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SHORT COMMUNICATION

Biofilm Production and Presence of *ica* and *bap* Genes in *Staphylococcus aureus* Strains Isolated from Cows with Mastitis in the Eastern Poland

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Abstract

The aim of the study was phenotypic and genotypic analysis of 132 *S. aureus* strains isolated from mastitis in eastern Poland in respect to their biofilm formation ability. The analysis of the size polymorphism of fragment X in the gene encoding protein A (*spa*) revealed high genetic differentiation of the analyzed group of isolates. The ability of biofilm formation by the isolates was tested using two phenotypic methods. The Congo Red plate assay was found to be irreproducible and very subjective. More objective results were obtained using the spectrophotometric, microtiter plate assay. Most of the isolates, namely 76/132 (57.6 %) were classified as biofilm producers depending on the value of absorbance in the microtiter plate test. All of the isolates tested were found to possess both *icaA* and *icaD* genes, while the *bap* gene was absent in all strains.

Key words: *Staphylococcus aureus*, biofilm, genes: *icaA*, *icaD*, *bap*, mastitis

Among animal diseases caused by S*taphylococci* one of the most important is bovine mastitis – subclinical or chronic infections of mammary glands. Although about 140 species of microorganisms have been identified as etiological agents of bovine mastitis (Watts *et al.*, 1988), *Streptococci*, Coliforms and S*taphylococci* are most often isolated (Tenhagen *et al.*, 2006; Piepers *et al.*, 2007; Malinowski and Klossowska, 2010; Smulski *et al.*, 2011).

It has been proved by many authors, that in the case of bovine mastitis caused by *S. aureus* and other *Staphylococci*, the ability to produce biofilm (slime) is the most important reason for unusual problems with eradication of infection and recurrent infections of mammary glands (Melchior *et al.*, 2006b). Production of slime enables adhesion of bacteria to the epithelium of mammary glands. It also facilitates persistence of micro-organisms in the host tissue by protecting the bacterial cells against the mechanisms of the host defense. Importantly, it causes the evident reduction of susceptibility to antibiotics, due to altered growth rate and delayed penetration of antimicrobial agents within the biofilm structure (Melchior *et al.*, 2006a, 2007). Production of biofilm requires the presence of the gene cluster *icaADBC* (the intracellular adhesion locus) (Cramton *et al.*, 1999) and strains harboring the *icaADBC* cluster are potential biofilm producers.

The alternative, *icaADBC*-independent, mechanism of biofilm formation by *S. aureus* isolates causing mastitis has been described by Penadés and Lasa. In their research on the *S. aureus* bovine mastitis isolate V329, they proved that the biofilm – associated protein (Bap) instead of PIA was indispensable for the primary attachment and cells' accumulation (Cucarella *et al.*, 2001; Lasa and Penadés, 2006).

Several reports concerning production of biofilm by *S. aureus* strains causing mastis, isolated from different regions of the world, have been recently presented (Vasudevan *et al.*, 2003; Ciftci *et al.*, 2009; Dhanawade *et al.*, 2010; Milanov *et al.*, 2010). In Poland however, the ability of biofilm production by *S. aureus* causing mastitis has not been deeply investigated, except some phenotypic analysis (Krukowski *et al.*, 2008; Dziekiewicz-Mrugasiewicz *et al.*, 2008). The aim of our studies was the analysis of the ability to produce biofilm by *S. aureus* strains isolated in Poland, by means of known phenotypic and genotypic methods.

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One hundred and thirty two strains of *S. aureus* isolated from the milk of cows suffering from mastitis were tested. The bacterial strains were identified as *S. aureus* by using the PCR analysis to amplify the part of the *S. aureus* specific *nuc* gene, encoding termostable nuclease (Brakstad *et al.*, 1992). The *S. aureus* strain V329, used as a positive control for detection of *bap* gene, was kindly provided by dr JR Penadés (Valencia, Spain). DNA was purified from bacterial cells using the Bacterial & Yeast Genomic DNA Purification Kit (EurX; Poland) according to the manufacturer's instruction and lysostaphin (1 U; Sigma) solution was added to the cell suspension for enzymatic lysis of *S. aureus* cell wall mureine. Five different targets were amplified. In the case of detection of *nuc*, *icaA*, *icaD* and *spa* genes, the same composition of the reaction mixtures was used: 1 µl of dNTPs (2.5 mM each), 2.5 µl of 10 × PCR reaction buffer (100 mM Tris-HCl, pH 8.8, 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% Nonidet P40, 0.5% Tween 20), 2 µl of $MgCl_2$ solution (50 mM), 1 µl of each of the two required primer solutions (10 µM), 1 µl of DNA solution (prepared as described above), 0.2 µl (1 U) of polymerase *Delta* from *Pyrococcus woesei* (DNA-Gdańsk II SC, Poland), and deionized sterile water to complete the mixture volume to 25 µl. In the case of PCR composition for the detection of *bap* gene, due to results of PCR optimalization, the 10 × PCR buffer containing 100 mM Tris HCl, pH 8.8, 500 mM KCl and 0.8% (v/v) Nonidet P40 was used. The primer sequences applied are presented in Table I. The PCR conditions were the following: an initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30 s, specific temperature of annealing (Table I) for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. The ability of biofilm production was performed by cultivation of isolated *S. aureus* strains on Congo Red Agar (CRA) as described earlier by Freeman *et al.* (1989). Strains producing rough black colonies were classified as slime producers while the non-producing isolates remained red. The semi – quantitative measurement of an ability of *S. aureus* isolates to produce biofilm was carried out by the method described earlier by Cucarella *et al.* (2001) and Vasudevan *et al.* (2003).

