

**SLIME PRODUCTION AND BIOFILM FORMING ABILITY BY STAPHYLOCOCCUS AUREUS BOVINE MASTITIS ISOLATES**

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*Some Staphylococcus aureus strains, identified as causative agents of mastitis in cattle, exhibit the ability of producing a viscous extracellular polysaccharide layer (slime), which is nowadays considered to be a virulence factor, as it promotes bacterial adhesion onto the mammary epithelial cells and protects bacteria from opsonization and phagocytosis. Some strains of this genus are believed to exist in the form of a biofilm in the udder tissue, which may partly explain frequent therapeutic failures and a chronic course of infection. In this study we investigated the ability of slime production and biofilm formation in 70 subclinical and clinical bovine mastitis isolates S. aureus. Slime production was determined from colony morphology of isolates in Congo red agar. The ability of biofilm formation was assessed in a quantitative assay using a microtiter-plate test involving crystal violet staining and for selected strains, scanning electron microscopy on stainless steel coupons was done.*

*Eight S. aureus isolates (11.42%) formed black colonies of dry consistency, characteristic for slime-producing strains. According to the result of microtiter plate test, 9 isolates (12.86%) were categorized as strong biofilm producers, 21 (30%) as moderate, and 40 (57.14%) as weak biofilm producers. Scanning electron microscopy revealed differences between investigated isolates with respect to their ability to colonize stainless steel surfaces and to form a three-dimensional biofilm structure.*

*Key words: biofilm, mastitis, slime, Staphylococcus aureus*

**INTRODUCTION**

*Staphylococcus aureus* is an opportunistic pathogen and causative agent of a number of human and animal infections, ranging from superficial skin infections to septicemias. This microorganism is one of the most frequent mastitis agents in herds of dairy cows, causing severe health and economical problems. The bacteria adhere to the ductular and alveolar epithelium in the gland producing

epithelial cell damage, inflammation of the mammary gland, impairment of the host immune system, macrophage activation, neutrophil migration from the blood into the milk resulting in increased somatic cell counts (Cucarella *et al.*, 2004). Even in cases when *Staphylococcus aureus* isolates from milk exhibit good sensitivity to antibiotics *in vitro*, the treatment of such infections mostly remains ineffective, i.e. there is a discrepancy between the two parameters (Melchior *et al.*, 2006). The ability of this organism to persist in the mammary tissue as a biofilm is one of the possible sources of chronic or persistent infections (Vaseduvan *et al.*, 2003). A biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donland and Costerton, 2002). Bacteria in the biofilm exhibit increased resistance to components of the host's immune system and antimicrobial agents, as compared to their planktonic counterparts (Mah and O'Toole, 2001; Lewis, 2001; Dunne, 2002). Biofilm-forming ability has been increasingly recognized as an important virulence factor in staphylococci (Oliveira *et al.*, 2006).

Formation of a biofilm begins with the attachment of bacteria to the host cells. This specific adhesion pattern is mediated by bacterial cell wall structures containing adhesins, which is a genetically determined feature of bacterial species. Adherence of *Staphylococcus* species to the host cells is mediated by specific cell-surface proteins such as fibronectin, fibrinogen and collagen. In coagulase negative staphylococci (CoNS), an exopolysaccharide containing layer (slime) is nowadays investigated as a possible major determinant of bacterial adherence to host cells and inert surfaces. The slime is viscous extracapsular layer, weakly immunogenic and of labile structure which is lost or partially lost on *in vitro* subcultures (Aguilar *et al.*, 2001). Slime production is a virulence marker for clinically significant coagulase-negative staphylococci isolates and it is associated with intravenous-catheter-related bacteremia and other prosthetic device infection (Ishak *et al.*, 1985). Similarly, slime production was detected also in human and ruminant mastitis strains of *Staphylococcus aureus*. Slime-producing strains are considered to have increased ability of colonizing host tissue and better protection from opsonization and phagocytosis (Baselga *et al.*, 1993; Aguilar *et al.*, 2001; Arslan and Özkardes, 2007).

