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In vitro biofilm activity of *Candida* species isolated from Anatolian buffaloes with mastitis in Western Turkey

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ABSTRACT

In the present study, identification of 66 *Candida* isolates obtained from mastitic Anatolian buffalo quarter milk samples was achieved using the API 20 C AUX system. Among the isolates, the most common species were *Candida krusei* (27.3%), followed by *Candida rugosa* (16.7%), *Candida kefyr* (12.1%) and *Candida tropicalis* (10.6%). The other species were detected as *Candida albicans* (9.1%), *Candida zeylanoides* (6.1%), *Candida parapsilosis* (6.1%), *Candida guilliermondii* (4.5%), *Candida famata* (3.0%), *Candida glabrata* (3.0%) and *Candida ciferrii* (1.5%). A total of 53 (80.3%) *Candida* isolates produced the biofilm. All *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii* strains were determined as biofilm positive. However, *C. ciferrii* did not produce any biofilm. This is the first study to investigate *in vitro* biofilm activity of different *Candida* species isolated from Anatolian buffalo mastitic milk in Turkey. We consider that this study may be a helpful tool for future research into mastitis caused by *Candida* species.

Key words: biofilm activity, buffalo, *Candida*, mastitis

Introduction

Mastitis is the most common and the most costly disease of the dairy industry all over the world that affects both the quality and quantity of milk. Several groups of microorganisms such as bacteria, yeast, fungi and algae from the *Prototheca* genus have been reported as etiological agents of mastitis (COSTA et al., 1993; COSTA et al., 2004; KRUKOWSKI et al., 2006; SPANAMBERG et al., 2008; TENHAGEN et al., 2006; WATTS, 1988). The prevalence of mastitis related to fungi and yeasts is usually low as compared with other agents of mastitis (WATTS, 1988). However, sometimes they have

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been associated with clinical mastitis in dairy herds (COSTA et al., 1993). *Candida* species are the most common microorganisms among the mycotic mastitis agents isolated from infected mammary glands (COSTA et al., 1993; KRUKOWSKI et al., 2006; SPANAMBERG et al., 2008; WATTS, 1988). It has been reported that *Candida* species can cause clinical mastitis characterized by pain, prolonged fever, tenderness, inflammatory reaction in the mammary gland and associated lymph nodes and reductions in milk yield and quality in animals (SINGH et al., 1998). They can also be isolated from animals with subclinical mastitis (ÖZENÇ et al., 2008; ŞEKER, 2010).

Besides the well-recognized virulence determinants such as hydrolytic enzymes, toxins, dimorphism, and hemolysin production (HAYNES, 2001; YANG, 2003), one of the major contributions to *Candida* virulence is its versatility in adapting to a variety of different habitats and the formation of surface-attached microbial communities known as biofilms (COSTERTON et al., 1995; DOUGLAS, 2003). Biofilms are organized into structured communities embedded within a matrix of extracellular material that is produced by the biofilm cells (DONLAN, 2002). Generally, biofilm matrix composition contains water, carbohydrates, proteins, phosphorus, glucose and hexosamines (BAILLIE and DOUGLAS, 2000; SILVA et al., 2009). Biofilms facilitate the adherence to the tissue and colonization of the *Candida* species and protect the microorganism from host defenses. Also, the formation of *Candida* biofilms has important clinical repercussions because of their increased resistance to antifungal therapy (MUKHERJEE and CHANDRA, 2004; RAMAGE et al., 2005). Therefore, biofilm formation is believed to play an important role in the pathogenesis of candidal infections in humans (DOUGLAS, 2003; RAMAGE et al., 2005; YANG, 2003). While many previous studies have focused on biofilm formation in various human clinical isolates and its importance in terms of human candidal infections (FURLANETO-MAIA et al., 2008; GOKCE et al., 2007; SHIN et al., 2002), biofilm activity exhibited by different *Candida* species isolated from animals or their products has not been reported.

Buffaloes have economic significance among livestock animals in terms of milk and meat yields, as well as for work purposes. Anatolian buffaloes are classified as a river subtype of the Mediterranean water buffalo group (BORGHESE, 2005). According to the 2009 data of the Turkish Statistical Institute (TurkStat), a population of 87,207 Anatolian buffaloes exists in Turkey. Afyonkarahisar is located in the western region of Turkey and is becoming significant as a breeding area for water buffaloes owing to its suitable conditions. This province has approximately 3% (2,558) of the total buffalo population. Although this rate seems to be low, buffalo milk is very important in Afyonkarahisar and the milk products, especially traditional clotted cream, obtained from these animals are sought-after Turkish products.

Although buffaloes have been traditionally considered less susceptible to mastitis than cattle (WANASINGHE, 1985), some researchers have shown similar mastitis frequencies for the two species (BACHAYA et al., 2005; SHARIF and AHMAD, 2007). Studies of the ecology and prevalence of mastitis in Anatolian buffaloes in Turkey are limited (ÖZENÇ et al., 2008). Investigations into the identification of *Candida* isolates at the level of species from Anatolian buffaloes have not been reported so far in Western Turkey.