The CRA method was found to be irreproducible, due to its high subjectivity in interpretation of obtained results. It was difficult to find a borderline in the color of the growing colonies that should be classified as slime producers or non-producers. Unreliability of the CRA method as a reference test for slime production was earlier presented by other authors (Rohde *et al.*, 2001, Vancraeynest *et al.*, 2004, Atshan and Shamsudin, 2011) and now confirmed in our studies. Because of the mentioned problems, it was impossible to make a reliable and reproducible selection of slime producers among the analyzed group of isolates.

Much better results were obtained using the semi-quantitative phenotypic test. The spectrophotometric assay revealed that 76 (57.6%) of strains tested produced biofilm, while remaining 56 demonstrated negative response in this test. Depending on the value of absorbance of the stained biofilm, the strains were classified as: weak biofilm producers (absorbance in the range 0.1–0.25), medium biofilm producers (absorbance in the range 0.25–0.5) and strong biofilm producers (absorbance above 0.5). According to these criteria, 46/76 isolates, were identified as weak biofilm producers and only 9/76 could be classified as strong slime producers. All of 132 strains tested were shown to harbor the *icaA* and *icaD* genes, what makes them potential biofilm producers. Amplification of a part of the *bap* gene resulted in obtaining the expected 971 bp band in the case of the positive control strain (V329), but a negative response was obtained for all 132 strains of the tested group.

The genotypic diversity of the tested group of strains was revealed by the size polymorphism analysis of a X fragment of the *spa* gene (Frenay *et al.*, 1994). The number of repeats of the 24 nucleotide sequence varied

Table I
Sequences of starters and thermal conditions of PCR analysis

Lp.	Detected gene	Primer sequences	Size of the PCR product (bps)	Temperature of annealing	References
1	*nuc*	NucF: 5'-GCGATAGATGGTGATACGGTT NucR: 5'-AGCCAAGCCTTGACGAACTAAAGC	270	55°C	Brakstad *et al.*, 1992
2	*icaA*	icaAF: 5'-ACACTTGCTGGCGCAGTCAA icaAR: 5'-TCTGGAACCAACATCCAACA	188	56°C	Rohde *et al.*, 2001
3	*icaD*	icaDF: 5'-ATGGTCAAGCCCAGACAGAG icaDR: 5'-AGTATTTTCAATGTTTAAAGCAA	198	56°C	Arciola *et al.*, 2001
4	*bap*	bapF: 5'-CCCTATATCGAAGGTGTAGAATTGCAC bapR: 5'-GCTGTTGAAGTTAATACTGTACCTGC	971	55°C	Cucarella *et al.*, 2001
5	*spa*	SpaF: 5'-CAAGCACCAAAAGAGGAA SpaR: 5'-CACCAGGTTTAACGACAT	100–388	57°C	Frenay *et al.*, 1996

Table II
Diversity of the 24 bp repeats in region X of the *spa* gene in the analyzed group of strains

Number of 24 bp repeats in region X	Size of the PCR product (bp)	Number of strains (percentage share of strains, %)
2	100	10 (7.6)
3	124	3 (2.3)
4	148	4 (3.0)
5	172	2 (1.5)
6	196	4 (3.0)
7	220	8 (6.1)
8	244	6 (4.5)
9	268	2 (1.5)
10	292	26 (19.7)
11	316	27 (20.5)
12	340	18 (13.6)
13	364	12 (9.1)
14	388	7 (5.3)
Lack of the PCR product	–	3 (2.3)

between 2 and 14 in the analyzed group of isolates. The most prevalent strains, constituting 19.7%, 20.5% and 13.6% of all strains tested, had 10, 11 or 12 repeats, respectively (Table II).