Slime production and biofilm forming ability of *Staphylococcus aureus* isolates from bovine clinical and subclinical mastitis was investigated in this study. Slime production was evaluated from colony morphology of isolates on Congo red agar. The ability of biofilm formation was assessed using a microtiter-plate test involving crystal violet staining and scanning electron microscopy.

## MATERIALS AND METHODS

### *Bacterial isolates*

The investigation was carried out on 70 mastitis isolates of *Staphylococcus aureus*. The isolates were identified by their cultural characteristics, microscopic

appearance in Gram stained preparations, positive catalase reaction, hemolysin, coagulase test with rabbit plasma and biochemical analysis utilizing Microbact™ Staphylococcal identification system (Oxoid LTD, UK). Until examination the isolates were stored on Tryptone Soya broth (TSB, Oxoid LTD, UK), to which 20% sterile glycerol was added, at -80°C. Immediately before testing the isolates were multiplied on blood agar with 5% ovine blood.

#### *Detection of slime production*

Slime production of all isolates was evaluated by Congo red agar method (CRA), according to the protocol of Freeman *et al.* (1989). The medium was prepared with 37 g/L brain heart infusion broth (BHI, Biokar Diagnostics), 50 g/L sucrose, 10 g/L agar and 0.8 g/L Congo red (MP Biomedicals, LCC, France). The medium was autoclaved at 121°C for 15 minutes. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents, and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24h at 37°C, followed by storage at room temperature for 48h. A positive result was indicated by black colonies with a dry crystalline consistency.

#### *Microtiter-plate test*

##### *Preparation of the inoculum:*

All *S. aureus* isolates were multiplied in pure culture on blood agar during 24 hours of incubation at 37°C. Groups of three single colonies were inoculated in 3 mL Tryptone Soya broth. Suspensions were incubated for 24h at 37°C and then diluted at 1:40 in a fresh TSB ( $2-7 \times 10^7$  cfu/mL). This dilution was used as the inoculum in the microtiter plate test.

##### *Test design:*

Microtiter plate test was performed by the method of Stepanović *et al.* (2000) with certain modifications. For each *S. aureus* isolate 200- µL aliquots of prepared suspension were inoculated into four wells of the 96-well tissue culture plates (NunclonDelta, Nunc, Roskilde, Denmark). Each culture plate included a negative control and four wells with TSB. The plates were incubated at 37°C for 24h. Afterwards, the well content was removed by pipetting and the wells were rinsed three times with 250 µL sterile physiological saline. The plates were dried in inverted position. The attached bacteria were fixed for 15 minutes at room temperature by adding 200-µL-volumes of methanol into each well. The plates were stained with 160 µL 0.5% aqueous solution of crystal violet (Crystal Violet, Fluka) for 15 minutes at room temperature. Following staining, the plates were rinsed under running water until there was no visible trace of stain. The stain bound to bacteria was dissolved by adding 160 µL of 95% ethanol. Optical density (OD) was measured spectrophotometrically (Labsystems Multiscan® MCC/340) using 595 nm filter.

Cut-off OD (OD<sub>c</sub>) is defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows:

- non-biofilm producers (OD ≤ OD<sub>c</sub>);

- weak biofilm producers ( $ODc < OD \leq 2 \times ODc$ );
- moderate biofilm producers ( $2 \times ODc < OD \leq 4 \times ODc$ );
- strong biofilm producers ( $4 \times ODc < OD$ ).

#### *Scanning electron microscopy*

Three *S. aureus* isolates were selected for scanning electron microscopy. The isolates were selected according to results obtained in the microtiter plate test, i.e. strong, moderate and weak biofilm producers. For biofilm formation sterile stainless steel coupons (1 x 1 x 0.2 cm) were used. The coupons were placed separately into the wells of the Nunc polystyrene plate with 12 wells (Roskilde, Denmark). 100  $\mu$ L -volumes of *S. aureus* isolates were inoculated onto the coupon surface. Bacterial adherence was enabled by incubation at room temperature over three hours, with subsequent pipetting of the suspension. The coupons were rinsed with sterile physiological saline and dipped in 2 mL of fresh TSB. Coupons were incubated over 5 days at 37°C and washed at 2-days intervals, and wells were inoculated with 2 mL of fresh TSB-YE.

