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Investigation of the Efficacy and Antifungal Drug Resistance of Non-Albicans *Candida* Species in Mycotic Mastitis

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Abstract: This research aimed to use a conventional and molecular approach to determine Candida species isolated from cattle clinical mastitis and examine their antifungal susceptibility. In this research, 100 milk samples with mastitis were collected from dairy farms in five different Izmir districts. On chromogenic agar, 23 *Candida* isolates were isolated from milk samples with mastitis, and 20 (87.0%) isolates were identified as *C. krusei*, two (8.6%) isolates as *C. albicans*, and one (4.3%) isolate as *C. tropicalis*. PCR analysis verified that all *Candida* isolates were *C. krusei*, *C. albicans,* and *C. tropicalis*. According to sequence analysis, it was determined that 11 of the *C. krusei* isolates had >97% similarity to "*Pichia kudriavzeii* ZKI117 5.8S ribosomal gene"; six of the *C. krusei* isolates to "*Pichia kudriavzeii* isolate L-012 small subunit ribosomal RNA gene"; two *C. krusei* isolates to "*Pichia kudriavzeii* isolate 3 internal transcribed spacer 1"; and one *C. krusei* isolates to "*Pichia kudriavzeii* LL11_078 18S ribosomal gene"; two *C. albicans* isolates to "*Candida albicans* isolate B02 5.8S ribosomal gene"; one *C. tropicalis* isolate to "*Candida tropicalis* isolate CTR1201 18S ribosomal gene". In the antifungal susceptibility test results, all *Candida* isolates (%100) were resistant to metronidazole and flucytosine. Consequently, it was determined that identifying *Candida* agents and determining antifungal susceptibility in farms with mycotic mastitis will ensure the application of correct treatment protocols and reduce economic losses due to fungal mastitis.
Keywords: *Antifungal drug resistance, Candida, Cattle, DNA sequence, Mastitis.*

Mikotik Mastitislerde Non-Albicans *Candida* Türlerinin Etkinliğinin ve Antifungal İlaç Dirençlerinin Araştırılması

Özet: Bu çalışmanın amacı, sığır klinik mastitislerinden izole edilen *Candida* türlerinin konvansiyonel ve moleküler yöntemlerle tanımlanması ve antifungal duyarlılıklarının belirlenmesidir. Bu araştırmada İzmir'in beş farklı ilçesindeki sığır çiftliklerinden 100 mastitisli süt örneği alınmıştır. Mastitisli süt örneklerinden 23 *Candida* izolatı izole edildi ve kromojenik agar ile 20 (%87.0) izolat *C. krusei*, iki (%8.6) izolat *C. albicans* ve bir (%4.3) izolat *C. tropicalis* olarak tanımlandı. Tüm *Candida* izolatları PCR analizi ile *C. krusei*, *C. albicans* ve *C.tropicalis* olarak doğrulandı. Sekans analizine göre *C. krusei* izolatlarından 11 tanesinin "*Pichia kudriavzeii* ZKI117 5.8S ribosomal gene" ile; altı tanesinin "*Pichia kudriavzeii* isolate L-012 small subunit ribosomal RNA gene" ile; iki tanesinin "*Pichia kudriavzeii* isolate 3 internal transcribed spacer 1", bir tanesinin "*Pichia kudriavzeii* LL11_078 18S ribosomal gene" ile; 2 *C. albicans* izolatının "*Candida albicans* isolate B02 5.8S ribosomal gene" ile; bir *C.tropicalis* izolatının "*Candida tropicalis* isolate CTR1201 18S ribosomal gene" ile >%97 benzerlik gösterdiği belirlendi. Tüm *Candida* izolatlarının antifungal duyarlılık testi sonuçlarında metronidazol ve flusitozine karşı %100 dirençli olduğu saptandı. Sonuç olarak, mikotik mastitisli çiftliklerde *Candida* etkenlerinin identifikasyonu ve antifungal duyarlılıklarının belirlenmesinin doğru tedavi protokollerinin uygulanmasını sağlayacağı ve mikotik mastitise bağlı ekonomik kayıpları önleyeceği kanaatine varıldı.
Anahtar Kelimeler: *Antifungal ilaç direnci, Candida, DNA sekansı, İnek, Mastitis.*

