerio M, Bouza E, Guinea J, Sukiennik T, Guimarães T, et al. Prognostic factors of *Candida* spp. bloodstream infection in adults: A nine-year retrospective cohort study across tertiary hospitals in Brazil and Spain. Lancet Reg Health Am. 2022;6:100117. [PMID] [PMCID] [DOI:10.1016/j.lana.2021.100117]
6. Pfaller MA, Huband MD, Flamm RK, Bien PA, Castanheira M. Antimicrobial activity of manogepix, a first-in-class antifungal, and comparator agents tested against contemporary invasive fungal isolates from an international surveillance programme (2018-2019). J Glob Antimicrob Resist. 2021;26:117-27. [DOI:10.1016/j.jgar.2021.04.012] [PMID]
7. Zastempowska E, Grajewski J, Twarużek M. Food-Borne Pathogens and Contaminants in Raw Milk - A Review. Ann Anim Sci. 2016;16(3):623-39. [DOI:10.1515/aoas-2015-0089]
8. Du J, Wang X, Luo H, Wang Y, Liu X, Zhou X. Epidemiological investigation of non-albicans Candida species recovered from mycotic mastitis of cows in Yinchuan, Ningxia of China. BMC Vet Res. 2018;14(1):251. [PMID] [PMCID] [DOI:10.1186/s12917-018-1564-3]
9. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev. 2000;13(1):122-43.[PMID][PMCID] [DOI:10.1128/CMR.13.1.122]
10. Arastehfar A, Gabaldón T, Garcia-Rubio R, Jenks JD, Hoenigl M, Salzer HJF, et al. Drug-Resistant Fungi: An Emerging Challenge Threatening Our Limited Antifungal Armamentarium. Antibiotics. 2020;9(12):877. [PMID] [PMCID] [DOI:10.3390/antibiotics9120877]
11. Fromtling RA. Overview of medically important antifungal azole derivatives. Clin Microbiol Rev. 1988;1(2):187-217. [DOI:10.1128/CMR.1.2.187] [PMID] [PMCID]
12. Berkow EL, Lockhart SR. Fluconazole resistance in Candida species: a current perspective. Infect Drug Resist. 2017;10:237-45. [DOI:10.2147/IDR.S118892] [PMID] [PMCID]
13. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in Candida albicans biofilms: phase-specific role of efflux pumps and membrane sterols. Infect Immun. 2003;71(8):4333-40. [PMID] [PMCID] [DOI:10.1128/IAI.71.8.4333-4340.2003]
14. Krishnan S, Manavathu EK, Chandrasekar PH. Aspergillus flavus: an emerging non-fumigatus Aspergillus species of significance. Mycoses. 2009;52(3):206-22. [PMID] [DOI:10.1111/j.1439-0507.2008.01642.x]
15. Sanglard D, Odds FC. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infec Dis. 2002;2(2):73-85. [DOI:10.1016/S1473-3099(02)00181-0] [PMID]
16. Villasmil ML, Barbosa AD, Cunningham JL, Siniossoglou S, Nickels JT, Jr. An Erg11 lanosterol 14-α-demethylase-Arv1 complex is required for Candida albicans virulence. PLoS One. 2020;15(7): e0235746. [DOI:10.1371/journal.pone.0235746] [PMID] [PMCID]
17. Jensen RH. Resistance in human pathogenic yeasts and filamentous fungi: prevalence, underlying molecular mechanisms and link to the use of antifungals in humans and the environment. Dan Med J. 2016;63(10):1-34.
18. Namvar Z, Sepahy AA, Tabatabaei RR, Rezaie S. Antifungal susceptibility of non-albicans Candida spp. isolated from raw milk and human blood in Alborz and Tehran provinces. Iran J Microbiol. 2019;11(6):520-6. [DOI:10.18502/ijm.v11i6.2224]
19. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002;30(9):e36. [DOI:10.1093/nar/30.9.e36] [PMID] [PMCID]
20. Han G, Liu N, Li C, Tu J, Li Z, Sheng C. Discovery of Novel Fungal Lanosterol 14α-Demethylase (CYP51)/Histone Deacetylase Dual Inhibitors to Treat Azole-Resistant Candidiasis. J Med Chem. 2020;63(10):5341-59. [DOI:10.1021/acs.jmedchem.0c00102] [PMID]
21. Aslam S, Qazi JI. Isolation of acidophilic lactic acid bacteria antagonistic to microbial contaminants. Pak J Zool. 2010;42(5):567-73.
22. Lee SC, Billmyre RB, Li A, Carson S, Sykes SM, Huh EY, et al. Analysis of a food-borne fungal pathogen outbreak: virulence and genome of a Mucor circinelloides isolate from yogurt. mBio. 2014; 5(4):e01390-14. [PMID] [PMCID] [DOI:10.1128/mBio.01390-14]
23. Goncalves SS, Souza ACR, Chowdhary A, Meis JF, Colombo AL. Epidemiology and molecular mechanisms of antifungal resistance in Candida and Aspergillus. Mycoses. 2016;59(4):198-219. [DOI:10.1111/myc.12469] [PMID]
24. De Aguiar Cordeiro R, Pereira de Alencar L, Nogueira Brilhante RS, de Souza Collares Maia Castelo-Branco D, Cordeiro Teixeira CE, de Brito Macedo R, et al. Antifungal susceptibility of emerging opportunistic yeasts and yeast-like fungi from Rhea americana. Can J Microbiol. 2013;59(8):577-80. [PMID] [DOI:10.1139/cjm-2013-0176]
25. Voidarou C, Antoniadou M, Rozos G, Tzora A, Skoufos I, Varzakas T, et al. Fermentative Foods: Microbiology, Biochemistry, Potential Human Health Benefits and Public Health Issues. Foods. 2020;10(1):69. [DOI:10.3390/foods10010069] [PMID] [PMCID]
26. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. Candida glabrata, Candida parapsilosis and Candida tropicalis: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol Rev. 2012;36(2):288-305. [DOI:10.1111/j.1574-6976.2011.00278.x] [PMID]
27. Demir KK, Butler-Laporte G, Del Corpo O, Ekmekjian T, Sheppard DC, Lee TC, et al. Comparative effectiveness of

