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Identification of *Candida* strains with nested PCR in bovine mastitis and determination of antifungal susceptibilities

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Abstract: Mastitis is one of the biggest problems of dairy cattle industries. In recent years, fungal agents have been frequently reported among the causative agents for mastitis. Among the fungal agents, *Candida* species are the most common. This study's aims were the isolation, identification, and determination of antifungal susceptibility of *Candida* species causing mycotic mastitis in cattle. A total of 260 mastitic milk samples were collected from different farms. Identification was conducted by rapid diagnostic tests and nested PCR method; 6 different antifungal agents were examined. *Candida* sp. was detected in 46 (17.7%) of the 260 mastitic milk samples. Based on API 20 C AUX and nested PCR test results, 6 different species of *Candida* were identified. *C. tropicalis* was the predominant one (26.1%), followed by *C. parasilosis* (21.7%), *C. kefyr* and *C. krusei* (17.4% each), *C. rugosa* (13%), and *C. glabrata* (4.4%). A total of 46 strains were confirmed by PCR. According to antifungal test results, the isolates were found to be susceptible to ketoconazole (78.3%) and resistant to flucytosine (91.3%), amphotericin (82.6%), miconazole and nystatin (73.9%), and fluconazole (69.5%).

Key words: Mastitis, nested PCR, antifungal, *Candida* sp.

1. Introduction

Mastitis is the most important health problem in bovine dairy herds. Multifarious microorganisms have been implicated as causative agents of bovine mastitis, including bacteria and fungi (1). Fungal infections of the mammary gland are mostly caused by yeast, the main genus of which is *Candida* (2). Mastitis infections caused by fungi of the genus *Candida* have long been known in animals and Fleischer was presumably the first researcher to describe a case of mycotic mastitis in 1930 (3).

The prevalence of mycotic mastitis is usually very low as compared to other agents of mastitis, but it has significantly increased during the last decade. Several species of yeast or yeast-like microorganisms have been reported to cause bovine mastitis (4). *Candida*, *Cryptococcus*, *Rhodotorula*, and *Trichosporum* have been associated with mastitis in dairy cattle. Intramammal infection caused by fungi as mastitis is usually self-limiting with spontaneous recovery, and the infection generally has no systemic results. *Candida* species are the most common microorganisms among the mycotic mastitis agents isolated from infected mammary glands (5). *Candida* species have been isolated from bovine milk with and without signs of mastitis (6). *Candida* is usually the most frequently isolated genus, with great variations in prevalence among herds and identified species (1).

Recently, rapid identification and molecular biology-based tests have begun to be used more easily and effectively than conventional tests in the identification of fungal pathogens. Diagnostic polymerase chain reaction (PCR) tests have been widely applied in laboratories for identification of many fungal species due to their speed, high sensitivity, and specificity (7). Since PCR was first identified, many modifications of the original procedure have been developed. As with nested PCR, the standard procedure can be made more specific and sensitive with simple modifications. In nested PCR, PCR products are subjected to a second amplification with the second pair of primers located in the middle of the first primers (8).

The DNA topoisomerase II gene is found in all eukaryotes, and the nucleotide sequence consists of very conserved regions distributed in species-specific regions. Sequence analysis of the DNA topoisomerase II (DNA gyrase) gene in bacterial strains is not only used to determine phylogenetic relationships, but also for the development of diagnostic identification systems for PCR of large bacteria and fungi species with medical importance (9). In many studies, DNA topoisomerase II

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gene nucleotide sequences of pathogenic *Candida* strains have been identified and phylogenetic relationships and characteristics according to nucleotide sequences have been reported (9–11).

Fungal mastitis has been described as related to treatment directed against other pathogens using contaminated syringes, cannulas, or contaminated antibiotic preparations. Teat injuries may predispose to establishment of a yeast infection. Yeast intramammary infections have been reported to be responsible for at least 10% of all clinical cases seen in veterinary practice; the majority of cases are usually mild. Although antimycotic drugs have been used for treatment of yeast mastitis, there is no clear evidence of the effectiveness of this therapy (12). Yeast resistance to antimycotics has been a major problem in recent decades, *Candida* being the most important pathogenic potential yeast (2).

The aim of the present study was to identify *Candida* species by rapid identification tests and nested PCR from milk samples of cows with mastitis, and to determine the antifungal susceptibilities of the species.