Introduction

Mastitis is an infection caused by various microorganisms and reduces milk yield and increases antibiotic treatment and farm costs. It also has a worldwide economic impact since it costs the global dairy industry 35 billion dollars (Du et al., 2018; Khalaf et al., 2021). In mastitis, an immunological response occurs in the mammary gland, which causes a physical change in milk due to the deterioration of the permeability of the milk-blood barrier (Khalaf et al., 2021). Fleischer first described mycotic mastitis caused by Candida species in 1930. Antibacterial misuse, treatment with contaminated antibiotic solutions, and contaminated objects that contact the mammary canal help yeast colonize the gland. Candida-induced udder infections account for around 10% of all mastitis cases in cattle (Du et al., 2018). *Candida glabrata* and *Candida krusei* are the most common *Candida* species in mycotic cattle mastitis. In cases of subclinical mastitis, *Candida tropicalis, Candida viswanathii*, *Candida guilliermondii*, *Candida norvegica*, and *Candida zeylanoides* are also identified (Bakr et al., 2015).

Candida species are identified using bacterioscopy, morphological diagnosis, and biochemical testing, often taking more than three days to complete. Chromogenic-based and standard media such as SDA have been used to diagnose *Candida* species. Diagnostic methods such as API 20C AUX and MALDI TOF are also used. However, in the differential diagnosis and typing of *Candida* species, molecular diagnostic techniques (PCR) and sequencing approaches have begun to assume a prominent role (Bakr et al., 2015).

It has been stated that most antimycotic drugs are not effective in treating *Candida* infections. Antifungal treatments have been developed due to the rise in mycotic infections in recent years. However, it has been noted that the increased usage of antifungal drugs reduces the endogenous fungal flora, resulting in the emergence of more resistant strains while susceptible strains are suppressed (Erbas et al., 2017). Antifungal-resistant *Candida* species must be identified fast and precisely, and antifungal susceptibility tests must be used to guide therapy (Hizlisoy et al., 2019).

This research aims to isolate *Candida* species that cause fungal mastitis in cattle and to investigate their susceptibility to antifungals.

Materials and Methods

Sampling: In this research, 100 milk samples with clinical mastitis were collected from dairy farms of 50 or more heads in five different districts in the province of Izmir (Beydag, Bayindir, Odemis, Kiraz, Tire). Twenty milk samples with clinical mastitis were collected from each district. All milk samples were taken from the mammary lobe with clinical mastitis of the cattle on these farms. Clinical mastitis was diagnosed by the presence of discomfort, swelling, warmth, and an abnormal look in the milk (bloody milk, watery discharges, clots, pus). Ten ml milk samples were collected from cattle with the above clinical findings and transported to the lab in a cold chain. No ethical permission is required for this study.

Phenotypic Isolation: Milk samples (100 µl) were pre-enriched by inoculating them with Sabouraud dextrose broth (HiMedia Lab., India) and incubated at 37°C for 72 hours. Fifty µl of each broth culture were inoculated on SDA (HiMedia Lab., India), chloramphenicol (0,05 mg/ml), and incubated at 37 °C for 48 hours. The isolates were identified according to their macroscopic and microscopic morphologies. *Candida* suspect colonies were passaged onto chromogenic agar (HiCromeTM Candida Differential Agar, HiMedia Lab., India) and incubated for 24 hours at 30 °C. On chromogenic agar, colonies of *C. krusei* are purple, *C. tropicalis* are blue to purple, *C. albicans* are light green, *C. parapsilosis* are white-cream, and *C. glabrata* are cream-white. Identification of *Candida* species was made phenotypically with detection of different color-forming colonies. (Milanov et al., 2014).

Genotypic Identification: The isolates for which species identifications were made on chromogenic agar were passaged to SDA, and DNA extractions were performed from the obtained colonies with the DNA extraction kit (UltraClean® Microbial DNA isolation Kit, MoBio Lab., USA). *Candida* DNA samples were kept at -20°C. The genotypic identification of *Candida* isolates was performed utilizing ITS3 (5'-GCA TCG ATG AAG AAC GCA GC- 3') and ITS4 (5'-CCT CCG CTT ATT GAT ATG C-3') primers (Fujita et al., 2001). PCR reaction was performed in a total volume of 20 µl, including 10 µl of 2x Taq master mix, 0.5 µl $MgCl_2$ (50 mM), and 0.1 µl primers (100 pmol). PCR conditions; a pre-denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 1 min for 30 cycles and followed by a final elongation at 72°C for 4 min with using a SimpliAmp thermal cycler (Appliedbiosystems, Massachusetts, USA). PCR amplicons were electrophoresed on 1.5% agarose gel at 80V for 40 minutes. After electrophoresis, agarose gel was visualized on the Vilber Lourmat (Collegien, France) imaging system. Band images were evaluated according to Fujita et al. (2001).

