

## Research Article

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

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## Abstract

The starting objective of this research communication was to determine the prevalence of sub-clinical mastitis in buffalo in Turkey. We also sought to isolate and identify staphylococci, determine their antimicrobial susceptibilities and biofilm-forming abilities as well as investigating the presence of biofilm-related genes and microbial surface components recognizing adhesive matrix molecules. A total of 107 (66.9%) staphylococci (28 *S. aureus* and 79 coagulase-negative staphylococci, CoNS) were isolated from 160 mastitic milk samples collected from 200 lactating water buffalos. The staphylococci were especially resistant to beta-lactams except for cefoxitin but were less resistant to the other antimicrobials that were tested. Based on the Congo red agar method, 92.9% of the *S. aureus* and 70.9% of the CoNS isolates were positive for biofilm-forming ability, while all *S. aureus* and 97.5% of CoNS isolates were positive by a microtiter plate analysis. The presence of *icaA* and *icaD* genes was not always correlated with biofilm synthesis, and even in the absence of these genes, the isolates were able to synthesize biofilm.

In the dairy industry, mastitis is considered as the most common and economically important disease worldwide, due to reduced milk quality and production, milk discard, involuntary culling, veterinary services, treatment costs and increased labor costs (de Souza Santos *et al.*, 2020). Although mastitis is a multifactorial disease, involving interrelationships between microorganisms, host and environmental factors, microorganisms have been playing an important role in the emergence and dissemination of disease (Singha *et al.*, 2021). Among microorganisms, in particular, bacteria are the most common cause of mastitis cases, and staphylococci are the main etiological agents isolated from mastitic milk samples (Gomes *et al.*, 2016).

The increasing trend of antimicrobial resistance presents a growing burden for the prevention and treatment of mastitis due to widespread misuse of antimicrobials. 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 (Melchior *et al.*, 2011). Staphylococcal biofilm formation is a two-step process involving cell attachment and the formation of an extracellular matrix. For cell attachment, staphylococci can express a variety of bacterial surface molecules that interact with host tissues. These molecules are known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs: Foster and Höök, 1998).

The purpose of the current study was to determine the prevalence of subclinical mastitis, to obtain detailed data on staphylococci associated with subclinical mastitis, to determine the antimicrobial susceptibilities of the isolates, to evaluate the biofilm-forming ability of the isolates and finally to investigate biofilm-related genes and MSCRAMMs by polymerase chain reaction (PCR).

## Materials and methods

A detailed description of materials and methods is provided in the online Supplementary File. The study was approved by the Animal Ethical Committee of the International Center for Livestock Research and Training, Lalahan, Ankara (30.05.2018/152).

## Study area and sample collection

This study was carried out among 200 lactating Anatolian buffalo breeds over six months (from June 2018 to September 2018) in the province of Çorum, which is located in the

**Table 1.** Biofilm-associated and MSCRAMM genes among staphylococci

Strains	Number (%) of Biofilm Positive Isolates <sup>a</sup>	Biofilm-Associated and MSCRAMM Genes											
		<i>icaA</i>	<i>icaD</i>	<i>bap</i>	<i>ebpS</i>	<i>eno</i>	<i>cna</i>	<i>fnbA</i>	<i>fnbB</i>	<i>fib</i>	<i>clfA</i>	<i>clfB</i>	<i>bbp</i>
<i>S. chromogenes</i> (n = 38)	38	2 (5.3)	7 (18.4)	–	5 (13.2)	30 (78.9)	2 (5.3)	–	1 (2.6)	3 (7.9)	5 (13.2)	5 (13.2)	1 (2.6)
<i>S. epidermidis</i> (n = 18)	18	–	6 (33.3)	–	2 (11.1)	14 (77.7)	2 (11.1)	–	1 (5.6)	2 (11.1)	1 (5.6)	2 (11.1)	–
<i>S. xylosus</i> (n = 6)	6	–	1 (16.7)	–	1 (16.7)	5 (83.3)	1 (16.7)	1 (16.7)	1 (16.7)	1 (16.7)	1 (16.7)	–	–
<i>S. arlettae</i> (n = 5)	5	–	1 (20)	–	–	5 (100)	1 (20)	1 (20)	3 (60)	1 (20)	1 (20)	2 (40)	–
<i>S. sciuri</i> (n = 4)	4	–	–	–	–	1 (25)	–	–	–	–	1 (25)	1 (25)	–
<i>S. hyicus</i> (n = 3)	3	–	–	–	–	2 (66.7)	–	–	–	–	2 (66.7)	2 (66.7)	–
<i>S. simulans</i> (n = 1)	–	–	–	–	–	–	–	–	–	–	–	–	–
<i>S. vitulinus</i> (n = 1)	–	–	–	–	–	–	–	–	–	–	–	–	–
<i>S. succinus</i> (n = 1)	1	–	–	–	–	1 (100)	1 (100)	–	–	–	–	–	–
<i>S. saprophyticus</i> (n = 1)	1	–	–	–	–	1 (100)	–	–	–	–	–	–	–
<i>S. haemolyticus</i> (n = 1)	1	–	–	–	–	1 (100)	–	–	–	–	–	–	–
Total CoNS (n = 79)	77 (97.5)	2 (2.5)	15 (19)	–	8 (10.1)	60 (75.9)	7 (8.9)	2 (2.5)	6 (7.6)	7 (8.9)	11 (13.9)	12 (15.2)	1 (1.3)
<i>S. aureus</i> (n = 28)	28 (100)	6 (21.4)	18 (64.3)	–	19 (67.9)	23 (82.1)	–	–	8 (28.6)	18 (64.3)	25 (89.3)	20 (71.4)	1 (3.6)
All staphylococci (n = 107)	105 (98.1)	8 (7.5)	33 (30.8)	–	27 (25.2)	83 (77.6)	7 (6.5)	2 (1.9)	14 (13.1)	25 (23.4)	36 (33.6)	32 (29.9)	2 (1.9)

<sup>a</sup>The isolates were found to be biofilm-positive by MTP.