amphotericin B, azoles and echinocandins in the treatment of candidemia and invasive candidiasis: A systematic review and network meta-analysis. Mycoses. 2021;64(9):1098-110. [DOI:10.1111/myc.13290] [PMID]

28. Forastiero A, Mesa-Arango AC, Alastruey-Izquierdo A, Alcazar-Fuoli L, Bernal-Martinez L, Pelaez T, et al. Candida tropicalis antifungal cross-resistance is related to different azole target (Erg11p) modifications. Antimicrob Agents Chemother. 2013;57(10):4769-81. [DOI:10.1128/AAC.00477-13] [PMID] [PMCID]

29. Ghannoum MA, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev. 1999;12(4):501-17. [DOI:10.1128/CMR.12.4.501] [PMID] [PMCID]

30. Zhang J, Li L, Lv Q, Yan L, Wang Y, Jiang Y. The Fungal CYP51s: Their Functions, Structures, Related Drug Resistance, and Inhibitors. Front Microbiol. 2019;10:691. [DOI:10.3389/fmicb.2019.00691] [PMID] [PMCID]

31. Turner SA, Butler G. The Candida pathogenic species complex. Cold Spring Harb Perspect Med. 2014;4(9):a019778. [PMID] [PMCID] [DOI:10.1101/cshperspect.a019778]

32. Gómez-Gaviria M, Mora-Montes HM. Current Aspects in the Biology, Pathogeny, and Treatment of Candida krusei, a Neglected Fungal Pathogen. Infect Drug Resist. 2020;13:1673-89. [DOI:10.2147/IDR.S247944] [PMID] [PMCID]

33. West PT, Peters SL, Olm MR, Yu FB, Gause H, Lou YC, et al. Genetic and behavioral adaptation of Candida parapsilosis to the microbiome of hospitalized infants revealed by in situ genomics, transcriptomics, and proteomics. Microbiome. 2021;9(1):142. [PMID] [PMCID] [DOI:10.1186/s40168-021-01085-y]

34. Trofa D, Gácser A, Nosanchuk JD. Candida parapsilosis, an emerging fungal pathogen. Clin Microbiol Rev. 2008;21(4):606-25. [DOI:10.1128/CMR.00013-08] [PMID] [PMCID]

35. van Asbeck EC, Clemons KV, Stevens DA. Candida parapsilosis: a review of its epidemiology, pathogenesis, clinical aspects, typing and antimicrobial susceptibility. Crit Rev Microbiol. 2009;35(4):283-309. [DOI:10.3109/10408410903213393] [PMID]

36. Fernandes T, Silva S, Henriques M. Candida tropicalis biofilm's matrix--involvement on its resistance to amphotericin B. Diagn Microbiol Infect Dis. 2015;83(2):165-9. [PMID] [DOI:10.1016/j.diagmicrobio.2015.06.015]

37. Hazirolan G, Canton E, Sahin S, Arikan-Akdagli S. Head-to-head comparison of inhibitory and fungicidal activities of fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole against clinical isolates of Trichosporon asahii. Antimicrob Agents Chemother. 2013;57(10): 4841-7. [DOI:10.1128/AAC.00850-13] [PMID] [PMCID]

38. Milici ME, Maida CM, Spreghini E, Ravazzolo B, Oliveri S, Scalise G, et al. Comparison between disk diffusion and microdilution methods for determining susceptibility of clinical fungal isolates to caspofungin. J Clin Microbiol. 2007; 45(11):3529-33. [DOI:10.1128/JCM.00826-07] [PMID] [PMCID]

39. Jensen RH, Johansen HK, Soes LM, Lemming LE, Rosenvinge FS, Nielsen L, et al. Posttreatment Antifungal Resistance among Colonizing Candida Isolates in Candidemia Patients: Results from a Systematic Multicenter Study. Antimicrob Agents Chemother. 2015;60(3):1500-8. [DOI:10.1128/AAC.01763-15] [PMID] [PMCID]

40. Lupetti A, Danesi R, Campa M, Tacca MD, Kelly S. Molecular basis of resistance to azole antifungals. Trends Mol Med. 2002;8(2):76-81. [DOI:10.1016/S1471-4914(02)02280-3] [PMID]