Sanger Sequencing: The Sanger sequencing method was used to sequence *Candida* species identified by PCR analysis with ITS3-ITS4 primers (Sanger et al., 1977). Enzymatic purification (ExoSap-IT™, USA) was used to purify PCR amplicons. The purified amplicons were subjected to sequence PCR analysis using the ITS4 primer. After sephadex purification, the amplicons were loaded into the Beckman Coulter CEQ8000 (Fullerton, CA, USA) instrument. The sequences were loaded into a Nucleotide-Blast software, and isolates that showed similarity were classified as *Candida* species.

Antifungal Susceptibility Test: The antifungal susceptibility of *Candida* isolates was examined using the disk diffusion method (CLSI, 2018). Colonies were inoculated in brain heart infusion broth and density adjusted to 0.5 MacFarland. Ketoconazole (10 µg), metronidazole (5 µg), nystatin (100 U), flucytosine (1 µg), fluconazole (10 µg) and miconazole (10 µg) were used in this research. Evaluation of zone diameters of antifungals was done according to CLSI M44-A2 standards (CLSI, 2018).

Results

Phenotypic Identification: In this study, 100 milk samples from the mammary lobe with clinical mastitis of the cattles were inoculated into SDA, and 23 *Candida* suspect colonies were observed in a morphology-based examination for phenotypic isolation. Gram staining was used to investigate microscopic morphologies. All samples (n=23) were Gram-positive coccoid shaped due to Gram staining under the microscope. After microscopic examination, 23 *Candida* suspected isolates were passaged onto chromogenic agar plates. Colored colonies formed duo to passages were evaluated according to the color scale. *Candida krusei* colonies were purple, *C. albicans* colonies were light green, *and C. tropicalis* colonies were blue-purple on chromogenic agar. As a result, 20/23 (87.0%) isolates were identified as *C. krusei*, two (8.6%) isolates as *C. albicans*, and one (4.3%) isolate as *C. tropicalis*. In the Bayindir district, 10 *C. krusei* isolates and one *C. albicans* isolate were isolated from 20 milk samples for the region-based evaluation. In the Kiraz district, 10 *C. krusei* isolates were isolated from 20 milk samples. One *C. tropicalis* isolate was isolated from Beydag, and one *C. albicans* isolate was isolated from Odemis. In the Tire district, no mycotic growth was detected.

Genotypic Identification: A commercial kit was used to extract DNA from 23 *Candida* sp. isolates typed on chromogenic agar, and universal fungal primers (ITS3 and ITS4) were used in PCR reactions. As a result of PCR analysis, it was determined that 20 *C. krusei* isolates were banded at 335 bp, two *C. albicans* isolates at 339 bp, and one *C. tropicalis* isolates at 329 bp in the Vilber Lourmat imaging and analysis system (Fujita et al., 2001). ITS3-ITS4 primers were used to identify genotypically 23 *Candida* sp. isolates obtained by phenotypic methods.

Sanger Sequencing: Twenty-three isolates were identified as *C. krusei*, *C. albicans*, and *C. tropicalis,* with sequence analysis of 23 *Candida* isolates described as phenotypic and genotypic. It was determined that 11 of the *C. krusei* isolates showed >97% similarity to the previously described "*Pichia kudriavzevii* ZKI117 5.8S ribosomal gene", six of the *C. krusei* isolates to "*Pichia kudriavzevii* isolate L-012 small subunit ribosomal RNA gene", two *C. krusei* isolates to "*Pichia kudriavzevii* isolate 3 internal transcribed spacer 1", and one *C. krusei* isolates to "*Pichia kudriavzevii* LL11_078 18S ribosomal gene". The *C. albicans* strains showed >97% similarity to the "*Candida albicans* isolate B02 5.8S ribosomal gene". One *C. tropicalis* isolate had >97% homology to "*Candida tropicalis* isolate CTR1201 18S ribosomal gene".

