Antimicrobial susceptibility and biofilm forming ability of staphylococci from subclinical buffalo mastitis

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SUPPLEMENTARY FILE

Introduction

In recent years, high consumer preference has increased the demand for buffalo's milk not only in the world but also in Turkey, due to its rich nutrition (Atasever and Erdem, 2008). In Turkey, the number of water buffalo was 1 178 000 in 1970, it declined dramatically until the 2000s, an d decreased to 84 705 in 2008. In 2010, the Ministry of Agriculture and Rural Affairs beganto support buffalo breeding (Sarıözkan, 2011). As a result of the support program, the population of buffalo, the number of lactating buffalos, the average annual milk production (ton) and the ratio of milk in total milk production were 86 297, 36 670, 31 422 tons 0.26% in
2008; these figures in 2018 reached 178 397, 87 862, 75 742 and 0.3%, respectively. A recent study (Alkoyak and Öz, 2020) reported that the average lactation period in Anatolian buffaloes is 260 days. It is also important to manage this period in a healthy way, which is shorter than cows. The increasing trend of antimicrobial resistance presents a growing burden for the prevention and treatment of mastitis due to widespread misuse of antimicrobials (Oliver and Murinda, 2012). The emergence of antimicrobial resistance among mastitis pathogens is also a concern for public health because resistant bacteria can also be transmitted to humans through the food chain (Oliver and Murinda, 2012).

Biofilm forming ability of *Staphylococcus* spp. is one of the important virulence factors that facilitate adhesion and colonization on the mammary gland epithelium, leading to recurrent or persistent infections (Fox *et al*., 2005; Melchior *et al*., 2011). Biofilm is a structured community

of bacterial cells enclosed in a self-produced matrix attached to biotic or abiotic surfaces (Flemming & Wingender, 2010). Biofilm gives a lot of advantages to the bacteria such as (i) protection from the immune system and hostile environments within the host, (ii) decreasing diffusion of bactericidal concentrations of antibiotics or disinfectants inside the biofilm matrix, (iii) helping bacteria for adhesion and colonization on mammary gland tissue and persistence of infection (Costeron *et al*., 1999)

Staphylococcal biofilm formation is a two-step process involving cell attachment and the formation of an extracellular matrix. Staphylococci can express a variety of bacterial surface molecules called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) interacting with host tissues such as *ebpS* (elastin binding protein), *eno* (laminin-binding protein), *cna* (collagen-binding protein), *fnbA* and *fnbB* (fibronectin-binding proteins A and B), *fib* (fibrinogen binding protein), *clfA* and *clfB* (clumping factors A and B), and *bbp* (bone sialoprotein binding protein) (Foster and Höök, 1998). The next step is the development of biofilm, facilitated by the polysaccharide intercellular adhesin (PIA), called poly-N-acetylglucosamine (PNAG). PIA synthesis results in multi-layer cell clustering. PIA synthesis is regulated by the *ica* gene locus, which comprises an N-acetylglucosamine transferase (*ica*A and *ica*D), and a PIA deacetylase (*ica*B), a putative PIA exporter (*ica*C), and a regulatory gene (*ica*R) (Otto, 2008). However, it has been reported that PIA production is not mandatory for biofilm formation and biofilm-related infection (Rohde *et al*., 2005), and even there are no *ica* genes in some strains isolated from biofilm-related infections (Arciola *etal*., 2006).

Materials and methods

California Mastitis Test (CMT) and milk sampling

Milk samples were taken from CMT positive buffaloes according to previously described protocols (Quinn et al., 1999). After the teats of the buffaloes were cleaned and disinfected using 70% ethanol, the first three streams were discarded. Then, approximately 40ml milk samples were taken into sterile 50 ml centrifuge tube and kept on ice until transported to the laboratory. The California mastitis test (CMT) was used as the screening test for sub-clinical mastitis and performed as previously reported by Quinn *et al*. (1999). The CMT results were scored as 0 (negative), 1 (weak positive), 2 (distinct positive), and 3 (strong positive) based on gel formation. All CMT scores of 0 and trace were considered negative while CMT scores of 1, 2, and 3 were considered indicators of subclinical mastitis. If one-quarter of the animal was positive for CMT and showed no clinical signs of infection were considered to have subclinical mastitis. Milk samples were taken from CMT positive buffaloes according to previously described protocols (Quinn et al., 1999). After the teats of the buffaloes were cleaned and disinfected using 70% ethanol, the first three streams were discarded. Then, approximately 40ml milk samples were taken into sterile 50 ml centrifuge tube and kept on ice until transported to the laboratory.

