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Research Article / Araştırma Makalesi

Determination of Virulence Factors in Candida albicans Isolated from Cattles with Mastitis

Mastitisli İneklerden İzole Edilen Candida albicans İzolatlarında Virülens Faktörlerinin Belirlenmesi

Orçun SAV[1], Dilek ÖZTÜRK[2]*

[1]Veterinarian, Antalya, Turkey
[2]Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology, Burdur, Turkey

Abstract: In recent years, the importance of yeast in bovine mastitis etiology is increasing. The aim of this study was to determine the prevalence of *Candida albicans* in milk samples collected from cows with mastitis, to identify the phenotypic and genotypic methods and to determine the virulence factors. According to the results of the surveys, 686 milk samples were collected from 178 cows with mastitis problems from 20 dairy cattle farms. Forty nine yeast isolates were obtained from these samples. Five isolates were identified as *C. albicans* with phenotypic tests (germ tube test, chlamydospor formation, chromogenic medium and reproduction at 45°C). Five isolates identified as C. albicans by phenotypic tests were confirmed by PCR using specific primers for detection CALB1 gene. It was determined that 2 (40%) of the *C. albicans* isolates had *ALS*1 and *PLB*1, 1 (20%) had *ALS*1 and 1 (20%) had *PLB*1 genes and no gene was found in 1 (20%) isolate. When the biofilm formation properties of *C. albicans* isolates were examined by tube adherence method, it was determined that 3 (60%) isolates were strong positive, 1 (20%) was weak and 1 (20%) isolate was negative. In conclusion, *C. albicans* isolates which were isolated from cows with mastitis had *ALS*1 and *PLB*1 genes and biofilm formation.

Keywords: *Candida albicans*, Mastitis, Virulence genes.

Öz: Son yıllarda sığır mastitis etiyolojisinde mayaların önemi giderek artmaktadır. Bu çalışmada mastitisli ineklerden toplanan süt örneklerinde *Candida albicans*'ın neden olduğu mastitis prevalansının belirlenmesi, *C. albicans*'ın fenotipik ve genotipik yöntemlerle identifikasyonu ve virülens faktörlerinin saptanması amaçlandı. Bu amaçla anket sonuçlarına göre mastitis problemi bulunan 20 süt sığırı işletmesinden 178 inekten 686 süt örneği toplandı. Bu örneklerden 49 maya izolatı elde edildi. Fenotipik testlerle (germ tüp testi, klamidospor oluşumu, kromojenik besiyerinde ve 45°C'de üreme) 5 izolat *Candida albicans* olarak identifiye edildi. Fenotipik testlerle *C. albicans* olduğu saptanan 5 izolat, *C. albicans* CALB1 genine spesifik primerler kullanılarak yapılan PZR ile doğrulandı. *C. albicans* izolatlarında adhezyondan (*ALS*1) ve fosfalipaz (*PLB*1) üretiminden sorumlu virülens genleri araştırıldı. İzolatların 2 (%40)'sinde *ALS*1 ve *PLB*1, 1 (%20)'inde *ALS*1 ve 1 (%20)'inde *PLB*1 genleri saptanırken, 1(%20) izolatta her iki genin de bulunmadığı belirlendi. *C. albicans* izolatlarının tüp adherens yöntemiyle biyofilm oluşturma özellikleri incelendiğinde 3 (%60) izolatın güçlü pozitif, 1 (%20) izolatın zayıf ve 1 (%20) izolatın ise negatif olduğu belirlendi. Sonuç olarak, bu çalışma ile mastitisli ineklerden izole edilen *C. albicans* izolatlarında önemli virülens genleri olan *ALS*1 ve *PLB*1 genleri ve biyofilm oluşumu belirlendi.

Anahtar Kelimeler: *Candida albicans*, Mastitis, Virülens genleri.