The phenotypic, genotypic, and sequencing results of Candida isolates are shown in Table 1.

Antifungal Susceptibility Test: As a result of the antifungal susceptibility tests, all *Candida* isolates (n=23) were resistant to metronidazole, flucytosine 100%, and flucanasole and micanasole 95%. On the other hand, it was determined that all *Candida* isolates (n=23) were sensitive to nystatin and ketoconazole at different percent degrees. The distribution of resistance of *Candida* isolates to antifungal agents is given in Figure 1.

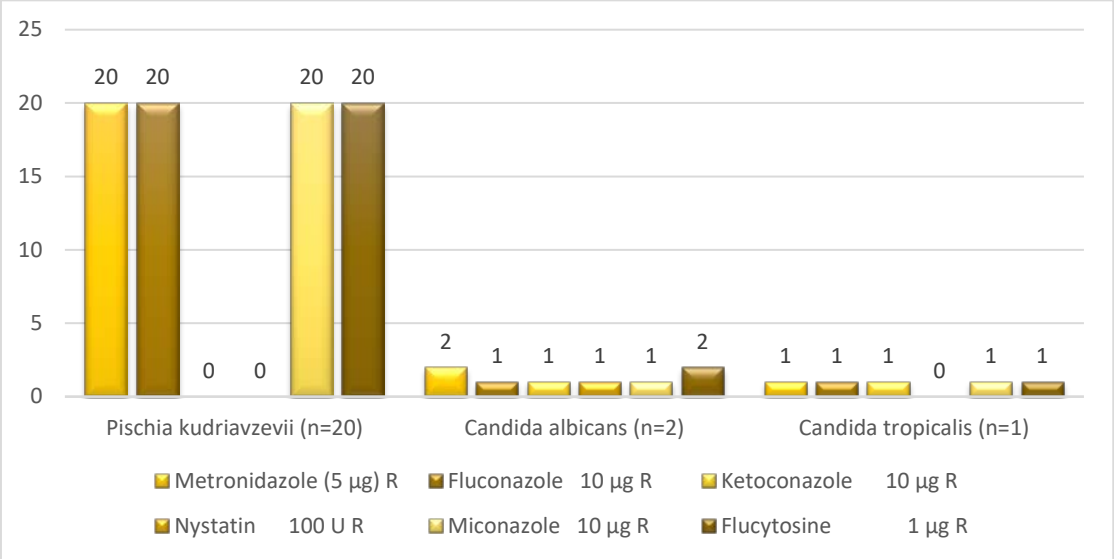

Figure 1. Antifungal resistance of *Candida* isolates

Table 1. Phenotypic, genotypic and sequencing results of *Candida* isolates

Sample No	Sample Name	Growth on SDA	Growth on Chromogenic Agar	Identification with Chromogenic Agar	ITS3/ ITS4 PCR Results	Sequencing Results
1	BA1	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
2	BA 3A	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
3	BA 6	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
4	BA 8	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
5	BA 9	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
6	BA 10	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
7	BA 11	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
8	BA 13	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
9	BA 14B	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
10	BA 15	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
11	BA 18	Positive	Positive	*C. albicans*	Positive	*Candida albicans*
12	BYD 20	Positive	Positive	*C. tropicalis*	Positive	*Candida tropicalis*
13	K 2	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
14	K 4	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
15	K 7	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
16	K 8	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
17	K 10	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
18	K 12	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
19	K 13	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
20	K 14	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
21	K 17	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
22	K 20	Positive	Positive	*C. krusei*	Positive	*Pischia kudriavzevii*
23	OD 1	Positive	Positive	*C. albicans*	Positive	*Candida albicans*

BA: Bayindir; BYD: Beydag; K: Kiraz; OD: Odemis

Discussion and Conclusion

The yeast has evolved to be a successful commensal in the healthy host. It displays variable characteristics required for survival on mucosal surfaces. The same features become virulence characteristics in abnormal conditions, increasing the yeast's invasive capacities. (Sartori et al., 2014). *Candida krusei* and *Candida kefyr* (non-albicans) are the most isolated species from bovine mastitis. *Candida krusei* has been identified as a cause of bovine mastitis since the 1970s. *Candida krusei* is considered an environmental pathogen, and the occurrence of mastitis is often linked to inadequate hygiene (Dworecka-Kaszak et al., 2012; Turkyilmaz and Kaynarca, 2010; Wawron et al., 2010; Zaragoza et al., 2011).

