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Original article

Comparison of biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* strains isolated from sheep milk using three diagnostic methods

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Abstract

In this study we investigated 24 strains of *Staphylococcus aureus* and 33 strains of *Staphylococcus epidermidis* isolated from milk of sheep with clinical mastitis, for their ability to form biofilms. Three methods for the determination of a biofilm were used. When evaluating the growth on Congo Red agar (CRA), 79.2% *S. aureus* strains and 72.7% *S. epidermidis* strains were positive for biofilm formation. The quantitative method of biofilm detection on a Microtitre Plate (MTP) revealed positive results for 75.0% of *S. aureus* samples and 75.8% for *S. epidermidis* samples. Using PCR method for determination of the presence of genes that affect formation of biofilms, the most frequently determined genes were *eno* in both *S. aureus* (18/24; 75.0%) and *S. epidermidis* strains (20/33; 60.6%). The genes *icaAB* and *ebpS* were detected in both *S. aureus* and *S. epidermidis* strains, and similarity between these strains was 12.5% – 15.1% and 4.2% – 6.0%, respectively. The *bap* was recorded only in *S. epidermidis* (3.0%). Statistical comparison of the level of biofilm formation was performed using Chi square test. There were no statistically significant differences in the amount of biofilm formation between two methods for detection of biofilm CRA and MTP ($p > 0.05$). Comparison of all six monitored parameters showed no dependence of characteristics of the tested strains *S. aureus* and *S. epidermidis* at significance level $\alpha = 0.05$. Biofilm formation by the bacteria isolated from 57 cases of clinical mastitis in sheep was confirmed. Sensitivity and specificity of the CRA method for *S. aureus* were 94.44% and 66.66%, respectively, and for *S. epidermidis* 92.0% and 87.5%, respectively. Both CRA and MTP methods can be recommended for the detection of biofilm production by *S. aureus* and *S. epidermidis* strains isolated from milk of sheep with clinical mastitis.

Key words: biofilm production, Congo Red agar, Microtitre Plate, genes coding for biofilm, PCR

Introduction

The sale and consumption of milk and products from sheep milk is more or less dependent on season and despite the legislative measures, especially as the traditional yard sales of non-pasteurized milk are concerned, may raise relevant public health issues. Staphylococci are the most important pathogenic bacteria causing ovine mastitis. The development of multidrug resistance in *Staphylococcus* spp. is a problem of concern in animal production.

Similar to the situation in sheep, Bochniarz et al. (2014) reported that the major problems in the therapy of infections caused by coagulase-negative staphylococci (CNS) in dairy cows involve the ability of bacterial agents to form biofilm and the mechanisms of acquiring the drug-resistance (Łopaciuk and Dzierżanowska 2002).

Biofilm formation is considered to be one of the virulence factors in staphylococci, which helps staphylococci adhere to its target tissues, mainly implants and other foreign body materials, through adhesive mechanisms (Rupp et al. 1999, Götz 2002). Some of the strains become invasive under favourable conditions, while others do not cause disease. Early detection and management of potentially pathogenic staphylococci is the essential step to prevent device-associated infections (Jain and Agarwal 2009).

Biofilm formation is an important factor of the virulence of the pathogen of the mammary gland, which allows bacteria to survive outdoors, and at the same time protects them from host immune defences. The pathogen is often involved in the emergence and persistence of subclinical and latent forms of mastitis in dairy cows and sheep (Melchior et al. 2005). The predominant species isolated at these infections are *Staphylococcus epidermidis* and *Staphylococcus aureus*, their major pathogenic factor being their ability to form biofilm on polymeric surfaces (Kloos and Bannerman 1994). Fabres-Klein et al. (2015) reported, that the majority of bacteria characterized in their study formed a biofilm, which suggested that biofilm formation has an important role in the virulence of *S. aureus* isolated from bovine intramammary infections. Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide (slime), which facilitates the adherence of these microorganisms to biomedical surfaces and protects them from host immune defences and antimicrobial therapy (O Gara and Humphreys 2001).

Biofilm formation starts with the adherence of bacteria to the surface, followed by accumulation, maturation and detachment phases essential for the dissemination of staphylococci (Fey and Olson 2010). This reaction is caused by a protein family of

Staphylococcal Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (Foster and Hook 1998). Staphylococcal adhesins, which promote adhesion to host cells, are important for cellular interactions with cell surface proteins such as collagen, fibrinogen-, fibronectin-, elastin-, and laminin-binding proteins and clumping factors (Götz 2002). Considering the role of a biofilm in the adherence and colonisation of staphylococci on the mammary gland epithelium, evasion of the immunological defences, resistance to antibiotic therapy and difficulty of pathogen eradication, the findings have high significance (Dhanawade et al. 2010). It is now well documented that biofilm is notoriously difficult to eradicate and often resistant to systemic antibiotic therapy, so the removal of infected devices becomes necessary (Lewis 2001).