*Corresponding author : Dilek ÖZTÜRK e-mail : dozturk@mehmetakif.edu.tr
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Introduction

Mastitis is the most prevalant and economically important problem of dairy cattle in the worldwide (Eldesouky et al., 2016). A wide variety of microorganisms including bacterial and fungal agents have been found as causative agents of bovine mastitis (Erbaş et al., 2017). Although mycotic mastitis is less common in cattle compared to bacterial infections, *Candida* species

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are usually isolated from these cases. *Candida albicans (C. albicans)* is the most frequently isolated *Candida* species (Costa et al., 1993; Şeker and Özenç, 2011; Pachauri et al., 2013). Machine or manual milking of contaminated milk, indiscriminate and frequent use of antibiotics by animal owners and veterinarians, intramammary administration of contaminated antibiotics, cannulas and injectors cause yeast infections (Dworecka-Kaszak et al., 2012). *C. albicans* and its' spores are also important for public health as they can resist pasteurization heat (Schmitt, 1971; Tarfarosh and Purohit, 2008).

C. albicans is identified by conventional methods and biochemical tests, but these tests may take a long time from days to weeks. Furthermore, identification by phenotypic tests may sometimes fail to identification of *C. albicans* (Tarini et al., 2010). Molecular detection, especially Polymerase chain reaction (PCR) is rapid, sensitive and specific on detection of fungal DNA sequences (Pincus et al., 2007). *C. albicans* has several virulence factors that appear critical for pathogenicity such as adhesion and hydrolytic enzymes secretion. Phospholipase B1 (*PLB*1) is considered as one of important virulence factors and phosphalipases damage the cell membrane by hydrolyzing membrane lipids. So far, it has been reported that only *PLB*1 is detected in animal candidiasis cases (Novarro-Garcia et al., 2001; Eldesouky et al., 2016; Mousa et al 2016). Agglutinin-like Seguence (ALS) protein encodes large cell surface glycoproteins in *C. albicans* and provides adhesion to host surfaces. Upto date, nine ALS proteins have been identified in *C. albicans* isolates (Hoyer, 2001).

The aim of this study was to determine the prevalence of *C. albicans* in dairy cattle herds and to detect virulence genes of *C. albicans* isolated from bovine mastitis.

Materials and Methods

Samples

This study was conducted between August 2017 and June 2018 in dairy cattle farms in Burdur province of Turkey. Firstly, a questionnaire including questions about whether there was mastitis problem or not, treatment, antibiotics used, or not were made by owner. Enterprises with mastitis problems were selected to the study according to the results of the survey. The herd sizes were ranged from 13 to 60 animals. A total of 686 milk samples were taken from 178 cows in 20 different farms (Table 1).

This research was carried out with the approval of Mehmet Akif Ersoy University, Research Animal Local Ethics Committee (MAKÜ-HADYEK / 2017-315).

Milk samples were collected from cattle herds with clinical and subclinical mastitis problems. Before taking the milk sample, teat ends were cleaned and wiped using 70% alcohol. After the first few drops were thawed and discharged, 10 ml of the milk samples were taken into separate sterile tubes and send to Burdur Mehmet Akif Ersoy University Faculty of Veterinary Medicine, Department of Microbiology Laboratories.

Isolation and Identification of Candida albicans

Milk samples were cultured in Sabouraud Dextrose Agar (SDA) (Oxoid, Hampshire, UK) were suplemented with containing chloramphenicol (Oxoid, Hampshire, UK) and Blood agar (Merck, Germany) with 7% sheep blood. Petri dishes were incubated at 25°C for 24-72 hours aerobically Yeast identification were conducted by conventional methods such as Gram staining, germ tube, chlamydospor formation, reproduction at 45 °C and chromogenic medium (Quinn et al., 2011; Arda, 2015).

Germ Tube Formation

The first test used in identification of *Candida* species is germ tube . Each isolate was inoculated into a tube containing 1ml human serum and

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incubated at 37°C for 2 hours. One drop of suspension was placed to slide. Hyphae like extentions were accepted to be positive for germ tube formation at the microscopic examination (Quinn et al., 2011).

Table 1. Number of samples and enterprises.

Farm No	Location	Number of Animals	Number of Samples	Herd Size
1	Kayaaltı	7	27	45
2	Kayaaltı	4	15	21
3	Kayaaltı	7	27	30
4	Suludere	10	38	27
5	Suludere	10	40	24
6	Çine	10	38	32
7	Kuruçay	9	35	17
8	Düğer	11	42	55
9	Taşkapı	11	44	40
10	Taşkapı	8	30	20
11	Ardıçlı	7	26	30
12	Askeriye	6	22	23
13	Akyaka	10	38	48
14	Centrum	10	40	13
15	Yazıköy	10	39	32
16	Yazıköy	10	36	35
17	Yazıköy	9	35	60
18	Yazıköy	11	42	33
19	Yazıköy	8	32	25
20	Kemer	10	40	59
Total		**178**	**686**	**669**