Central Anatolia region of Turkey. All farms ( $n = 42$ ) included in the study were family-sized, and the herd size of the selected farms varied from 5 to 30 water buffalos. The milk yield of the buffalos ranged from 3 to 7 kg per day, with on average 1160 kg of annual milk yield. The lactation period was between 230 and 240 d. All animals were subjected to clinical examination and California mastitis test (CMT), then the CMT positive milk samples were microbiologically examined.

### Isolation and identification of staphylococci

Isolation of staphylococci was conducted as previously described (Quinn *et al.*, 1999). The identification of staphylococci at the species level was performed using a MALDI Bruker Biotyper system (Bruker Daltonics Inc., Billerica, MA, USA).

### 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, 2022) guidelines.

### Determination of biofilm formation

The biofilm-forming ability of the isolates was investigated by two different methods: Congo Red Agar (CRA) Method and Microtiter Plate (MTP) Method. All methods are detailed in the online Supplementary File.

## Results and discussion

In this study, 24% (48) of the water buffaloes were diagnosed with subclinical mastitis. Özenç *et al.* (2008) reported a prevalence rate of 16.8% in Afyon province in Turkey. While Sharif and Ahmed (2007) found the prevalence of sub-clinical mastitis as 37.75% in Pakistan, Elhaig and Selim (2015) reported the prevalence of sub-clinical mastitis as 43.3% in Egypt.

Out of 160 milk samples, 107 (66.9%) *Staphylococcus* spp. including 28 *S. aureus* and 79 coagulase-negative staphylococci (CoNS) were isolated. Özenç *et al.* (2008) also reported that staphylococci were the most common bacteria with a rate of 52.95%. Preethirani *et al.* (2015) reported staphylococci as major pathogens isolated from subclinical bovine mastitis cases of buffaloes in India.

As can be seen in online Supplementary Tables S1 and S2, among the staphylococci, *S. aureus* showed a higher resistance rate to penicillin, ampicillin, amoxicillin-clavulanic acid (53.6–67.9%), and showed low resistance rates to trimethoprim-sulfamethoxazole, ciprofloxacin, ceftiofloxacin (3.6–14.3%), while no resistance was observed to gentamicin and chloramphenicol. On the other hand, CoNS displayed intermediate resistance to penicillin and ampicillin (41.8–51.9%), low resistance to trimethoprim-sulfamethoxazole (16.5%) and least resistance to chloramphenicol, ceftiofloxacin, erythromycin, tetracycline, amoxicillin-clavulanic acid (1.3% to 7.6%). The CoNS were all sensitive to gentamicin and ciprofloxacin. The high resistance for beta-lactams was not surprising, because beta-lactams are widely prescribed agents in Turkey. These are consistent with previous reports of beta-lactam resistance rates in staphylococci isolated from buffalo mastitis (Medeiros *et al.*, 2011; Preethirani *et al.*, 2015).

The results for biofilm forming ability obtained by the CRA method and MTP method are comparatively given in online

Supplementary Table S3. While the *bap* gene was not detected in any staphylococci, various biofilm-associated and MSCRAMM gene profiles was observed among staphylococci (online Supplementary Table S4). The frequency of genes associated with adhesion and biofilm formation in *S. aureus* and CoNS strains isolated from mastitis cases vary according to the geographical area studied (Felipe *et al.*, 2017). Overall, 70.9% ( $n = 56$ ) and 97.5% ( $n = 77$ ) of the isolates were determined to be biofilm producers by the CRA method and MTP, respectively. The contradictory results between phenotypic methods and genotypic methods have been reported in previous studies (Ciftci *et al.*, 2009; Darwish and Asfour, 2013). Biofilm forming ability of the isolates that were negative for *icaA* and *icaD* genes could be attributed to *ica* gene independent control of slime production/adhesion mechanism (Liberto *et al.*, 2009). In contrast, the inability to form biofilm of the isolates having *icaA* and *icaD* genes could be due to point mutations in the *ica* locus or any other unidentified factors that negatively regulate polysaccharide intercellular adhesion synthesis or influence biofilm formation (Cramton *et al.*, 1999). Some researchers also indicated that the process of biofilm formation is complex involving many genes or many unknown variables (Darwish and Asfour, 2013; Tremblay *et al.*, 2013). The results obtained from this and other studies indicated the importance of other mechanisms of biofilm-forming independent of the *ica* operon. Biofilm-associated and MSCRAMM genes observed among staphylococci are given in Table 1.

In conclusion, the prevalence of subclinical mastitis cases in buffaloes was found to be 24% in the family-sized farms where no preventive control programs for mastitis were applied. The findings of the study also showed that staphylococci isolated from buffalo mammary glands have the ability to develop biofilm with different degrees despite the presence or absence of biofilm and MSCRAMMs genes. Finally, despite high rates of resistance to beta-lactam antibiotics, staphylococci generally exhibited low resistance to other individual antibiotics, indicating that prudent use of antibiotics is necessary to prevent the emergence and dissemination of antibiotic-resistant bacteria in buffalo mastitis cases.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0022029923000080>.

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