After 5 days of incubation the coupons were removed from the well and washed by mild pipetting with 3 mL of sterile saline to remove the medium and non-adhered cells. The coupons were then fixed overnight in the refrigerator in 4% formaldehyde, double-washed in sterile distilled water and dehydrated in serial dilutions of 30, 50, 60, 70, 90% ethanol for 5 min each, followed by three 10-min rinses in aetanolum concentratum (95-96 vol %). Air-dried preparations were immediately sputter-coated with gold (Sputter Coater SCD 005, BALTEC SCAN, WD = 50 mm, 90s, 30 mA) and examined using scanning electron microscope (JMS SEM 6460 LV).

## RESULTS AND DISCUSSION

*S. aureus* is one of the most prevalent mastitis agents in dairy herds. It is believed that slime-producing *S. aureus* strains have a higher capacity of colonizing the mammary gland than its non-slime-producing variants (Baselga *et al.*, 1993; Cucarella *et al.*, 2004). Adhesion to mucosal surfaces may be a significant step in establishing persistent infections (Fox *et al.*, 2005). In this study, slime production was examined qualitatively, from the colony morphology of 70 bovine mastitis isolates of *S. aureus* produced on Congo red agar. Originally, CRA was established as a method for slime detection in coagulase-negative staphylococci (Freeman *et al.*, 1989). However, it has also been applied for investigating the slime production in human and animal *S. aureus* isolates (Knobloch *et al.*, 2002; Citak *et al.*, 2003; Oliveira *et al.*, 2006; Arslan and Özkardes, 2007; Krukowski *et al.*, 2008; Jain and Agarwal, 2009).

The examined *S. aureus* isolates produced four different colony phenotypes on CRA: 1. black colonies of dry consistency and with rough surface and edges (Fig. 1); 2. black colonies with a smooth, round and shiny surface (Fig. 2, left); 3. red colonies of dry consistency and with rough edges and surface (Fig. 3) and 4. red colonies with a smooth surface, round and shiny (Fig. 4).

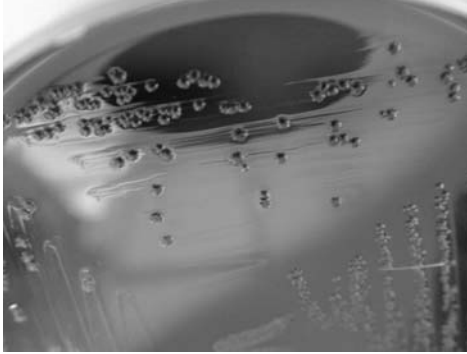


Figure 1. CRA – positive result:  
black colonies with a dry  
crystalline consistency

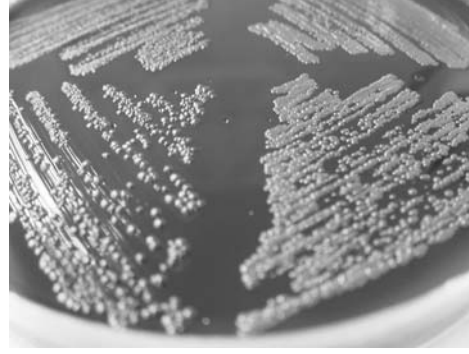


Figure 2. CRA – indeterminate result:  
black-smooth colonies (left)

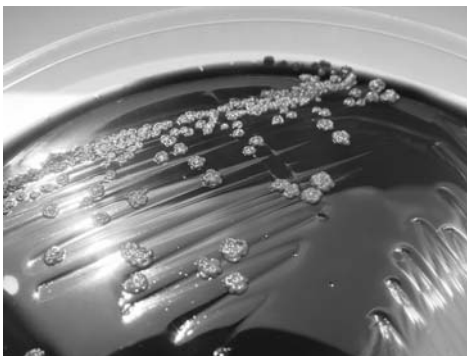


Figure 3. CRA – indeterminate result: red  
colonies a dry crystalline consistency