Many studies suggested that bovine staphylococci isolates can adapt to the environment found in the udder, with milk influencing the biofilm production and therefore promoting bacterial survival (Seixas et al. 2015). Under dynamic conditions, a low degree of biofilm formation by staphylococci was observed. This can be explained by the fact that dynamic conditions may impair planktonic bacterial adhesion, the first step of biofilm formation (Stepanović et al. 2001). Several studies revealed that dynamic conditions affect Gram-negative and Gram-positive biofilm-producing isolates, emphasizing the importance of experimental conditions for the final outcome (Stepanović et al. 2001, Seixas et al. 2014). A positive reaction was obtained with CNS from the milk of the cows with both clinical and subclinical form of mastitis.

The aim of this study was to compare the results obtained by three methods of detection of biofilm formation and the occurrence of the selected genes in 24 strains of *S. aureus* and 33 strains of *S. epidermidis* isolated from sheep with clinical mastitis.

Materials and Methods

Bacterial strains

The study investigated bacterial strains *S. aureus* (n = 24) and *S. epidermidis* (n = 33) isolated from sheep with clinical mastitis (acute, subacute), collected during two milking seasons. All isolates were characterized by conventional microbiological methods, by primo-cultivation on 5% blood agar and consistent cultivation on specific culture media. The staphylococcal species were identified by two different identifying methods: on the basis of biochemical enzymatic properties of bacteria by STAPHYtest 24, using

an identifying software TNW 7.0 (Erba-Lachema, Brno, Czech Republic), with precision of detection exceeding 90.0%. Second used method for evaluation of concretely strains, was detection on the basis of determination of proteins spectrum by Maldi-Biotyper (Bruker, USA). Results which provided Maldi score values in the range of 2.300-3.000, were indicated as highly probable species identification.

Formation of biofilm was determined by phenotypic and genotypic methods of detection. The following three methods were used: Growth on CongoRed agar (CRA); Microtitre plate method (MTP); PCR genotypic detection of genes coding for biofilm formation. *S. epidermidis* ATCC 35984 was used as the control biofilm-producing strain and *S. epidermidis* ATCC 12228 served as the control biofilm non-producer.

Growth on CongoRed agar (CRA)

Production of slime by all strains was studied by cultivation of the strains on CRA according to Arciola et al. (2001). CRA plates (0.8 g of CRA and 36 g of saccharose in 1 litre of brain heart infusion agar) were incubated at 37°C for 24 h and subsequently overnight at room temperature. The slime producing strains form black colonies on CRA, whereas the non-producing strains develop red colonies. In some cases, when pink sub colonies emerged in the centre of the black colonies, small pink or black samples were picked from both pink and black areas and provided dubious results.

Tissue Culture Plate (TCP) methods for detection of biofilm

The TCP biofilm assay described by Christensen et al. (1985) and Simojoki et al. (2012) was used with slight modification. Briefly, isolates were incubated at 37°C for 18h – 24h in tryptone soy broth (TSB). The analysis was performed for each isolate with glucose and lactose broth supplementation. Lactose was used to substitute saccharide supplementation, so that the amount of lactose was comparable with that in milk, 5% in TSB (0.25% lactose, Merck, Darmstadt Germany). Next, 0.1 ml of broth suspension was added to 10 ml TSB (1:100) and another 100 µl aliquot of this solution was pipetted onto flat-bottomed tissue culture plates. The plates were incubated at 37°C for 24 h, washed three times with 200 µl phosphate buffered saline (PBS), dried and incubated at 37°C for 30 min in an inverted position. The plates were then stained with 200 µl of 0.4% crystal violet for 5 min, washed

five times with 200 µl distilled water and fixed with 200 µl solution of ethanol:acetone (80:20). Then a MRX Microplate Reader (DYNEX Technologies, Inc., Chantilly, VA, USA) was used to determine absorbance at 570 nm. Each plate included a pure TSB sample (blank) and positive and negative controls, all in triplicate. The absorbance of the blanks was subtracted from the absorbance of the isolates.

The strains were categorized using a scale based on the average optical density (OD) of the blank wells plus 3 times the standard deviation of the mean (ODs), according to Moore (2008). A strain was considered negative if the optical density was below the cut off value (ODs), and weakly positive if the OD was between the cut off value and two-fold of this number (2x ODs). Any strain with an optical density greater than two-fold of the cut off value was categorized as strongly positive.