In research by Seker (2010), 207 (12.7%) *Candida* sp. isolates were found in 1620 subclinical and clinical mastitis milk samples in Turkey (Izmir and Afyon). *Candida* isolates were identified as *C. krusei*, *C. kefyr*, *C. albicans*, and *C. tropicalis*, respectively (34.8%), (12.6%), (10.1%), (10.1%). Turkyilmaz and Kaynarca (2010) determined that 41 of 339 milk samples with subclinical mastitis in Aydin province in Turkey were positive for yeast, and 34 of the isolates were *Candida* species that were isolated as *C. krusei* (36.6%), *C. kefyr* (29.4%), *C. guilliermondii* (7.3%), *C. famata* (4.9%), *C. rugosa* (2.4%) and *C. utilis* (2.4%). In a study by Zaragoza et al. (2011), they examined 1095 milk samples and isolated yeast in 282 (25.75%) samples. They isolated 23.39% of yeast from healthy cows, 9.92% from cows with subclinical mastitis and 43.27% from cows with clinical mastitis. In the

clinical mastitis group, 40.24% *C. krusei* and 23.17% *C. glabrata* were identified.

Sartori et al. (2014) examined 428 milk samples from cattle with clinical or subclinical mastitis in Brazil and found that 55 (12.8%) samples were *Candida* positive. Du et al. (2018) isolated 256 (51,1%) pathogenic yeast isolates from 482 cattle with clinical mastitis in China. *Candida* sp. was isolated in 60 (23.44%) of the 256 yeast isolates, and *C. krusei* and *C. parapsilosis* were the most common species in clinical mastitis. Sonmez and Erbas (2017) analyzed 100 milk samples with mastitis in Tekirdag, Turkey, and obtained 25 *Candida* isolates. *C. krusei* (32%), *C. albicans* (20%), *C. boidinii* (12%), *C. kefyr* (12%), *C. famata* (12%), *C. spherical* (8%) and, *C. thermophilia* (4%) were identified.

Candida krusei prevalence was reported as 11.25% by Zaragoza et al (2011), 34.6% by Sartori et al (2014), and 23% by Du et al (2018). In studies conducted in Turkey, *C. krusei* prevalence was reported as 34.8% by Seker (2010), 36.6% by Turkyilmaz and Kaynarca (2010), and 32% by Sonmez and Erbas. In these studies, *C. krusei* is the most common species.

In this research, *C. krusei* was found in 87% of the 23 *Candida* isolates. This study found that *C. krusei* isolation was higher in the study region than other species, which appears to be consistent with the previous investigations.

In Poland, Dworecka-Kaszak et al. (2010) obtained 55 *Candida* sp. from 66 mastitis milk samples. It was stated that 25 (45%) isolates were *C. parapsilosis*, 15 (27%) were *C. krusei*, 5 (9%) isolates were *C. lusitaniae*, 5 (9%) isolates were *C. famata*, 3 (5%) isolates were *C. guilliermondii*, 1 (2%) isolate was *C. albicans* and 1 (2%) isolate was *C. tropicalis*. Milanov et al. (2014) conducted a study with 332 milk samples with mastitis, and *Candida* sp. were isolated in 20 samples. It was reported that they were identified as 8 (40%) *C. kefyr*, 6 (30%) *C. rugosa,* and 6 (30%) *C. krusei*. Eldesouky et al. (2016) found that the prevalence of *Candida* sp. was 27.3% (n=41) in 150 milk samples with mastitis in Egypt. The prevalence of *C. albicans* (29%), *C. tropicalis* and *C. guilliermondii* (19.5%), *C. glabrata* (14.6%), and *C. krusei* (12.2%), and *C. kefyr* (4.9%) has been found among the identified isolates. Erbas et al. (2017) isolated 17.7% *Candida* sp. from milk samples with mastitis in Aydin, Turkey. It was stated that the isolates were identified as *C. tropicalis, C. parapsilosis*, *C. kefyr*, *C. krusei*, *C. rugose*, *C. glabrata* respectively (26.1%), (21.7%), (17.4%), (17.4%), (13%), (4.4%).