Chlamydospor Formation

Chlamydospore formation test was performed on Corn Meal agar (Oxoid, Hamshire, UK) suplemented with Tween 80 (Merck, Merck Millipore Corporation, Almanya). According to this, a loop yeast colony was pierced to medium at a point of about 30 degrees, pressed to the bottom of the medium and pushed forward in a line and withdrew. The lines are covered with coverslip and petri dishes were incubated at 26 ºC for 72 h aerobically The petri dishes were examined at magnification for 10x ve 40x by light microscopy. Detection of big, thin wall and round, and hyphae blastospores at the junction of chlamydospores which were evaluated to be positive for *C. albicans* (Yücel and Kantarcıoğlu, 1999).

Molecular Identification

DNA extraction was performed using a commercial yeast DNA extraction kit (Yeast DNA Preparation Kit, Jena Bioscience, Germany). DNA samples were stored at -20 °C until use. The specific primers (*CALB1*) for the ITS region of *C. albicans*, were determined by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 2). PCR was performed in 25 µl reaction mixture (5 µl target DNA, 12.5 µl PCR master mix (2X) (Thermo Fisher Scientific, Inc., USA), 1 µl each

primer (10 Mm), 5.5 µl ddH$_2$O) (Eldosouky et al 2016). An initial denaturation (at 95 °C, 5 min) was followed by 35 cycles containing denaturation in 94 °C, 1 min, annealing in 52 °C, 1 min and extention in 72 °C, 1 min and chain extension at 72 °C, 5 min. PCR products (5 µl) were stained with ethidium bromide in TAE (Thermo Scientific, USA) containing 1.5% agarose gel and visualized under the UV light (Edas 290, Eastman Kodak Company, Rochester, NY, USA). The bands in 273 bp were evaluated as *C. albicans* positive (Susever and Yegenoglu, 2012). *C. albicans* ATCC 90028 strain as positive control and sterile bidistile water as negative control in this study.

Detection of Virulence Genes

The amplification of *PLB1* gene was performed as described by Eldesouky et al (2016). In this study, it was used spesific primers for *PLB1* gene described by Mukherjee et al. (2001) (Table 2). Amplification was performed in 25 µl PCR reaction mixture containing 5 µl target DNA, 12.5 µl 2X master mix, 5.5 µl ddH$_2$O, 1 µl primer F (10 µM) and primer R (10 µM). Amplification was performed in an initial denaturation step at 94°C for 5 minutes and followed by 35 cycles (at 94 °C for 1 minutes, 47 °C for 1 minutes and at 72 °C for 1 minutes) and a final extention at 72 °C for 5 minutes).

Table 2. Primers coded *C. albicans* and virulence factors.

Target genes	Primer sequences	DNA sizes
CALB1	Forward 5'-TTTATCAACTTGTCACACCAGA-3' Reverse 5'-ATCCCGCCTTACCACTACCG-3'	273 bp
*PLB*1	Forward 5'-ATGATTTTGCATCATTTG-3' Reverse 5'-AGTATCTGGAGCTCTACC-3'	751 bp
*ALS*1	Forward 5'-GACTAGTGAACCAACAAATACCAGA-3' Reverse 5'-CCAGAAGAAACAGCAGGTGA-3'	318 bp

bp: base pair.

PCR for *ALS1* gene was done in 25 µl PCR reaction mixture (5 µl target DNA, 12.5 µl 2X master mix, 1 µl of primer F (10 µM) and primer R (10 (M), 5.5 µl ddH$_2$O) as described by İnci et al 2013. The spesific primer for *ALS1* was shown in Table 2. Amplification was done in an initial denaturation at 94°C for 4 minutes followed by 35 cycles (at 94 °C for 30 second, 52 °C for 1 minutes and at 72 °C for 2 minutes) and a final extention at 72 °C for 5 minutes.

PCR products were electrophoresed in 1.5 % agarose gel with ethidium bromide. *C. albicans* ATCC 90028 strain as positive control and sterile bidistile water as negative control were used.