PCR methods for detection of genes coding for production of biofilm

Four adhesion genes were analysed by PCR. Genomic DNA of all *Staphylococcus* spp. isolates were extracted using a DNA purification QiAMP tissue kit (QIAGEN, Hilden, Germany). Three different PCR reactions were set up to detect the four adhesion genes (Schlegelová et al. 2008, Seo et al. 2008, Simojoki et al. 2012). PCR 1 was designed to detect *eno*, *ebpS* genes except *bap*, which was detected by PCR 2. The *icaAB* gene was detected by PCR 3. The names of target genes and nucleotide sequences of primers are shown in Table 1.

In the reaction mixture, 100 µL Cheetah Taq DNA Polymerase (BIOTIUM, Inc., Hayward, USA) and 10 mM dNTP (Invitrogen, Carlsbad, USA) were used. The nucleotide sequence of the primer used in this study was the same as that used in the previous reports (Cucarella et al. 2001). The thermo cycler programme for PCR 1 started with an initial denaturation step of 5 min at 94°C, followed by 25 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (1 min at 72°C). The reactions were finalized by polymerization for 10 min at 72°C. PCR 2 was performed with an initial denaturation step of 30 s at 94°C, followed by 30 cycles of denaturation (45 s at 94°C), annealing (1 min at 62°C), and extension (1 min at 72°C). The reactions were finalized by polymerization for 7 min at 72°C. The reaction PCR 3 was run under the following regime: 2 min at 96°C, 10 s at 96°C, 10 s at 55°C, 40 s at 72°C, 45 cycles with final extension (2 min 72°C). The PCR products were analysed by electrophoresis in 1% agarose gel, and were visualized under a UV trans-illuminator. Detection of

Table 1. PCR primers used for detection of biofilm encoding genes in *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Genes	Encoding protein	Nucleotide sequence of primers	Amplicon size (bp)
<i>bap</i>	<i>biofilm associated protein</i>	3'-CCCTATATCGAAGGTGTAGAATT-5' 5'-GCTGTTGAAGTTAATACTGTACCTGC-3'	971
<i>icaAB</i>	<i>intercellular adhesin protein AB</i>	3'-CTGTTGACGCCGTAACCTATT-5' 5'GTTTAACGCGAGTGCGCTAT- 3'	546
<i>eno</i>	<i>laminin binding protein</i>	3'-ACGTGCAGCAGCTGACT-5' 5'-CAACAGCATCTTCAGTACCTTC-3'	302
<i>ebpS</i>	<i>elastin binding protein</i>	3'-GTCAAGCAGTTATTAACACCAGAC-5' 5'-AATCAGTAATTGCACCTTTGTCCACTG-3'	423

The primers were used according to Tristan et al. (2003), except for *icaAB* for which they were used according to Schlegelová et al. (2008), and for *bap* for which they were used according to Seo et al. (2008)

Table 2. Comparison of the biofilm formation by *S. aureus* and *S. epidermidis* strains using three methods.

Methods used for testing the biofilm formation potential	<i>S. aureus</i> (n = 24)			<i>S. epidermidis</i> (n = 33)			Test* G	
	positive	%	negative	positive	%	negative		
A Congo red agar (CRA)	19	79.2	5	24	72.7	9	0.308 ¹	
B Microtitre plate (MTP)	18	75.0	6	25	75.8	8	0.005 ¹	
C	<i>Bap</i>	0	0.0	24	1	3.0	32	0.737 ¹
	<i>IcaAB</i>	3	12.5	21	5	15.1	28	0.517 ¹
	<i>Eno</i>	18	75.0	6	20	60.6	13	1.295 ¹
	<i>ebpS</i>	1	4.2	23	2	6.0	31	0.098 ¹

A – qualitative testing by Congo red agar method (CRA); B – quantitative testing by Microtitre plate method (MTP); C – genes detected by PCR – Polymerase chain reaction method; * – Chi-quadrat test (significance level $\alpha = 0.05$ (5%); critical value $\chi^2 = 3.841$; G – test value), ¹ – dependence of individual characteristics at significance level $\alpha = 0.05$ was not rejected.

amplification products of 546 bp, 302 bp, 971 bp, 423 bp demonstrated the positivity of isolates for genes *icaAB*, *eno*, *bap* and *ebpS*, respectively.