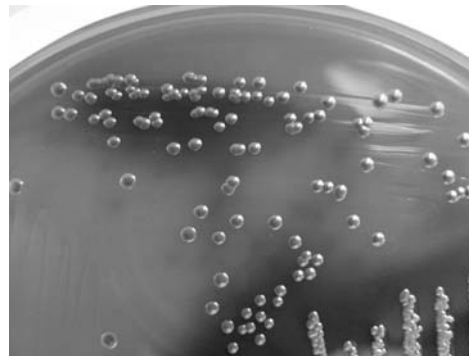


Figure 4. CRA – negative result: red-smooth  
colonies

According to Freeman *et al.* (1989), slime producing strains produce black colonies with a dry crystalline consistency on Congo red agar. An indeterminate result was indicated by a darkening of the colonies, but with the absence of a dry crystalline colonial morphology. In this study, isolates that formed black/rough colonies were characterized as slime producing, whereas isolates forming red/smooth colonies were described as non-slime producers. The remaining two types were considered *indeterminate result*. The results are displayed in Table 1.

Out of 70 bovine mastitis *S. aureus* isolates examined in CRA test, slime production was established in eight (11.42%) isolates, which produced characteristic black colonies of dry crystalline consistency. Similar results were obtained by Baselga *et al.* (1993) who detected 12% positive strains out of 92 bovine strains tested. Citak *et al.* (2003) established slime production in 5.1% *S.*

*aureus* isolated from raw milk samples. Higher percentage of slime producing *S. aureus* mastitis isolates was reported by Oliveira *et al.* (2006) (37.5%) and Krukowski *et al.* (2008) (42.37%). Results of Vasudevan *et al.* (2003) revealed that out of 35 *S. aureus* mastitis isolates, 32 produced typical black colonies within 24-48h.

Table 1. Slime production by *Staphylococcus aureus* in CRA method

No. of strains tested	Negative result	Indeterminate result		Positive result
	red-smooth colonies	black-smooth colonies	red colonies a dry crystalline consistency	black colonies with a dry crystalline consistency
70	39	15	8	8

Some differences between researches were apparent with respect to interpretation of CRA test results. In that respect, both bright black colonies (Citak *et al.*, 2003) and black colonies (Oliveira *et al.*, 2006; Arslan and Özkardes, 2007; Jain and Agarwal, 2009) were considered as a positive result. In our research 15 isolates (21.43%) produced black colonies of smooth and shiny surface, which was characterized as indeterminate result. Furthermore, in our study, red colonies with rough surface were described as indeterminate result. However, Cucarella *et al.* (2004) describe dry crystalline surface (rough colony phenotype) as a positive result, disregarding the color (black or pink). Finally, Knobloch *et al.* (2002) reported that 128 human isolates of *S. aureus* tested in CRA with brain heart infusion (BHI) base produced only red (ranging from pink to orange) colonies, as well as that particular strains formed black colonies on CRA using a TSB base. A dry crystalline morphology of these red colonies was observed after 24h for the five *S. aureus* strains which were described as slime producing. Such discrepancy when interpreting the results may possibly be due to the fact that the test itself was not originally designed for investigating *Staphylococcus aureus* isolates.

The ability of biofilm formation of *S. aureus* isolates was examined in the microtiter plate test. The results are presented in Table 2.

Table 2. Results of microtiter-plate test

No. of strains tested	No. of isolates showing			
	Non biofilm producer OD ≤ 0.178	Weak biofilm producer 0.178 < OD ≤ 0.356	Moderate biofilm producer 0.356 < OD ≤ 0.712	Strong biofilm producer 0.712 < OD
70	–	40	21	9

According to measured extinctions, nine *S. aureus* isolates (12.85%) were identified as strong biofilm producers. In a microtiter plate test Oliveira *et al.* (2006)



also obtained a lower percentage (18.75%) of biofilm producers among *S. aureus* mastitis isolates. However, results of Vasudevan *et al.* (2003) in the tissue culture plate test revealed 24 biofilm producing strains of the total 35 mastitis *S. aureus* isolates investigated.