Biofilm Formation

Biofilm production ability of *C. albicans* isolates were determined by tube adherens methods as described by Christensen et al (1995). A loop ful of *C. albicans* from the SDA was inoculated into tube containing sterile 10 ml TSB medium with glucose. The tubes were incubated at 37 °C, for 24 hours. At the end of this period, the tube contents were discharged into the jar with disinfectant, washed 3 times with phosphate buffer saline (PBS) (pH 7.2). Dried tubes were stained with 1% crystal violet for 3 hours. The stain was removed from tubes and the tubes were inverted and allowed to dry. Tubes were observed for biofilm formation. The presence of colored layers in the inner wall of the tubes was evaluated as "positive". Test was performed in

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twice and repeated for three times. *C. albicans* ATCC 90028 as positive control and sterile TSB as negative control were used.

Results

Isolation and Identification of C. albicans

Out of 686 milk samples, 49 (7.14 %) yeast isolates were detected in this study. The isolates were identified as *Candida* spp. to according cultural and morphological characteristics. 5 (10.20%) of yeast isolates were described as *C. albicans* by germ tube test and chlamydospore formation. While these isolates were shown blue-green color in Chromogenic agar, non-*C. albicans* isolates were shown pink color. Also, the isolates were growth in 45 °C.

Molecular Identification of C. albicans

The isolates identified by conventional methods as *C. albicans* were confirmed by using spesific gene (*CALB*1) encoded ITS region (Fig 1).

*ALS*1 and *PLB*1 genes were detected in 3 (60%) each of these isolates (Fig. 2,3). Only two isolates were included *PLB*1 and *ALS*1 genes. *PLB*1 and *ALS*1 genes were not determined in one isolates. Only one isolate was included *ALS*1 and 1 isolate *PLB*1gene.

Biofilm Results

Biofilm production was detected to be strict positive in 3 *C. albicans* isolates, weak in 1 isolate and negative in 1 isolate (Table 3).

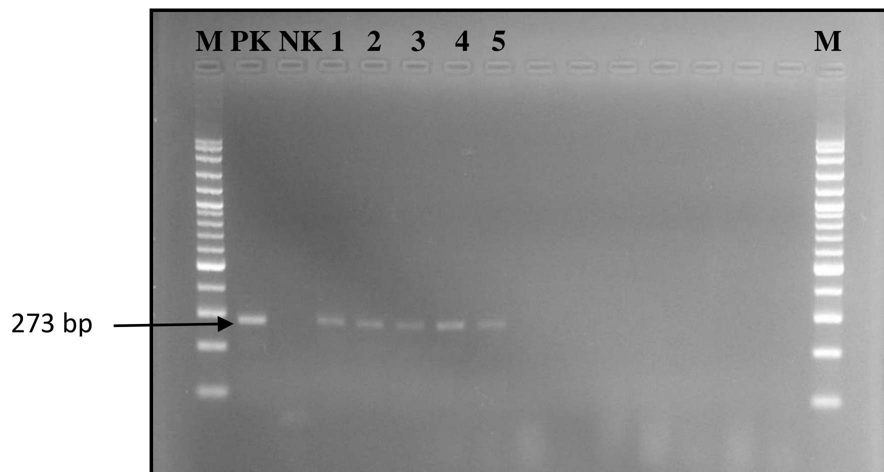

Figure 1. *CALB1* genes in *C. albicans* isolates [M: marker (100 bp); PC: positive control, *C. albicans* 90028 strain; NC: negative control, bidistilled water; 1-5: *C. albicans* isolates].

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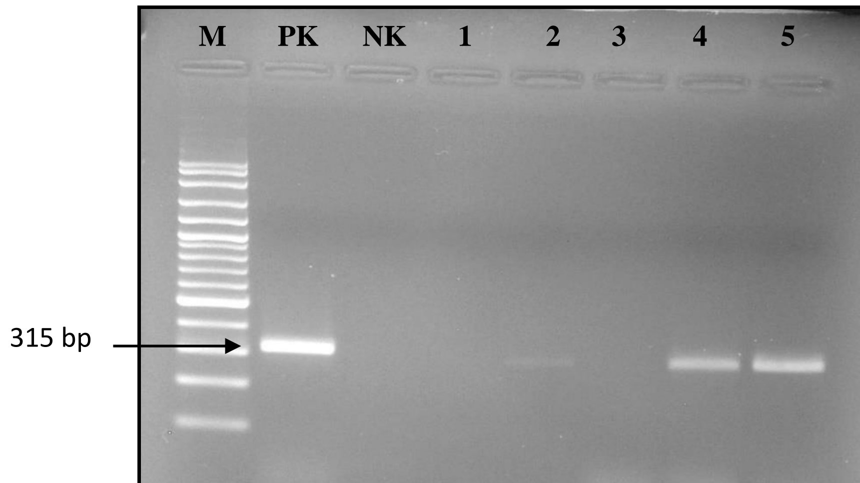