The aim of this study was to identify *Candida* species isolated from Anatolian buffalo mastitic milk and determine their *in vitro* biofilm activity in Western Turkey. To our knowledge, this is the first study identifying *Candida* isolates from Anatolian buffaloes and determining biofilm production among *Candida* species in Turkey.

Materials and methods

Collection of samples and identification of Candida species. The total of 66 *Candida* isolates used in the present study were isolated from 324 quarter milk samples belong to Anatolian buffaloes with clinical and subclinical mastitis between January 2007 and December 2009 from Afyonkarahisar, located in Western Turkey. Before sampling, the teat ends were cleaned using 70% alcohol and dried. The first streams of foremilk were discharged, and then 10 ml of milk was collected aseptically in sterile tubes. Samples were immediately transported to the laboratory in a cool box on ice. Ten microliters of each milk sample was inoculated onto Sabouraud dextrose agar (SDA, Oxoid CM0041) supplemented with chloramphenicol and gentamicin. The plates were incubated under aerobic conditions for 24-48 h at 37 °C. After the incubation, the isolates were previously classified at the level of genus according to their macroscopic and microscopic morphology (QUINN et al., 2002). Identification of *Candida* isolates was done using the API 20 C AUX system (bioMérieux, Marcy-l'Etoile, France).

Determination of biofilm formation. Biofilm formation was determined by using a modification of the test established for Coagulase-negative staphylococci (CHRISTENSEN et al., 1985). For this purpose, a loopful of organisms from the surface of SDA plate was inoculated into a polystyrene falcon conical tube containing 10 ml Sabouraud liquid medium (SLM, Oxoid CM0147) supplemented with glucose (final concentration 8%). The tubes were incubated at 37 °C for 48 h. After the incubation, the broth in the tubes was gently aspirated, and tubes were washed with distilled water twice and then stained with 1% safranin for 10 min, after which they were examined for the presence of an adherent layer. Biofilm production was scored as negative, weak (+), moderate (++), or strong (+++) positive (CHRISTENSEN et al., 1985). Biofilm producer *Staphylococcus epidermidis* ATCC 35984 was used as a positive control. Each isolate was tested at least three times.

Results

Identification findings. The most frequently isolated *Candida* species from Anatolian buffaloes with mastitis were *Candida krusei* (27.3%), followed by *Candida rugosa* (16.7%), *Candida kefyr* (12.1%) and *Candida tropicalis* (10.6%). The other species identified from Anatolian buffalo quarter milk samples were shown in Table 1.

Table 1. *Candida* species identified from Anatolian buffalo quarter milk samples in this study and their biofilm activity

			Biofilm activity					
			(+++)[a]	(++)[b]	(+)[c]		(-)[d]	
Species	n	%	n	n	n	%	n	%
C. krusei	18	27.3	15	3	-	100	-	-
C. rugosa	11	16.7	4	3	1	72.7	3	27.3
C. kefyr	8	12.1	2	2	1	62.5	3	37.5
C. tropicalis	7	10.6	5	1	1	100	-	-
C. albicans	6	9.1	-	2	1	50.0	3	50.0
C. zeylanoides	4	6.1	1	1	1	75.0	1	25.0
C. parapsilosis	4	6.1	1	2	1	100	-	-
C. guilliermondii	3	4.5	2	1	-	100	-	-
C. famata	2	3.0	-	-	1	50.0	1	50.0
C. glabrata	2	3.0	1	-	-	50.0	1	50.0
C. ciferrii	1	1.5	-	-	-	-	1	100
Total	66	100	31	15	7	80.3	13	19.7

a: strong positive; b: moderate positive; c: weak positive; d: negative

Biofilm formation. A total of 53 (80.3%) of 66 *Candida* species obtained from mastitic milk samples were determined as biofilm positive. All *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii* strains were biofilm positive. The only *C. ciferrii* did not produce any biofilm. The distribution of biofilm formation in different *Candida* species is shown in Table 1.

Discussion

In this study, we described for the first time the *Candida* species isolated from Anatolian buffalo mastitic milk samples and their *in vitro* biofilm activity in Turkey.

Fungal infections of dairy cow's mammary glands are predominantly caused by yeast of the *Candida* genus (COSTA et al., 1993; KRUKOWSKI et al., 2000; PENGOV, 2002; SPANAMBERG et al., 2008). It was reported that the distribution of *Candida* species isolated from bovine mastitic milk showed diversity in several surveys (KRUKOWSKI

et al., 2000; PENGOV, 2002; SANTOS and MARIN, 2005; WAWRON et al., 2010). PENGOV (2002) emphasized that *C. krusei, C. rugosa, C. tropicalis, C. albicans*, and *C. kefyr* were predominantly isolated from cows with mastitis. While KRUKOWSKI et al. (2000) showed that the most frequently isolated species were *C. kefyr*, *C. ciferrii*, and *C. krusei*, WAWRON et al. (2010) reported that *C. krusei, C. kefyr*, and *C. lusitaniae* were the most common species. In another study, *C. krusei* and *C. rugosa* were reported as the most common species isolated from bovine mastitic milk samples (SANTOS and MARIN, 2005). Similar to our previous study, which investigated *Candida* species isolated from cows with mastitis (ŞEKER, 2010), in this study, *Candida krusei* (27.3%), *Candida rugosa* (16.7%), *C. kefyr* (12.1%) and *C. tropicalis* (10.6%) were commonly isolated from Anatolian buffaloes with mastitis. The animal species and geographical variations may be the reason for the discrepancy in the distribution of species.