2. Materials and methods

2.1. Sample collection

In this study, 260 mastitic milk samples that were collected from 10 different private dairy cattle establishments in Aydın were included. Approximately 20–30 mastitic cattle on each of the 10 dairy farms were sampled and tested for identification of *Candida* from 2015 to 2016. Mastitis was diagnosed by veterinary practitioners from changes in the udder and milk. Changes in the udder included pain, swelling, warmth, and abnormal appearance of milk (blood-tinged milk, watery secretions, clots, pus). For collection of milk samples, teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2–5 mL of the milk samples was collected into sterile glass flasks. All milk samples were brought to Adnan Menderes University, Veterinary Faculty, Department of Microbiology laboratory, under cold chain.

The authors of this research hereby declare that collection of specimens was carried out in accordance with the guidelines laid down by the US National Institutes of Health (NIH) regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Isolation and identification of *Candida*

Milk samples (100 µL) were inoculated in Sabouraud dextrose broth (Merck 108339) and then incubated at 37 °C for 24 h for the enrichment period. Thereafter, 50 µL of each broth culture was plated on Sabouraud dextrose agar (SDA, Merck 105438) with chloramphenicol (0.05 mg/mL) and incubated at 37 °C for 24–48 h. After incubation, the isolates were classified to the genus level according to their macroscopic and microscopic morphology (13). Identification of *Candida* isolates was carried out using an API 20 C AUX system (bioMérieux, France).

2.3. DNA extraction

All identified *Candida* strains were inoculated on 4% SDA and incubated at 37 °C for 24 h. After that, genomic DNA was isolated with an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's protocol. The genomic DNA was used as template DNA for PCR amplification. Genomic DNA samples were stored in –20 °C deepfreeze until used for PCR.

2.4. Primers

PCR primers were designated as CDF28 and CDR148 and were used as common primers for *Candida* species in the first round of PCR (9,10).

The species-specific primers for each of the *Candida* species were designed to be within the region amplified by the common primer pair. For the design of the species-specific primers, the nucleotide sequences of the DNA topoisomerase II genes of *Candida* species were used (9,10) (Table 1).

All species-specific primers were pooled and designated as PsV, which consisted of 5 species-specific primer pairs. PsV should amplify DNA fragments of 227 bp, 310 bp, 532 bp, 672 bp, and 777 bp corresponding to *C. krusei*, *C. parapsilosis*, *C. kefyr*, *C. glabrata*, and *C. tropicalis*, respectively (9,10).

2.5. Standard serotypes

Candida krusei ATCC 6258, *Candida parapsilosis* ATCC 22019, *Candida kefyr* ATCC 42265, *Candida glabrata* ATCC 90030, and *Candida tropicalis* ATCC 750 standard serotypes were provided by the Adnan Menderes University Faculty of Medicine, Department of Medical Microbiology.

2.6. PCR amplifications

Genomic DNA purified from each fungal culture sample was amplified in a reaction mixture (25 µL) containing 12.5 µL of 2X HRM PCR master mix, 9 µL of Milli-Q, 0.75 µL of forward primer CDF28 (5 µM), 0.75 µL of reverse primer CDR148 (5 µM), and 2 µL of template DNA. The concentration of each template DNA was adjusted to 1 pg per PCR tube. The PCR cycle parameters were as follows: preheating at 96 °C for 2 min; then 30 cycles of 96 °C for 30 s, 57 °C for 3 s, and 74 °C for 30 s. PCR products were analyzed using agarose gel electrophoresis on the 1200-bp DNA fragment.

Table 1. The common and species-specific primers for *Candida* spp. (9,10).

Fungal species and primers	Direction	Sequence (5'–3')	Product (bp)
Common primers			
CDF28[a]	F	GGTGGWMGDAAYGGDTWYGGYGC	1,200
CDR148[a]	R	CCRTCNTGATCYTGATCBGYCAT	
Specific primers			
Candida krusei			
CKSF35	F	GAGCCACGGTAAAGAATACACA	227
CKSR57	R	TTTAAAGTGACCCGGATACC	
Candida parapsilosis			
CPP2F038	F	GGACAACATGACAAAAGTCGGCA	310
CPP2R069	R	TTGTGGTGTAATCTTGGGAG	
Candida kefyr			
CKFF35	F	CTTCCAAAGGTCAGAAGTATGTCC	532
CKFR85	R	CTTCAAACGGTCTGAAACCT	
Candida glabrata			
CGBF035	F	CCCAAAAATGGCCGTAAGTATG	672
CGBR102	R	AGTCGCTACTAATATCACACC	
Candida tropicalis			
CTR2F049	F	GGACAGTTTGGATGAAGATTTA	777
CTR2R126	R	GAGACCAGCCACGGACAAATTCAAC	

[a]: W: A or T; M: A or C; Y: C or T; R: A or G; D: A, G, or T; B: C, G or T; N: A, C, G, or T.