Figure 2. *ALS* genes in *C. albicans* isolates [M: Marker (100 bp); PC: Positive control, *C. albicans* 90028 strain; NC: Negative control, bidistilled water; 1,3: *ALS*1 negative *C. albicans* isolates; 2, 4-5: *ALS*1 positive *C. albicans* isolates].

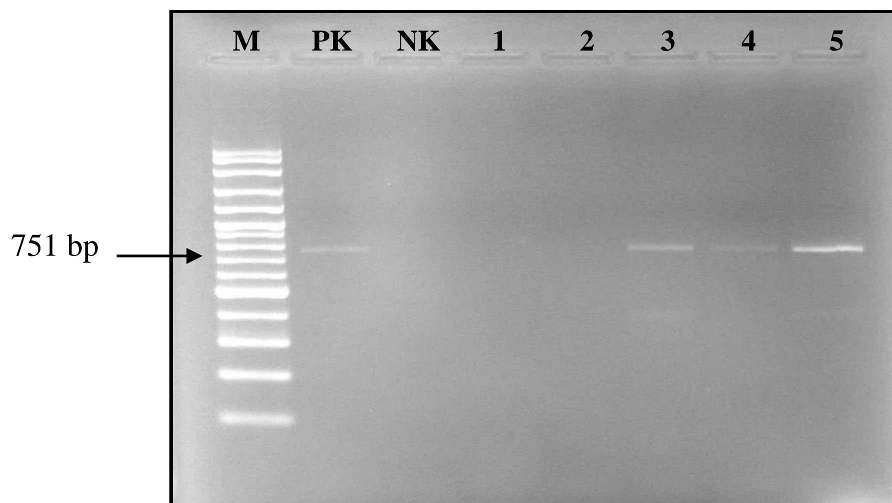

Figure 3. *PLB*1 genes in *C. albicans* isolates [M: Marker (100 bp); PC: Positive control, *C. albicans* 90028 strain; NC: Negative control, bidistilled water; 1-2: *PLB*1 negative *C. albicans* isolates, 3-5: *PLB*1 positive *C. albicans* isolates].

Table 3. Presence of biofilm formation and virulence genes in *C. albicans* isolates.

Isolates	***CALB*1**	***PLB*1**	***ALS*1**	**Biofilm**
1	+	-	-	+
2	+	-	+	+
3	+	+	-	+*
4	+	+	+	-
5	+	+	+	+
Total	**5 (100 %)**	**3 (60%)**	**3 (60%)**	**4 (%80)**

+: positive; -: negative *: weak positive

Discussion

C. albicans are often isolated from mycotic mastitis cases (Krukowski et al., 2000; Wawnon et al., 2010). In the present study, *Candida* spp. were isolated from 7.14% of milk samples. 10.20% of these isolates were identified as *C. albicans*. This

rate was found to be compatible with the results of studies conducted in Turkey and other countries (Tarfarosh and Purohit, 2008; Krukowski et al., 2000; Şeker, 2010; Türkyılmaz and Kaynarca, 2010; Wawron et al., 2010; Costa et al., 2012; Sartori et al., 2014). Some researchers (Costa et al., 1993; Krukowski et al., 2000; Santos and Marin, 2005; Mousa et al., 2016; Eldesouky et al., 2016; Erbaş et al., 2017) have reported that mycotic mastitis rate was varied between 17.7-79.4%. *Candida* species, especially *C. albicans* exists as a flora bacteria in the skin, digestive and genital systems of humans and animals (Santos and Marin, 2005). Unhygienic conditions may be one of the reasons for the high incidence of mastitis caused by *Candida* species. However, contaminated antibiotic preparations, cannulas and injector applications, and antibiotic resistance due to long-term use of antibiotics in mastitis cases can lead to *Candida* mastitis (Dworecka Kaszak et al., 2012).

