The Slime Production by Yeasts Isolated from Subclinical Mastitic Cows

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Abstract

The aim of this study was to isolate yeasts from subclinical mastitic cows and to investigate the slime production by the isolated yeasts. The material used in this study included 339 milk samples from 152 dairy cattle with subclinical mastitis. Milk was plated onto blood agar, MacConkey agar and Sabouraud dextrose agar. Forty-one samples (12.1% of total milk samples) were found positive for the yeast by API 20 C AUX identification system. The isolated yeasts were classified into four genera of *Candida, Trichosporon, Cryptococcus* and *Saccharomyces*. The *Candida* species were following: *C. krusei*, *C. kefyr*, *C. guilliermondii*, *C. famata*, *C. rugosa* and *C. utulis*. Other yeasts were identified as *Trichosporon mucoides*, *T. asahii*, *Cryptococcus laurentii*, *C. neoformans* and *Saccharomyces cerevisiae*.

Slime production was tested on Congo red brain heart infusion agar and evaluated according to Congo red phenomenon. Fifteen (36.6%) strains were slime factor positive: seven were *C. krusei*, four *C. kefyr*, one *C. guilliermondii*, one *C. famata*, one *T. asahii*, and one *C. laurentii*. The results of the present study indicate that yeast mastitis is significant for causing economic losses and slime production is mostly found in non-*albicans Candida* species. Therefore, non-*albicans Candida* species should be examined for slime production.

Mastitis, cows, milk, yeast, slime production

Mastitis, which has multiple and complex aetiology, is a common syndrome among bovines and inflicts losses on livestock owners. The mastitis cases are infectious in nature and are usually caused by bacteria, fungi or yeasts, some algae, and other microorganisms as viruses (Watts 1988; Chahota et al. 2001).

Mastitis either occurs with clinical symptoms (clinical mastitis) or without them (subclinical mastitis). The reduction in milk production attributed to subclinical mastitis may account for 70%-80% of the total losses (Philpot and Nickerson 1991). Somatic cell counts (SCC) in milk may be used to identify the presence of subclinical mastitis. California mastitis test (CMT), a qualitative measurement of the SCC in milk, is a screening test for mastitis that can be used easily. Although CMT and SCC are used for the determination of mastitis, the definitive test for the diagnosis of mastitis is bacteriological isolation and identification (Leslie et al. 2002).

Mycotic mastitis had been documented to be caused by various genera of yeasts. However, the most frequently encountered species are *Candida* spp., *Trichosporon* spp., *Cryptococcus* spp., *Saccharomyces* spp., *Aspergillus* spp. (Farnsworth et al. 1972; Costa et al. 1993; Bourtzi-Hatzopoulou et al. 2003; Santos and Marin 2005; Krukowski et al. 2006).

Fungi are usually considered an environmental mastitis and they are usual agent in cows' mastitis (Watts 1988; Sheena and Siegler 1995). Fungal infections are predominantly caused by yeasts of the genus Candida (Watts 1988) and several species of yeast have been reported in many countries as causative of mastitis. Bovine yeast mastitis was reported to be responsible for 10% in Brazil (Costa et al. 1993), 9.6% in Poland (Krukowski et al. 2000), 6.2% in Greece (Bourtzi-Hatzopoulou et al. 2003) of all mastitis cases.

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Candida spp. produces large quantities of viscid material in glucose containing solutions. A biofilm (or slime) is a community of microorganisms and their extracellular polymers that are attached to a surface. The ability to form biofilms is associated with the ability to cause infections and as such should be considered an important virulence determinant. Slime related infections are difficult to treat (Van Veen et al. 1992; Baillie et al. 1999; Cevahir et al. 2003; Cengiz et al. 2006).

Slime formation may be determined in several different ways, but most frequently it is demonstrated with the standard tube test, in which bacterial film lining a culture tube is stained with a cationic dye and visually scaled; with the microplate test, in which the optical density of the stained bacterial film is determined spectrophotometrically; or with the Congo red agar test (Christensen et al. 1982). Cevahir et al. (2003) investigated the slime production in *Candida* strains by these methods and they reported that the Congo red agar method is simple and practical for investigating slime production.

The aim of this study was to isolate yeasts from subclinical mastitic cows using standard methods in Aydin, Turkey, and to investigate the slime production from the isolated yeasts using Congo red agar method.

Materials and Methods

Diagnosis of mastitis

Clinical mastitis was diagnosed by changes in the udder and milk compositions. Changes in the udder included pain, swelling, warmth and abnormal appearance (blood tinged milk, watery secretions, clots, pus) of milk.

Cows that did not have clinical mastitis were subjected to further investigation for subclinical mastitis by using CMT. The procedures and interpretations were performed according to Quinn et al. (1994).

Milk samples

Milk samples were taken from the dairy farms located in Aydin. Aydin is a country of 8 007 km^2 by the Aegean Sea at the western part of Turkey. There are a total of 1 740 farms and 70% of these farms have $\leq$10 animals.