Cilvez and Turkyilmaz (2019) examined 400 milk samples with subclinical mastitis in Denizli, Turkey, and the presence of *Candida* sp. was determined in 96 (24%) samples. According to their sequence results, they were identified as *C. parapsilosis* (22.9%), *C. krusei* (21.9%), *C. keyfr* (19.8%), *C. albicans* (16.7%), *C. tropicalis* (9.38%), *C. glabrata* (4.2%), *C. guilliermondii* (3.1%), *C. lipolytica* (1.0%), *Trichosporon asahii* (1.0%) with >97% similarity rate at Genbank BLASTN. Onwuhafua et al. (2018) identified 12% yeast isolate in a study of subclinical cattle mastitis in Nigeria. Eleven of these isolates were analysed with DNA sequencing, and 7 of them were identified as *P. kudriavzevii* with a 93%-100% similarity rate at Genbank BLASTN. Hayashi et al. (2012) reported that *Pichia kudriavzevii* is the most common isolated species of cattle mastitis. It was stated that the sequence data they obtained were analyzed with the BLAST system of the National Biotechnology Information Center, and isolates with >99 % similarity were considered conspecific.

In the present research 23 *Candida* isolates were identified as *C. krusei*, *C. albicans*, and *C. tropicalis* with sequence analysis. It was determined that all of the *Candida* isolates showed a >97% similarity rate at Genbank BLASTN. The high similarity rates in this research are thought to be due to regional similarities and differences. When the present study is compared to the previous investigations, it is clear that the isolation rates of *C. krusei* differ and that the dominant *Candida* species vary. Changes in *C. krusei* isolation rates might be related to variances in the farms' sanitary conditions. At the same time, the different rates of isolation of *Candida* species between different geographical regions may result from various factors such as mistaken administration of intramammary antibacterial therapy, yeast-contaminated food or environment, and milking procedures.

Milanov et al. (2014) found that *Candida krusei* isolates were resistant to flucytosine and fluconazole. Du et al. (2018) reported that 14 *Candida krusei* isolates were resistant to fluconazole, ketoconazole, itracanazole and flucytosine. They also stated that 2 *Candida krusei* isolates were resistant to amphotericin and 8 *Candida krusei* isolates were resistant to nystatin. Sonmez and Erbas (2017) found that 25 *Candida* isolates were 100% resistant to amphotericin B, flucytosine, fluconasole, and miconazole in their study in Turkey. In another study by Erbas et al. (2017), it was reported that *Candida krusei* isolates were 100% resistant to flucytosine and fluconazole and 50% to miconazole. In this research 23 *Candida* isolates were resistant to 100% miconazole and flucytosine. *Candida krusei* (n=20) isolates were resistant to 100% fluconazole and metronidazole, except miconazole and flucytosine, which is critical for research results. Based on these data, *Candida krusei* is naturally resistant to fluconazole (Jamiu et al., 2021).

Resistance to other antifungals is considered to emerge due to the antifungals used in the therapy.

Bovine mastitis is one of the infections with the most significant economic impact on farms. In addition to health burden, it also causes irreversible damage to the udder that has lost its health. Studies that previously focused on *C. albicans* are now changing to the point that non-albicans *Candida* species (especially *Candida krusei*) play an essential role in fungal mastitis. This study revealed that *Candida* species from milk with mastitis differ according to regions, and the dominant species is *C. krusei,* both conventionally and molecularly. In antifungal susceptibility tests, it was determined that *Candida* isolates developed resistance to antifungals. Consequently, it was found that in farms with *Candida*-origin mastitis, thorough separation of the causative organisms and continued treatment operations with antifungal medicines indicated by antifungal susceptibility testing of the obtained isolates would be economically beneficial.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Similarity Rate

We declare that the similarity rate of the article is 12% as stated in the report uploaded to the system.

Author Contributions

Motivation / Concept: HTYD, SK, VÖ
Design: HTYD, SK, VÖ
Control/Supervision: HTYD, SK, VÖ
Data Collection and/or Processing: HTYD, SK,VÖ
Analysis and / or Interpretation: HTYD, SK, VÖ
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