Statistical analysis

Statistical analysis was performed using software Microsoft Excel 2007. Chi square test (χ^2 test) was used to compare the individual proportions (Kabrt 2013). The dependence of the individual signs was tested at a significance level $\alpha = 0.05$, with critical value $\chi^2 = 3.841$. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the CRA method were calculated using Bayesian analysis of 2x2 Table. The MTP method was used as a gold standard (Jain and Agarwal 2009).

Results

The present study allowed us to determine biofilm formation potential of *Staphylococcus* spp., namely *S. aureus* – 24 and *S. epidermidis* – 33, isolated from sheep milk. Three methods for the determination of

biofilm were used: the quantitative growth on Congo Red agar; quantitative determination by Microtitre Plates (MTP) method; determination of the presence of the selected genes coding for production of biofilm, *bap*, *eno*, *ebpS* and *icaAB*, by PCR.

Evaluation of the growth on CRA showed positivity in 79.2% (19/24) of *S. aureus* strains and in 72.7% (24/33) of *S. epidermidis* strains. By the quantitative method for detection of biofilm (MTP), the positive results were obtained for *S. aureus* in 75.0% (18/24) and for *S. epidermidis* in 75.8% (25/33) of strains. Phenotypic positivity by both methods of detection was confirmed in 40 cases, from these in 17 strains of *S. aureus*, and in 23 strains of *S. epidermidis*.

DNA was isolated from all 57 strains and used to determine the presence of selected genes that affect biofilm formation. We determined the presence of *bap*, *icaAB*, *eno*, and *ebpS* genes. The most frequently detected gene was *eno* in both *S. aureus* (18/24; 75.0%) and *S. epidermidis* (20/33; 60.6%). Genes *icaAB* and *ebpS* were detected in *S. aureus* and *S. epidermidis* with small difference between these strains (12.5% – 15.1% and 4.2% – 6.0%, respectively). Gene *bap* was found only in *S. epidermidis* (3.0%).

Table 3. Comparison of detection of biofilm formation by *Staphylococcus aureus* (n=24) using Congo Red agar and Microtitre Plate methods.

CRA vs. MTP		MTP method		Total
		positive	negative	
CRA method	positive	17	2	19
	negative	1	4	5
	total	18	6	24

CRA – Congo red agar; MTP – Microtitre plate method. Sensitivity – 94.44%; specificity – 66.66%; positive predictive value (PPV) – 89.47%; negative predictive value (NPV) – 80.0%.

Table 4. Comparison of detection of biofilm formation by *Staphylococcus epidermidis* (n=33) using Congo Red agar and Microtitre Plate methods.

CRA vs. MTP		MTP method		Total
		positive	negative	
CRA method	positive	23	1	24
	negative	2	7	9
	total	25	8	33

Sensitivity – 92.0%; specificity – 87.5%; positive predictive value (PPV) – 95.83%; negative predictive value (NPV) – 77.77%.

Statistical evaluation of biofilm formation and the presence of genes coding for this formation were carried out by Chi-squared Test (χ^2 test). Comparison of all six monitored parameters showed no dependence of characteristics of the tested strains *S. aureus* and *S. epidermidis* at significance level $\alpha = 0.05$. The value of the test criterion G ranged from 0.005 to 1.517 (Table 2) and in none of the evaluations reached the critical value of $\chi^2 = 3.841$, which resulted in the decision that the null hypothesis of independence of the characters was not refused. Thus it was concluded that the production of biofilm as a test criterion confirmed in both strains of staphylococci was not accidental.

Sensitivity and specificity of testing of the strains *S. aureus* (Table 3) and *S. epidermidis* (Table 4) by CRA and MTP methods were evaluated. Of the total 24 *S. aureus* isolates, 18 were biofilm positive and 6 biofilm negative by the MTP method. The CRA test gave 3 false results of which 1 was false positive and 2 false negative. Of the 33 *S. epidermidis* isolates, 25 were biofilm positive by the MTP method. When using the CRA test, 3 isolates gave false results, of which 2 were false positive and 1 was false negative.

Sensitivity and specificity of the CRA method for *S. aureus* were 94.44% and 66.66% respectively, and for *S. epidermidis* 92.0% and 87.5%, respectively.

Discussion

Nasr et al. (2012) compared the results of both the CRA and MTP methods and observed that despite

46% positivity of the total staphylococcal isolates detected by both methods, results correlated only in 10 (20%) isolates. A low correspondence between both methods was demonstrated also by Marthur et al. (2006). On the other hand, better correlation between both methods was reported by other investigators, as all staphylococci positive by one test were also positive by the other (Cafiso et al. 2004). Yazdani et al. (2006) also reported that 2 of 27 *Staphylococcus aureus* strains, which produced black colonies on CRA, were biofilm negative by MTP.