Within this study, CMT was done on 272 cows (1 088 quarters). Twenty three dairy farms (5-16 cows per herd) were investigated from January 2008 to January 2009 in Adnan Menderes University Veterinary Faculty Microbiology Laboratories. The milk samples were always aseptically collected by veterinarians.

Isolation of microorganisms

The samples were plated onto 7% sheep blood agar, MacConkey agar and Sabouraud dextrose agar. The plates were incubated at 37 ºC and examined for growth at 24, 48 and 72 h and at biweekly intervals for 4 weeks after which the plates showing no growth were considered negative.

Bacteria were identified by standard methods using morphological and biochemical characteristics (Holt and Kreig 1994; Carter and Cole 1995). When yeast growth was noticed, the yeast colonies were plated on Sabouraud agar, incubated at 37 ºC for 48 h and investigated by Gram stain. The yeasts were identified on the basis of morphological, physiological, and biochemical characteristics, including "germ tube test", urease production, and carbohydrate assimilation. The genera and species of yeast were identified by API 20 C AUX system (bioMerieux, France).

Detection of slime by Congo red agar (CRA) method

The method developed by Freeman et al. (1989) was used in this study. The composition of CRA was brain heart infusion broth (BHIB) 37 g/l, glucose 80 g/l, agar 10 g/l, and Congo red 0.8 g/l. The Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121 ºC for 15 min and was added when the agar had cooled to 55 ºC. Plates were inoculated and incubated aerobically at 35 ºC for 48 h. Slime production was evaluated according to the "Congo red phenomenon". Isolates that produced dark red colonies were regarded as slime positive, whereas those showing pink or white colonies were slime negative.

Results

Isolation of microorganisms

A total of 339 milk samples from 152 subclinical mastitic cows were detected microbiologically for aerobic pathogenic microorganism. From 339 milk samples, 297 aerobic microorganisms were isolated and identified. Coagulase negative staphylococci (24.5%), coagulase positive staphylococci (20.9%), yeasts (12.1%), *Escherichia coli* (9.8%), *Streptecoccus* spp. (6.2%), *Bacillus* spp. (5.6%), *Shigella* spp. (3.5%), *Pseudomonas* spp. (2.6%), other bacteria (2.4%) were isolated, and 12.4 showed no growth (Table 1). Bacteria, especially staphylococci, were most frequently isolated as the main aetiological agent of subclinical bovine mastitis in the examined milk samples.

Table 1. Aetiological agents of bovine mastitis

Aetiological agents	n	%
Coagulase negative staphyloccci	83	24.5
Coagulase positive staphylococci	71	20.9
Yeasts (**Candida* spp. **other yeasts)	41 (34 7)	12.1 (10.0 2.1)
Escherichia coli	33	9.8
Streptecoccus spp.	21	6.2
Bacillus spp.	19	5.6
Shigella spp.	12	3.5
Pseudomonas spp.	9	2.6
Other bacteria	8	2.4
No growth	42	12.4
Total	339	100

n - The number of isolated strains

Table 2. The genera and species of yeasts isolated from bovine mastitis

Genera	Species	n	%
Candida	*krusei*	15	36.6
	kefyr	12	29.4
	guilliermondii	3	7.3
	famata	2	4.9
	utulis	1	2.4
	rugosa	1	2.4
Trichosporon	*mucoides*	2	4.9
	asahii	1	2.4
Cryptococcus	*laurentii*	2	4.9
	neoformans	1	2.4
Saccharomyces	*cerevisiae*	1	2.4
Total	41		100

n: The number of isolated strains

Species of isolated yeasts

Yeasts were more commonly isolated agent than staphylococci. Forty one milk samples (12.1% of total) were found positive for yeasts. The isolated yeasts were classified into the genera *Candida, Trichosporon, Cryptococcus, Saccharomyces*. Of total of 41 yeast species, 15 (36.6%) were identified as *C. krusei*, 12 (29.4) *C. kefyr*, 3 (7.3%) *C. guilliermondii*, 2 (4.9%) *C. famata*, 1 (2.4%) *C. utilis* and *C. rugosa,* 2 (4.9%) *T. mucoides*, 1 (2.4%) *T. asahii, 2* (4.9%) *C. laurentii,* 1 (2.4%) *C. neoformans,* 1 (2.4%) *S. cerevisiae* (Table 2).

Slime production

Fifteen strains (36.6%) found as slime factor producer. Of the slime factor positive strains 7 were *C. krusei,* 4 *C. kefyr,* 1 *C. guilliermondii,* 1 *C. famata,* 1 *C. laurentii,* and 1 *T. asahii* (Table 3).

Discussion

Infections by yeasts have been known both in animals and humans for years. Although the majority of mycotic mastitis in cows are mild, some cases may result in death. Excessive and erratic use of antibiotics, corticosteroids, immunosuppressive drugs and chronic diseases are the major contributing factors in increasing the incidence of diseases due to yeasts (Watts 1988; Van Veen and Kremer 1992).

In this study, 12.4% (number/total number) of the bacteriological cultures were negative. In other studies it ranged from 7.3% to 38% (Arda ve İstanbulluoğlu 1979; Bartlett et al. 1992; Kuyucuoglu and Ucar 2001; Gianneechini et al. 2002; Tel et al. 2009). The reason may be attributed to a suspected anaerobic microorganism, a virus, mycoplasma.