The PCR products generated by the common primers were diluted at a ratio of 1:200 in distilled water and then used as DNA templates for the subsequent nested PCR using PsV. PsV contained 5 primer pairs (10 species-specific primers; Table 1), each of which was adjusted to 5 µM. With the exception of template DNAs and PCR primers, the nested PCR using PsV was carried out under the same parameters as those of the first round of PCR using the common primers. The reaction mixtures were amplified using a thermal cycler (Mastercycler Personal, Eppendorf Netheler Hinz GmbH, Germany).

2.7. Agarose gel electrophoresis

The PCR products were analyzed using agarose gel electrophoresis. The amplified products were electrophoresed in 1.2% agarose gel (Agarose-ME, classic type; Nacalai Tesque, Inc., Japan) with ethidium bromide at 100 V for approximately 45 min. The DNA bands were visualized with a gel documentation system (Infinity VX2, France) and photographed. PCR products were examined for *C. krusei* (227 bp), *C. parapsilosis* (310 bp), *C. kefyr* (532 bp), *C. glabrata* (672 bp), and *C. tropicalis* (777 bp).

2.8. Antifungal susceptibility testing

Antifungal susceptibility testing of all the yeast isolates was assessed using the agar disk diffusion method as per CLSI guidelines (14). Mueller-Hinton agar (Merck 105437), pH 7.2, supplemented with 2% glucose and 0.5 µg/mL methylene blue, was utilized as the culture medium. The yeast suspension was adjusted to 1–5 × 10^6 CFU/mL and was seeded onto the surface of the plates. In the present study, the antifungal agents used for the disk diffusion method were amphotericin B (20 µg, BD), ketoconazole (10 µg, BD), miconazole (10 µg, BD), fluconazole (10 µg, BD), flucytosine (1 µg, BD), and nystatin (100 units, BD). To determine whether the isolates were susceptible or had either intermediate resistance or resistance, the above antifungals' zone diameters were compared with the standard zone interpretive break points published in the CLSI M44-A2 guidelines (14).

3. Results

3.1. Isolation and identification

In this study, 46 (17.7%) *Candida* species were isolated from 260 milk samples with mastitis. Based on cultural and morphological characteristics and API 20 C AUX test results, 6 different species of *Candida* were identified. *C. tropicalis* was the predominant serotype (26.1%), followed by *C. parasilosis* (21.7%), *C. kefyr* and *C. krusei* (17.4% each), *C. rugosa* (13%), and *C. glabrata* (4.4%).

3.2. Molecular identification of *Candida* species

In this study, PCR based on common primers and species-specific universal oligonucleotide primers were used to confirm phenotypic identification of *Candida* species. According to the PCR results, a total of 46 strains were confirmed as *Candida* sp. by the first round of PCR. Species distribution of *Candida* isolates (except *C. rugosa*) was verified with the second round of PCR. Consequently, confirmation of all *Candida* isolates identified by API 20 C AUX was done by nested PCR. Some of the amplified PCR products are shown in the Figure.

3.3. Antifungal susceptibility

The isolates were found to be susceptible to ketoconazole (78.3%) and resistant to flucytosine (91.3%), amphotericin (82.6%), miconazole and nystatin (73.9%), and fluconazole (69.5%). The susceptibility of *Candida* species is given in Table 2.

The individual species of isolated *Candida* spp. showed significant differences in their susceptibility to the drugs used.

Candida kefyr was most sensitive to ketoconazole, miconazole, and fluconazole (100.0%) and nystatin (75% susceptible strains), and resistant to amphotericin and flucytosine (75%).

Candida tropicalis showed susceptibility to ketoconazole (83.3%); intermediate susceptibility to nystatin (83.3%), amphotericin (66.7%), and miconazole and fluconazole (50%); and resistance to flucytosine (83.3%).

Candida glabrata was susceptible to amphotericin (100%), intermediately susceptible to nystatin (100%), and resistant to ketoconazole, miconazole, fluconazole, and flucytosine (100%).