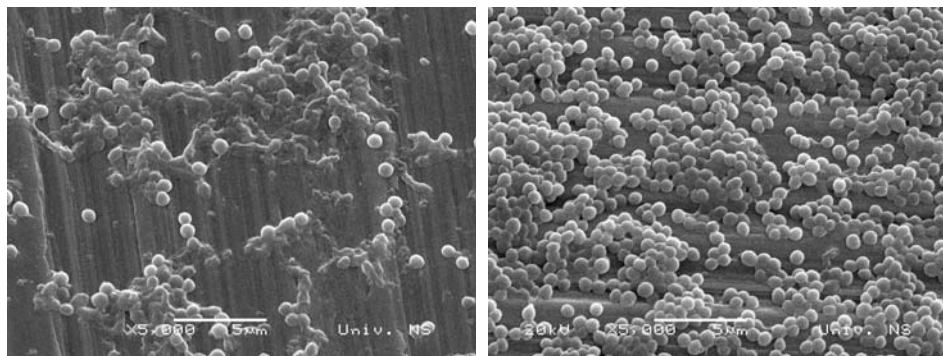
Out of nine strong biofilm producers confirmed in our microplate test, four proved positive in CRA test, 3 gave indeterminate results (red/dry crystalline consistency), and two were considered negative results. Positive or negative result of the CRA test does not necessarily imply that the isolate will prove as a biofilm producer in the microtiter plate test, which was confirmed by the research of Vasudevan *et al.* (2003), Oliviera *et al.* (2006), and Jain and Argawal (2009). Assessment of biofilm formation according to phenotypic expression (slime production on CRA, or biofilm formation on tissue culture plates) is highly susceptible to *in vitro* conditions (Baselga *et al.*, 1993; Stepanović *et al.*, 2000; Vasudevan *et al.*, 2003). Knobloch *et al.* (2002) emphasized that supplementation of TSB or BHI media with different sugars (TSB<sub>glc/suc</sub>, BHI<sub>suc</sub>, BHI<sub>glc/suc</sub>) increased biofilm formation significantly.

It is always recommendable to apply several *in vitro* methods in such research, but it must be kept in mind that attachment to abiotic surfaces might not be necessarily related to attachment to biotic surfaces (Cucarella *et al.*, 2001). The hypothesis that *S. aureus* isolates from cow's milk produce a biofilm more frequently (41.4%) than the *S. aureus* strains obtained from teats (24.7%), as well as from the milking equipment (14.7) was experimentally confirmed by Fox *et al.* (2005). Vasudevan *et al.*, (2003) believe that screening of biofilm forming ability of *S. aureus* isolates requires combining of phenotypic and genotypic methods. Though research results of these authors revealed biofilm formation *in vitro* in 24 out of 35 mastitis isolates of *S. aureus*, all isolates were found to possess *ica* gene locus (and *icaA* and *icaD* genes), which is considered to play an important role in biofilm formation by *S. aureus* and *S. epidermidis*.

Different abilities of selected *S. aureus* isolates to colonize stainless steel surfaces and form distinctive biofilm structures was confirmed by scanning electron microscopy. Whereas weak biofilm producers formed scant individual microcolonies (Figure 5a), moderate biofilm producers colonized the stainless steel surface to somewhat greater extent (Figure 5b), a strong biofilm producer completely covered the surface, forming massive, robust biofilm composed of densely packed bacterial colonies (Figure 5c and 5d).