Jain and Agarwal (2009) reported results of comparison of CRA and MTP methods for determination of biofilm formation by staphylococcal strains isolated from peripheral intravenous device (IVD), venous blood, site of IVD insertion and nasal mucosa of patients admitted to a pediatric ward with peripheral intravenous devices in place for more than 48 h. The total of 100 invasive, 50 colonizing and 50 commensal isolates were studied. Of 100 invasive isolates 74% (74/100) were biofilm positive while only 68% (34/50) colonizing and 32% (16/50) commensal isolates were biofilm positive. The difference in biofilm production by commensal, colonizing and invasive strains was significant ($p < 0.0001$). Sensitivity and specificity of the CRA test for detection of biofilm producers were 90.63% and 90.79%, respectively, for *Staphylococcus aureus* and 75.86% and 96.88%, respectively, for coagulase-negative staphylococci. CRA is a method that can be used to determine whether an isolate has the potential for biofilm formation or not.

In our experiment, comparison of all six monitored parameters characteristic of the tested

strains *S. aureus* and *S. epidermidis* failed to show any dependence at a significance level of $\alpha = 0.05$. The value of test criterion G ranged from 0.005 to 1.517, and in none of the evaluations reached the critical value of $\chi^2 = 3.841$, resulting in the decision that the null hypothesis of independence of the characters was not refused in any case. Sensitivity and specificity of the CRA method were 94.44% and 66.66%, respectively, for *S. aureus*, and 92.0% and 87.5%, respectively, for *S. epidermidis*.

Iorio et al. (2011) reported that of the 40 *S. aureus* isolates which were positive for the *ica* gene, 25 (62.5%) were positive by MPA and 27 (67.5%) by CRA, whereas both methods combined identified 34 (85%) isolates as biofilm producers. Among 12 *S. epidermidis* isolates carrying *ica* genes, 8 were positive by MPA and 5 by CRA. The combination of CRA and MPA methods provided a better prediction of the presence of *ica* genes in *S. aureus* isolates than did either method alone. We detected the *eno* gene in both *S. aureus* (18/24; 75.0%) and *S. epidermidis* (20/33; 60.6%). Genes *icaAB* and *ebpS* were detected in *S. aureus* and *S. epidermidis* with similarity between these strains (12.5% – 15.1% and 4.2% – 6.0%, respectively).

Bap-positive isolates are able to infect and persist in the bovine mammary gland and are less susceptible to antibiotics when forming biofilms *in vitro* (Cucarella et al. 2004). Results of some authors indicated that none of the isolates carried the *bap* gene (Seo et al. 2008, Simojoki et al. 2012). In our experiment, the presence of the *bap* gene was confirmed only in *S. epidermidis* isolated from acute mastitis. Li et al. (2012) described that the *bap* gene was amplified in 51 of 116 biofilm-positive strains, indicating that the *bap* gene was present in not all but some of the biofilm-positive strains.

The MSCRAMM gene most commonly detected in our isolates from mastitis was the *eno* gene encoding laminin-binding protein (Simojoki et al. 2012). This gene was also found in coagulase-negative staphylococci (CNS) originating from filtered air and from infections in dogs and swine (Seo et al. 2008). The MSCRAMM genes are frequently detected in *S. aureus*. Vanraeynest et al. (2004) detected 7 MSCRAMM genes (median) in isolates originating from rabbits, and Tristan et al. (2003) detected 6 MSCRAMM genes in isolates of human origin. Different MSCRAMM genes are associated with different levels of pathogenicity of *S. aureus* strains. The low prevalence of these genes in our CNS isolates, except for the *eno* gene detected with higher prevalence, is consistent with the study by Seo et al. (2008), who detected MSCRAMM genes in CNS from other sources but not in isolates from mastitis.

The process of bacterial attachment is characterized by a number of variables, including the species of bacteria, surface condition of supporter, environmental factors, growth medium, and essential gene products (Dunne 2002, Seo et al. 2008). To monitor the biofilm formation by bacteria, all these various factors should be considered together. The analysis of MSCRAMM genes by phenotypic assays may be an essential step for bacterial control in the environment.

Statistical differences in biofilm formation between CNS – *S. epidermidis* and CPS – *S. aureus*, isolated from clinical mastitis in sheep, were not confirmed. On the basis of our investigations that focused on comparison of methods for detection of biofilm formation potential of strains isolated from milk of sheep with clinical mastitis, and determination of specificity and sensitivity of both these methods, we can recommend simultaneous use of CRA and MTP methods.

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