In this study, 5 of the *Candida* isolates were found positive by germ tube test and were identified as *C. albicans*. Presumptive identification of *C. albicans* is generally done by germ tube test (Madhavan et al., 2011; Quinn et al., 2011). The germ tube test, which is the fastest, simplest and cheapest test for distinguishing *C. albicans* from other *Candida* species, is the gold standard (Pincus et al., 2007; Byadarally-Raju and Rajappa, 2011). However, some researchers (Mackenzie, 1962; Lipperheide et al., 1993; Kadry et al., 2018) have reported that 5-10% of *C. albicans* isolates do not form germ tubes. In the present study, it was observed that other yeast isolates formed germ tube-like structures. *C. tropicalis* and *C. parapsilosis* can form germ tube-like hyphae and these hyphae show narrowing in the region where they extend from the mother cell, which is important in distinguishing it from *C. albicans* (Yücel and Kantarcıoğlu, 1999). 5 isolates identified as *C. albicans* by germ tube test were confirmed to be *C. albicans* by detection chlamidiophore formation.

The phenotypic characterization of *C. albicans* may cause problems due to morphological, biochemical similarities with other *Candida* spp. (Yücel and Kantarcıoğlu, 1999; Kadry et al., 2018). Therefore, in recent years, the identification of *C. albicans* has been done by molecular methods. In particular, PCR is the most commondiagnostic method and in the present study, 5 isolates identified by phenotypic methods as *C. albicans* confirmed using *C. albicans* spesific CALB primers.

ALS proteins are responsible for adhesion and biofilm formation and are encoded by 8 genes (Hoyer, 2001). *ALS*1 gene was detected in 3 (80%) of *C. albicans* isolates in this study. *ALS*1 gene is important for attachingto the host and biofilm formation in the early phase of *C. albicans* infection (Kamai et al., 2002; Green et al., 2004). It is thought that other ALS proteins or adhesion mechanisms may play a role in adhesion in *ALS* negative *C. albicans* isolates. However, it is reported that the detection rate of the HWP1 adhesion gene in milk samples of cattle with mastitis is higher than *ALS*1 gene (Mousa et al., 2016). *ALS*1 and *PLB*1 could not be detected in 1 of the *C. albicans* isolates in this study. This isolate of biofilm forming ability was weakly positive. These genes are the most important virulence factors of *C. albicans* (Calderone and Fonzi, 2001). Therefore, these *C. albicans* isolates could be found in the flora. As a matter of fact, isolation of *C. albicans* from healthy animals has been reported, but virulence factors could not be detected in these isolates (Türkyılmaz and Kaynarca, 2010; Mousa et al., 2016). In this study, it was determined that 3 isolates formed strong biofilms and one isolate formed weak biofilms. Studies have shown that ALS genes are associated with biofilm (Green et al., 2004; İnci et al., 2013). When the presence of *ALS*1 gene and biofilm formation were compared, it was determined that one of the 3 isolates that formed strong positive did not carry the *ALS*1 gene. This could be explained by the presence of other ALS genes (Mousa ve ark., 2016).

There are several studies were reported that *PLB*1 gene was detected in all *C. albicans* isolates obtained from cows with clinical mastitis (Eldesouky et al.,

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2016; Mousa et al., 2016). Hakim et al (2013) were reported the presence of *PLB*1 gene in 5 *C. albicans* isolated from Kareish cheeses and Eldesouky et al (2016) were detected in all *C. albicans* isolates (n:4) isolated from bovine mastitis. Although the *PLB*1 gene was reported in *C. albicans* isolated from milk with mastitis, it was detected in only 3 of the *C. albicans* isolates. It has been reported that the *PLB*1 gene is an important virulence factor of *C. albicans*, and the virulence of the infection decreases when the mutant strains obtained by deletion of this gene are given to animals (Mukherjee ve ark, 2001).

In conclusion, the presence of *ALS*1 and *PLB*1 genes, which are important virulence genes, were detected in *C. albicans* isolated from milk samples with mastitis for the first time in Turkey. Also, it was determined that PCR was the fastest, most reliable and inexpensive method for the identification of *C. albicans*. Virulence factors detected in *C. albicans* isolates isolated from milk with mastitis were also detected in strains isolated from humans. This situation shows that these microorganisms can be transmitted to humans through raw milk and dairy products and cause public health problems.

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