Biofilms have been considered as an important virulence factor in the pathogenesis of infections because biofilm-associated microorganisms show an innate resistance to antibiotics, disinfectants and clearance by host defense mechanisms (COSTERTON et al., 1995; DONLAN, 2002). Although bacterial biofilms and their role in infections have been examined in detail over a number of years, much less is known about fungal or yeast biofilms. Generally, studies have focused on the biofilm activity of human clinical *Candida* isolates (FURLANETO-MAIA et al., 2008; GOKCE et al., 2007; KUMAR and MENON, 2006; SHIN et al., 2002) and bacterial isolates isolated from bovine mastitic milk (FOX et al., 2005; OLIVERIA et al., 2006). Researchers investigating biofilm production among human *Candida* isolates have detected higher levels of positivity among non-*albicans Candida* strains than of *C. albicans* strains (FURLANETO-MAIA et al., 2008; GOKCE et al., 2007; KUMAR and MENON, 2006; SHIN et al., 2002). These authors also reported that biofilm production was most frequently observed for isolates of *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. guillermondii* and *C. krusei*. We investigated biofilm activity of 66 *Candida* isolates obtained from Anatolian buffaloes with mastitis by the tube adherence method in this study. Similar to the findings of other researchers, all *C. krusei*, *C.tropicalis*, *C. parapsilosis* and *C. guillermondii* strains were biofilm positive. The biofilm activity of *C. zeylanoides* (75.0%), *C. rugosa* (72.7%) and *C. kefyr* (62.5%) strains was fairly high. Slight biofilm production was detected in other species, while *C. ciferrii* did not show any biofilm activity (Table 1). These findings were consistent with the biofilm activities of different *Candida* species obtained from various human clinical isolates.

In the present study, a total of 66 *Candida* isolates obtained from 324 quarter milk samples with mastitis were identified. The significant increase in the number of udder infections caused by *Candida* species in recent years will probably become a common problem for dairy herds in Turkey. The reasons for this increase may be intensive and prolonged antibiotic therapy without specific microbiological examinations of milk

from affected quarters, and inadequate milking hygiene. In this study, biofilm activity constituted by different *Candida* species isolated from Anatolian buffaloes with mastitis was also investigated for the first time in Turkey. Anatolian buffalo milk and milk products are sought-after foods in Turkey. Although many *Candida* species are considered as opportunistic pathogens, they can cause serious infections under some suitable conditions in the hosts, especially when they are immunocompromised. Thus, the consumption of raw milk and milk products may create a potential risk for immunocompromised patients. It has been emphasized that when bovine mastitis is associated with biofilm-producing bacteria, the colonization capacity of bacteria in the mammary gland increases and thus, biofilm formation may constitute an important selective advantage to producer strains for persistent infections (OLIVERIA et al., 2006).

In the present study, we detected a relationship between the severity of clinical signs and biofilm activity for a few biofilm positive *C. krusei, C. tropicalis, C. parapsilosis, C. guillermondii* and *C. albicans* isolates. However, this finding was not sufficient to make a comment. Therefore, our study may be improved for the detection of the relationship between the biofilm activity of different *Candida* species and clinical signs in mastitis. We believe that this study may be a helpful tool for further research into mastitis caused by *Candida* species.

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SAŽETAK

Identificirana su bila 66 izolata roda *Candida* izdvojena iz uzoraka mlijeka upaljenih četvrti vimena anatolijskih bivolica upotrebom sustava API 20 C AUX. Najčešće izdvojene vrste bile su *Candida krusei* (27,3%), zatim *Candida rugosa* (16,7%), *Candida kefyr* (12,1%) i *Candida tropicalis* (10,6%). Ostale izdvojene vrste bile su *Candida albicans* (9,1%), *Candida zeylanoides* (6,1%), *Candida parapsilosis* (6,1%), *Candida guilliermondii* (4,5%), *Candida famata* (3,0%), *Candida glabrata* (3,0%) i *Candida ciferrii* (1,5%). Ukupno su 53 (80,3%) izolata tvorila biofilm. Svi izolati vrsta *C. krusei*, *C. tropicalis*, *C. parapsilosis* i *C. guilliermondii* tvorili su biofilm. *C. ciferrii* nije tvorila biofilm. To je prvo istraživanje tvorbe biofilma *in vitro* različitih vrsta *Candida* izdvojenih iz sekreta upaljenog vimena anatolijskih bivolica u Turskoj. Ovo istraživanje može biti od koristi u budućim istraživanjima mastitisa uzrokovanoga vrstama roda *Candida*.

Ključne riječi: aktivnost biofilma, bivol, *Candida*, mastitis