Isolation and identification of staphylococci

CMT positive milk samples taken from buffalos were subjected to isolation of staphylococci, according to previously described protocols (Quinn *et al*., 1999). Briefly, 100 µl of milk from each quarter was streaked onto Blood agar supplemented with 5% defibrinatedsheep blood, and incubated at 37 ^{o}C for 24-48 h. The plates having the number of colonies >25CFU/100 µl was considered for the presence of mastitis infection. The colonies that were suspected to be staphylococci were subcultured on Blood agar plates and incubated for 24 h at37 ^{o}C. Colonies were characterized based on Gram staining, catalase, presence of hemolysis (Quinn *et al*.,

1999).

MALDI-TOF MS

The identification of staphylococci at the species level was performed using a MALDI Bruker Biotyper system (Bruker Daltonics Inc., Billerica, MA, USA) at the Center for Implementation and Research of Plant Health Clinic, University of Hatay Mustafa Kemal, Hatay, Turkey. Results were analyzed using Biotyper 3.1 software which included over 6903 reference spectra. For MALDI-TOF analysis, pure cultures of staphylococci were grown on 5% sheep blood agar for 24 h. A small amount of bacterial growth was transferred to the spots of an MSP 96 polished steel BC target plate (Microflex LT, Bruker Daltonics/BD, Germany, USA) using a disposable loop, then 1 µL of Matrix [α-cyano-4-hydroxycinnamic acid (HCCA)] was added and the plates were air-dried 1 to 2 min at room temperature. The resultant mass spectra were analyzed and compared with the reference spectra. Bacterial identification to the species level was achieved if the score value was equal to or greater than 2.00. A score value between 1.70 and 1.99 was accepted as accurate genus-level identification and presumptive species-level identification.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of the isolates were determined using the disc diffusion method and the results were interpreted according to Clinical and Laboratory Institute (CLSI, 2021) guidelines. The discs used were: penicillin (1 U), ampicillin (AMP, 10 µg), amoxycillin-clavulanic acid (AMC, 20/10 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), gentamicin (CN, 10 µg), tetracycline (TE, 30 µg), cefoxitin (FOX, 30 µg) and erythromycin (15 µg). *S. aureus* ATCC 25213 was included as a reference strain.

Determination of biofilm formation

The biofilm-forming ability of the isolates was investigated by two different methods.

Congo Red Agar Method. Qualitative detection of biofilm production was determined by the cultivation of *S. aureus* strains on Congo Red Agar (CRA) method plates as previously described by Freeman *et al*. (1989). Based on colony morphology, staphylococci were classified into four classes: the isolates with dry black colonies were evaluated as strong, the isolates with smooth black colonies were classified as moderate, the isolates with dry red colonies were considered as weak, and the isolates showing smooth red colonies were accepted as negative for the biofilm-forming ability.

Microtiter Plate (MTP) Method. Quantitative biofilm determination was carried out using the microtiter plate (MTP) method described by Christensen *et al*. (1985) in tissue culture plates with 96 flat-bottomed wells. Each culture plate included a negative control, three wells with Tryptone Soya Broth (TSB). All the experiments were repeated at least three times, and the values of optical density were then averaged. The optical density (OD) of each well was measured using a microplate ELISA reader (BioTek, Quant, USA) at 570 nm. Cutt-off OD (ODc) is defined as three standard deviations above the mean OD of the negative control. Strains were interpreted as follows: (i) non-biofilm producers ($OD \leq ODc$), (ii) weak biofilm producers ($ODc < OD \leq 2 \times ODc$), (iii) moderate biofilm producers ($2\times ODc < OD \leq 4\times ODc$), and (iv) strong biofilm producers ($4 \times ODc < OD$).

Results

Isolation and species-level identification by MALDI-TOF MS

Out of 200 lactating water buffalo, 24% (48) were subclinical mastitis. 160 milk samples were collected from 64 animals. All quarters were sampled in 26 animals, whereas three, two, and one quarter were sampled from 11, 7, and 2 animals, respectively. Out of 160 milk samples, 107 (66.9%) *Staphylococcus* spp. were isolated. Analysis of the 107 staphylococci by MALDI-TOF MS revealed the presence of 12 different species. Distribution of staphylococci were as

follow: *S. chromogenes* (n=38), *S. aureus* (n=28), *S. epidermidis* (n=18), *S. xylosus* (n=6), *S. arlettae* (n=5), *S. sciuri* (n=4), *S. hyicus* (n=3), *S. simulans* (n=1), *S. vitulinus* (n=1), *S. succinus* (n=1), *S. saprohyticus* (n=1) and *S. heamolyticus* (n=1).