Yeasts are microorganisms found in nature and isolated from diseased humans and animals. Among the yeast, *Candida* species are eukaryotic pathogens that are most commonly isolated from infections of dairy cows' mammary glands. Mycotic mastitis was first described in Turkey in 1979 (Arda and İstanbulluoğlu 1979). To our knowledge, slime production in yeasts isolated from bovine mastitis has not been studied in Turkey yet.

Table 3. The distribution of slime positivity according to the isolated strains

Species	Positive/n
C. krusei	7/15
C. kefyr	4/12
C. guilliermondii	1/3
C. famata	1/2
C. utulis	0/1
C. rugosa	0/1
T. mucoides	0/2
T. asahii	1/1
C. laurentii	1/2
C. neoformans	0/1
S. cerevisiae	0/1
Total (%)	15/41 (36.6)

Positive: the slime positive strains
n: the number of isolated strains

The percentage of yeasts causing mastitis varies between countries. The percentage of yeast isolation carried out in many countries varies considerably, with 6.3% in Greece (Bourtzi-Hatzopoulou et al. 2003), 10%-17.3% in Brazil (Costa et al. 1993; Santos and Marin 2005) and 4.2%-9% in Poland (Krukowski et al. 2000; Krukowski et al. 2006). In the present study, it was 12.1%.

There are many regional differences in yeast species causing mastitis. Many yeast species have been reported as causative agents of mastitis (Costa et al. 1993; Bourtzi-Hatzopoulou et al. 2003; Santos and Marin 2005; Krukowski et al. 2006; Seker 2010). Bourtzi-Hatzopoulou et al. (2003) reported that the yeasts isolated were classified into the genera of *Candida, Geotrichum, Rhodotorula*. Krukowski et al. (2000) reported that all the isolated yeasts were *Candida, Trichosporon, Rhodotorula* and in another study Krukowski et al. (2006) reported *Candida, Trichosporon, Saccharomyces,* and *Rhodotorula*. In the present study the yeasts isolated were classified into the genera of *Candida, Trichosporon, Cryptococcus* and *Saccharomyces.*

In studies conducted in Turkey, *Candida* species were isolated from 1-8.8% of cows with mastitis (Arda and İstanbulluoğlu 1979; Beytut et al. 2002; Turutoglu and Mudul 2002; Tel et al. 2009). In the present study, the high isolation ratio (10.0%) for *Candida* species may be attributed to the excessive humidity during the year and uncontrolled antibiotic use in Aydin region.

C. krusei is the most commonly isolated strain in the present study. It is usually considered an agent responsible for environmental mastitis due to poor animal hygiene (Watts 1988). *C. krusei* was also the predominant species demonstrated by Farnsworth and Sorensen (1972), Santos and Marin (2005) and Seker (2010). This result was in accordance with our study. But Sheena and Siegler (1995) reported that *C. krusei* was isolated sporadically in bovine mastitis. In several countries, it was demonstrated that *C. krusei, C. kefyr, C. rugosa* and *C. albicans* were often responsible for mycotic mastitis (Farnsworth and Sorensen 1972; Costa et al. 1993; Sheena and Siegler 1995; Santos and Marin 2005; Seker 2010). The geographical variations may be the reason for discrepancy in the distribution of species.

The yeasts were isolated in pure culture or mixed with bacteria. In the study 90% isolation rate as pure culture was higher than in other researches (Costa et al. 1993; Krukowski et al. 2000; Santos and Marin 2005).

Although *C. albicans* is frequently isolated, isolation of non-*albicans Candida* species strains has been frequently encountered in the past few decades. It has been known that non-*albicans Candida* strains have produced slime. It has been reported that the slime production by *C. albicans* was less frequent (42.9%), than that by non-*albicans Candida* (63.4%) (Vinitha and Ballal 2007). There is little information about slime production by the yeasts except *C. albicans* and *C. parapsilosis* in various clinical samples in humans in Turkey (Yakupoğullari and Toraman 2004). Kocazeybek et al. (2000) reported that the most frequently isolated species were *C. albicans* and *C. krusei* in mortal cases and in intensive care units in humans, respectively. Vinitha and Ballal (2007) reported that 34 *Candida* spp. were isolated from human blood, and *C. krusei* was the most commonly isolated species.

Slime production by non-*albicans Candida* species was found in 25% (Kalkancı et al. 1999) and in 35% (Birinci et al. 2005). In this study, 15 strains of *C. albicans* (36.6%) produced slime.

In conclusion, yeasts such as *Candida* spp., *Trichosporon* spp., *Cryptococcus* spp., and *Saccharomyces* spp. were isolated from subclinical mastitic cows in Aydin in Turkey. *C. krusei* and *C. kefyr* were the most common isolated strains. It was detected that *T. asahii, C. famata, C. laurentii, C. krusei, C. kefyr, C. guilliermondii* strains had strong slime production. Increased slime production can lead to serious problems during therapy of infections caused by these yeast species.

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