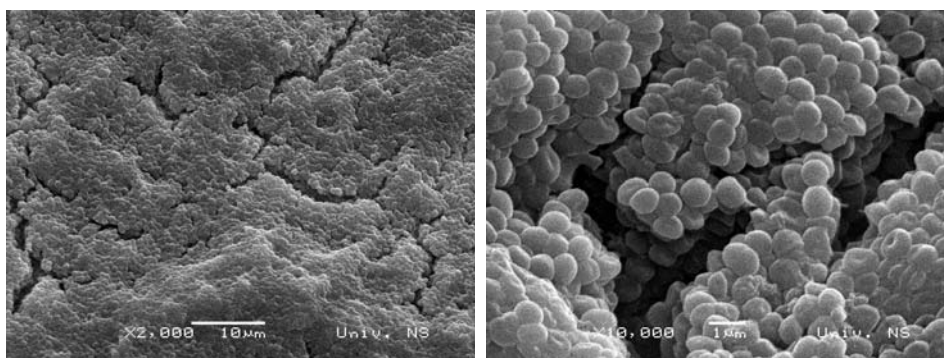
Results of numerous studies demonstrated the ability of *S. aureus* mastitis isolates to produce slime and form a biofilm. This property helps the bacteria to survive hostile environments within the host, and considered to be responsible for chronic or persistent infection. Antimicrobial therapy of mastitis is usually based on the results of susceptibility tests *in vitro*, applying the disc diffusion or dilution method. However, antibiotics are effective in inhibiting planktonic bacterial population, whereas bacteria in biofilm survive the treatment and provide material for further growth. *In vitro* research confirmed different antimicrobial susceptibility of the planktonic and biofilm population of *S. aureus* (Amorena *et al.*, 1999). Thus, bovine mastitis caused by *S. aureus* still remain an open research field for

investigating the genetic basis of biofilm formation and finding new options of effective prophylaxis and treatment of such infections.



a) weak biofilm producer (5 000x)

b) moderate biofilm producer (5 000x)



c) strong biofilm producer (2 000x)

d) strong biofilm producer (10 000x)

Figure 5. Scanning electron microscopy – biofilms of *S. aureus* isolates on stainless steel after 5 days incubation in TSB at 37°C

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**SPOSOBNOST "SLIME" PRODUKCIJE I FORMIRANJA BIOFILMA KOD  
STAPHYLOCOCCUS AUREUS IZOLOVANIH IZ MLEKA KRAVA SA MASTITISOM**

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SADRŽAJ

Pojedini sojevi *Staphylococcus aureus* koji su uzročnici mastitisa krava, imaju sposobnost produkcije viskoznog, ekstracelularnog, polisaharidnog sloja ("slime") koji se u novije vreme razmatra kao faktor virulencije, jer olakšava adheziju bakterija na epitelne ćelije mlečne žlezde i štiti ih od opsonizacije i fagocitoze. Takođe se smatra da neki sojevi ove bakterijske vrste egzistiraju u formi biofilma u tkivu vimena, čime se između ostalog, objašnjava čest neuspeh u terapiji i hronični tok infekcije. U ovom radu je ispitana sposobnost "slime" produkcije i formiranja biofilma kod 70 izolata *S. aureus* poreklom iz mleka krava sa kliničkim i subkliničkim mastitisom. "Slime" produkcija je ispitana na osnovu morfologije kolonija koje su izolati formirali na "Congo red" agaru, a sposobnost formiranja biofilma kvantitativnim testom na mikrotitracionim pločama upotrebom boje kristal-violet i skening elektronskom mikroskopijom na kuponima od nerđajućeg čelika (za odabrane sojeve).

Crne kolonije suve konzistencije, karakteristične za "slime" produkujuće sojeve, formiralo je osam izolata *S. aureus* (11,42%). Prema rezultatima testa na mikrotitracionim pločama, devet izolata (12,86%) je klasifikovano u jake, 21 (30%) u umerene, a 40 (57,14%) u slabe biofilm producere. Skening elektronskom mikroskopijom potvrđene su razlike među ispitivanim izolatima u njihovoj sposobnosti kolonizacije površine nerđajućeg čelika i formiranja trodimenzionalne biofilm strukture.