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of the isolates were given in supplementary Table S1. Among the staphylococci, *S. aureus* showed higher resistance rates to penicillin (67.9%), ampicillin (67.9%), and amoxicillin-clavulanic acid (53.6%). Low or lower resistance rates to tetracycline (17.6%), trimethoprim-sulfamethoxazole (14.3%), cefoxitin (7.1%) and ciprofloxacin (3.6%), no resistance to gentamicin and chloramphenicol was observed among *S. aureus* isolates. Similarly, CoNS displayed high resistance rates to penicillin (51.9%) and ampicillin (41.8%), but they were least resistant to trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, tetracycline, erythromycin, cefoxitin, chloramphenicol (1.3% to 16.5%), and were all susceptible to gentamicin and ciprofloxacin. In addition, 16 resistance phenotype were detected among the staphylococci (supplementary Table S2).

Biofilm formation

Slime production on Congo Red Agar (CRA)

Out of 28 *S. aureus* isolates, 26 (92.9%) were positive for biofilm production with different degrees, and 42.9%, 50%, and 7.1% of the isolates were strong, intermediate, and negative biofilm producers, respectively. Among the 79 CoNS isolates, 25.3%, 45.6% and 29.1% of the isolates were strong, intermediate, and negative biofilm producers, respectively.

Out of 23 biofilm negative CoNS isolates by CRA method, 21 isolates were positive by MTP method with different degrees of biofilm production (4 moderate, 17 weak). Only two CoNS isolates were negative by both methods.

Biofilm formation by MTP method

The biofilm production of *S. aureus* isolates (100%) was slightly higher than that of CoNS isolates (97.5%). While 42.9% of the biofilm positive *S. aureus* isolates were strong producers,

53.6% and 3.6% were moderate and weak biofilm producers, respectively. In the biofilm-positive CoNS isolates, 17.7%, 27.8%, and 51.9% were strong, moderate, and weak producers, respectively. CRA method showed low correlation with MPT method, only two CoNS isolates were true negative by both methods. Comparison of CRA method and MTP method for the detection of biofilm formation by *S. aureus* and CoNS was given in supplementary Table S3.

Genotypic analysis of biofilm-related and MSCRAMM Genes

Among the CoNS isolates, both *ica*A and *ica*D genes were only detected in one isolate (1.3%), the *ica*D gene was alone detected in 14 (17.7%) isolates, and *ica*A was only detected in one isolate (1.3%). The remaining 63 isolates didn't harbor neither *ica*A nor *ica*D genes. While the *eno* (60, 75.9%) gene was the most frequently detected among 79 CoNS, the other MSCRAMM genes were less frequently detected: 13.9% (11) were positive for *clf*A, 12.7% (10) were positive for *clf*B, 10.1% (8) were positive for *ebp*S, 10.1% (8) were positive for *cna*, 7.6% (6) were positive for *fnb*B and 7.6% (6) were positive for *fib*. Thirteen of 79 isolates carried none of the MSCRAMM genes. Out of 28 *S. aureus* isolates, four were positive for both *ica*A and *ica*D, 14 were only positive for *ica*D. The rest of the isolates were negative for the presence of *ica*A and *ica*D genes. The most common MSCRAMM gene was *clf*A (89.3%) among *S. aureus* isolates, followed by *eno* (71.4%), *clf*B (71.4%), *ebp*S (67.9%), *fib* (64.3%), *fnb*B (28.6%) and *bpb* (3.6%), respectively. Two *S. aureus* isolates harbored none of the MSCRAMM genes. The *bap* gene was not detected in any staphylococci. MSCRAMM and biofilm gene profiles determined among staphylococci were given in supplementary Table S4.

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Table S1. Antimicrobial resistance of the staphylococci

Antimicrobial	**CoNS (n=79)**	***S. aureus* (n=28)**
Penicillin	41 (51.9%)	19 (67.9%)
Ampicillin	33 (41.8%)	19 (67.9%)
Amoxicillin-clavulanic acid	6 (7.6%)	15 (53.6%)
Trimethoprim-sulfamethoxazole	13 (16.5%)	5 (14.3%)
Gentamicin	0 (0)	0 (0)
Ciprofloxacin	0 (0)	1 (3.6%)
Chloramphenicol	1 (1.3%)	0 (0)
Cefoxitin	2 (2.5%)	2 (7.1%)
Tetracycline	6 (7.6%)	5 (17.6%)
Erythromycin	2 (2.5%)	0 (0)