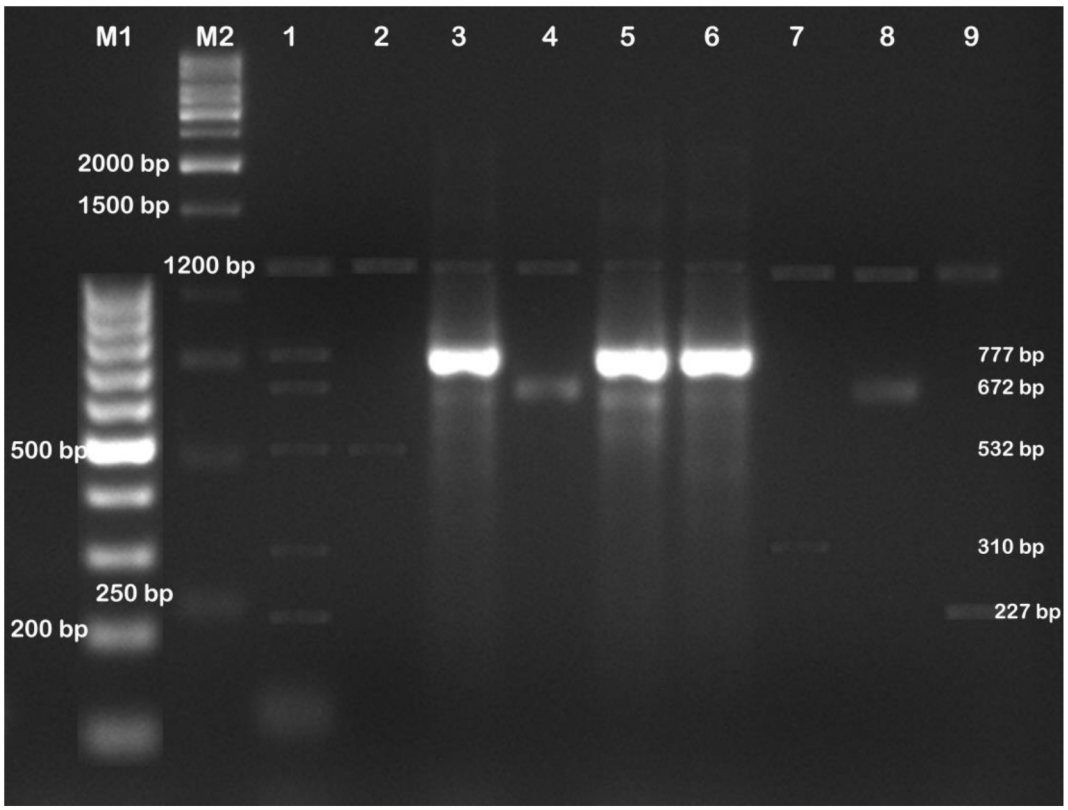

Figure. PCR results of *Candida* serotypes. M1 and M2: 100-bp DNA ladders; 2–9: *Candida* sp.-positive samples for 1200-bp fragment; 1: DNA mixture of *Candida* standard strains; 2: *Candida kefyr*-positive sample; 3, 5, 6: *Candida tropicalis*-positive samples; 4, 8: *Candida glabrata*-positive samples; 7: *Candida parapsilosis*-positive sample; 9: *Candida krusei*-positive sample.

Table 2. Antifungal susceptibility results of *Candida* species.

Species	Antifungals[a]																	
	KCA			MCL			AMB			FCN			NY			FY		
	S[b]	I[b]	R[b]	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Candida kefyr (n = 8)	8	-	-	8	-	-	-	2	6	8	-	-	6	2	-	2	-	6
Candida tropicalis (n = 12)	10	-	2	2	6	4	4	8	-	4	6	2	2	10	-	2	-	10
Candida glabrata (n = 2)	-	-	2	-	-	2	2	-	-	-	-	2	-	2	-	-	-	2
Candida parapsilosis (n = 10)	8	-	2	2	6	2	-	10	-	2	-	8	-	10	-	-	-	10
Candida krusei (n = 8)	8	-	-	-	4	4	-	2	6	-	-	8	2	6	-	-	-	8
Candida rugosa (n = 6)	2	2	2	-	2	4	2	4	-	-	-	6	2	4	-	-	-	6
Total (n = 46)	36	2	8	12	18	16	8	26	12	14	6	26	12	34	-	4	-	42

[a]: KCA: Ketoconazole (10 µg); MCL: miconazole (10 µg); AMB: amphotericin (20 µg); FCN: fluconazole (10 µg); NY: nystatin (100 U); FY: flucytosine (1 µg).
[b]: S: Susceptible, I: intermediately susceptible, R: resistant.