Table S2. Resistance phenotypes detected among the staphylococci

Resistance Phenotype	**CoNS**	***S. aureus***
P, AMP, AMC, SXT, TE, E	1	-
P, AMP, AMC, SXT, FOX	-	2
P, AMP, AMC, TE, SXT	1	-
P, AMP, AMC, TE	-	3
P, AMP, AMC, SXT	-	2
P, AMP, FOX	2	-
P, AMP, SXT	3	-
P, AMP, AMC	4	8
P, AMP, E, C	1	-
P, AMP, TE	3	1
SXT, TE	1	-
P, AMP	18	3
SXT	7	1
CIP	-	1
TE	-	1
P	9	-
Sensitive	29	6
Total	**79**	**28**

Table S3. Comparison of CRA method and MTP method for the detection of biofilm formation by *S aureus and CoNS.*

	S. aureus **(n=28)**	**CoNS (n=79)**	**Total (n=107)**
CRA*			
Negative	2 (7.1%)	23 (29.1%)	25 (23.4%)
Weak	4 (14.3%)	22 (27.8%)	26 (24.3%)
Moderate	10 (35.7%)	14 (17.7%)	24 (22.4%)
Strong	12 (42.9%)	20 (25.3)	22 (20.6%)
MTP**			
Negative	-	2 (2.5%)	2 (1.9%)
Weak	3 (10.7%)	41 (51.9%)	44 (41.1%)
Moderate	14 (50%)	22 (27.8%)	36 (33.6%)
Strong	11 (39.3%)	14 (17.7%)	25 (23.4

CRA: Congo Red agar method. MTP: Microtiter plate method

Table S4. MSCRAMM and biofilm gene profiles among 79 CoNS and 28 *S. aureus* isolates

Biofilm Associated Genes			MSCRAMM Genes	MP					
				Weak		Moderate		Strong	
***ica*A**	***ica*D**	***bap***		***S. aureus***	**CoNS**	***S. aureus***	**Co NS**	***S. aureus***	**CoNS**
(-)	*ica*D	(-)	*eno*, *ebp*S, *clf*A, *clf*B, *fib*, *fnb*B			2		1	
(-)	(-)	(-)	*eno*, ebpS, *clf*A, *clf*B, *fib*, *fnb*B					2	
*ica*A	*ica*D	(-)	*eno*, *ebp*S, *clf*A, *clf*B, *fnb*B, *fib*				1	1	
(-)	*ica*D	(-)	*eno*, *ebp*S, *bbp*, *clf*B, *clf*A, *fib*	1					
*ica*A	*ica*D	(-)	*eno*, *fnb*B, *clf*A, *clf*B, *fib*					1	
*ica*A	*ica*D	(-)	*eno*, ebpS, *clf*A, *clf*B, *fib*			1			
(-)	*ica*D	(-)	*eno*, *ebp*S, *clf*A, *clf*B, *fib*			1			1
*ica*A	*ica*D	(-)	*eno*, *fnb*B, *clf*A, *clf*B, *fib*			1			
(-)	*ica*D	(-)	*eno*, *ebp*S, *clf*A, *clf*B, *fib*			2		1	
(-)	(-)	(-)	*eno*, *ebp*S, *clf*A, *clf*B, *fib*				1		
(-)	*ica*D	(-)	*eno*, *ebp*S, *clf*A, *clf*B			1			
(-)	(-)	(-)	*eno*, *ebp*S, *clf*A, *clf*B					2	
(-)	(-)	(-)	*eno*, *cna*, *fnb*B, *clf*B						1
(-)	(-)	(-)	*eno*, *ebp*S, *clf*A			1			
(-)	(-)	(-)	*eno*, *fnb*A, *fnb*B				1		1
(-)	*ica*D	(-)	*eno*, *clf*A, *clf*B, *fib*			2			1
(-)	*ica*D	(-)	*eno*, *ebp*S, *clf*A, *fib*			1			
(-)	(-)	(-)	*eno*, *ebp*S, *clf*A					1	
(-)	*ica*D	(-)	*eno*, *ebpS*, *clfA*				1		
*ica*A	(-)	(-)	*eno*, *ebp*S, *clf*A					1	
(-)	(-)	(-)	*eno*, *clfA*, *clfB*		2				
(-)	(-)	(-)	*eno*, *fib*, *fnb*B				1		
(-)	(-)	(-)	*eno*, *fnb*B, *clf*B						1
*ica*A	(-)	(-)	*clf*A, *fib*		1				
(-)	(-)	(-)	*clf*A, *clf*B					1	2
(-)	(-)	(-)	*eno*, *ebp*S		1				
(-)	(-)	(-)	*eno*, *clf*A					1	