Candida parapsilosis was sensitive to ketoconazole (80%), intermediately susceptible to amphotericin and nystatin (100%) and miconazole (60%), and resistant to flucytosine (100%) and fluconazole (80%).

Candida krusei was sensitive to ketoconazole (100%), intermediately sensitive to nystatin (75%), and miconazole (50%), and resistant to fluconazole and flucytosine (100%) and amphotericin (75%).

Candida rugosa was resistant to fluconazole and flucytosine (100%).

4. Discussion

The prevalence of mastitis related to yeasts is usually low in comparison with other agents of mastitis. However, a high incidence of mycotic mastitis, especially that caused by *Candida* species, has been noticed recently (15). Along with the high incidence of fungal infections, an increase in the resistance to antifungal agents was reported (16,17).

Presence of *Candida* spp. in mastitic milks show differences in various parts of the world, e.g., 6.1% in Egypt (18), 1.3% in Denmark (19), and 12.08% in Brazil (6). Şeker (20) reported the *Candida* isolation rate in mastitic milks to be 12.7% in Turkey. In this study, the presence of *Candida* species in clinical cases of mastitis was determined to be 17.7%. This high incidence rate may be due to the extensive and prolonged use of antibiotics nowadays in Turkey, not only for treatment of mastitis but also as a prophylaxis during the dry periods.

In Turkey, Şeker (20) reported that the most frequently isolated species were *C. krusei*, *C. rugosa*, *C. kefyr*, *C. albicans*, and *C. tropicalis*, respectively. Some other studies have had similar results (3,21,22). According to Krukowski et al. (12), the most frequently isolated species in Poland were *C. kefyr*, *C. cirferi*, and *C. krusei*. In our research, the most frequently isolated species was *C. tropicalis* (26.1%), followed by *C. parapsilosis* (21.7%), *C. kefyr* (17.4%), *C. krusei* (17.4%), *C. rugosa* (13%), and *C. glabrata* (4.4%). *C. albicans* was not isolated. Simaria and Dholakia (23) and Sukumar and James (24) reported relatively more frequent isolation of *C. tropicalis* from mastitic udders than other species of *Candida*. In this study, *C. tropicalis* was the most frequently isolated yeast from the mastitic milk samples examined. In addition, when the data are examined, the distribution of the *Candida* percentages and species isolated in cow mastitis varies considerably. This difference is thought to be caused by long-term antibiotic usage or inappropriate hygiene conditions on the farms from which the specimens were collected. In addition, these differences may vary from year to year and may be based on isolation and identification methods.

In our study, we used the API 20 C AUX system, which is a rapid diagnostic test to distinguish *Candida* species. As indicated by Kanbe et al. (9,10), species-level differences were also detected by nested PCR. Both rapid diagnostic tests and PCR results were found to be the same in the study (except for *C. rugosa*, because primer pairs of *C. rugosa* could not be provided). When the results were evaluated, it was seen that more accurate and clear results were obtained with nested PCR. However, since nested PCR is more expensive and takes a long time, especially for routine diagnostic laboratories, the rapid diagnostic test kit is more useful.

A very detailed antifungal resistance formation was investigated in this study. The resistance statuses of 6

Candida species against 6 different antifungals are given in Table 2. According to our results, when all isolates were evaluated, 36 of the 46 strains (78%) were found to be susceptible to ketoconazole, which was the most effective agent against the *Candida* species. Nystatin was the second most effective antifungal option following ketoconazole in mastitis cases that were due to *Candida*. According to Wawron et al (25), nystatin was also the most active antifungal, along with tioconazole.

Some of the *Candida* species are naturally resistant to some antifungals, e.g., there is intrinsic fluconazole resistance in *C. krusei*. It was shown that many *Candida* isolates have resistance to amphotericin B. As resistance to flucytosine in *C. albicans* (10%–30%) is known, increasing resistance in non-*albicans Candida* species has risen. Natural or acquired resistance to fluconazole was confirmed in *C. krusei* and *C. glabrata* (26). Our findings with *C. krusei* and *C. glabrata* supported the literature results.

When the results of this research were evaluated, resistance differences among *Candida* species to antifungals were demonstrated. Therefore, when *Candida* species are isolated in cases of mastitis, it is necessary to perform identification for appropriate antifungal selection. For identification, rapid identification tests are useful and not expensive for the routine laboratories. However, especially for scientific research, molecular-based tests involve more precise data.

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