The results of presented study are in accordance with those presented previously by Vasudevan and coworkers who detected the presence of both genes in the whole tested population of 35 strains of *S. aureus* isolated from milk of cows suffering from mastitis (Vasudevan *et al.*, 2003). Several authors also showed presence of the *ica* locus genes in all *S. aureus* clinical isolates analyzed in their studies (Fowler *et al.*, 2001, Rohde *et al.*, 2001, Knobloch *et al.*, 2002, Atshan and Shamsudin, 2011). The groups of 15, 80, 128 and 200 isolates were tested by the above mentioned authors, respectively. In contrast, some other authors exploring either strains isolated from bovine mastitis or clinical isolates, found a number of strains lacking some genes of the *ica* locus. It was found in 74 out of 99 strains isolated from mastitis in Netherlands (Melchior *et al.*, 2009). Ciftci and coworkers examined the group of 59 isolates from mastitis and found only 16 *ica*A-positive strains, 38 strains harbouring the *ica*D gene and 15 of them containing both genes (Ciftci *et al.*, 2009). Among the group of 102 *S. aureus* mastitis isolates from India, only 36 revealed the presence of both genes (Dhanawade *et al.*, 2010). In the case of strains isolated from human infections, 36/46 isolates from auricular infections in Tunisie were *icaA* and *icaD* positive (Zmantar *et al.*, 2010), while Grinholc and coworkers were not able to detect the presence of *icaD* gene in the case of 27 strains among the tested group of 302 clinical MRSA isolates, whereas all of them harbored the *icaA* gene (Grinholc *et al.*, 2007). Recently Murugan and coworkers examined the group of 24 *S. aureus* isolates from conjunctivitis patients. The sequencing analysis of the amplified *icaA* gene revealed high sequences similarity but some differences were observed (Murugan *et al.*, 2010). This finding confirms that some mutations of the genes of the *icaADBC* operon are possible and this polymorphism may be the reason for problems with their amplification. In our opinion, more accurate PCR tests for checking polymorphism of the sequence of the *icaADBC* operon, instead of simple detection of individual genes would be very useful. It would be also a good idea to supplement the test for the presence of the genes of the *ica* operon by any genotypic method, for example polymorphism analysis of sequences of genes coding for protein A or coagulase (Jakubczak *et al.*, 2007). Such method would be essential, especially, when a large number of isolates from one geographical region or even single clinic is tested. In this situation it is highly possible that most of analyzed isolates originate from one initial strain. The analysis of only the presence of the genes coding any of virulence factor (*ica* operon in presented investigation) could lead to the false conclusions – for example when most of tested strains possess the gene but all of them belong to the same genotype group, on the other hand the rest of analyzed population representing different genotypes do not exhibit the presence of particular gene. Our research revealed substantial genetic differentiation of the analyzed group of isolates, what seems to exclude their origin from the same initial strain, however all of them were *icaA* and *icaD* positive. Previous investigations of ability of biofilm formation by *S. aureus* isolates from mastitis carried out in Poland were based on the results of phenotypic tests. Among the group of 59 isolates, Krukowski and coworkers found 28 (47 %) slime producers using the Christensen method (Christensen *et al.*, 1985) and 25 (42%) using the CRA method (Krukowski *et al.*, 2008). The other authors tested the group of 45 strains and found 19 (42%) and 29 (64%) biofilm producers using the CRA and spectrophotometric method, respectively (Dziekiewicz-Mrugasiewicz *et al.*, 2008). The results of phenotypic test presented in our research are very similar, as 57% of analyzed isolates were positive. However the results of genetic investigations presented above revealed that all tested strains possess the *ica* operon and should be considered as able to produce biofilm. In our studies, none of the analyzed strains harbored the gene encoding the Bap protein. These results are in agreement with the previous reports on *S. aureus* isolates of different origin, such as human, sheep, goat, bovine, pig, poultry, horse and rabbit (Vancraeynest *et al.*, 2004, Nitzsche *et al.*, 2007, Vautor *et al.*, 2008,

Melchior *et al.*, 2009), where the *bap* gene was absent in the *S. aureus* isolates. Moreover, our results confirm the hypothesis proposed by Vautor and coworkers, that the *bap* gene had not spread yet among the *S. aureus* isolates of animal and human origin and its prevalence is very low (Vautor *et al.*, 2008).

Results of the phenotypic tests obtained in our studies do not correlate with the presence of *icaA* and *icaD* genes. A similar discrepancy was reported in other studies concerning the ability of biofilm production by *S. aureus* isolates of different origin. It has been previously demonstrated that the phenotypic expression of biofilm production ability is influenced by numerous factors, such as: medium composition (Atshan and Shamsudin, 2011), presence and concentration of glucose (Rodrigues *et al.*, 2010), pH and hydrogen peroxide (Zmantar *et al.*, 2010). These factors seem to be the most important reason of observed differences in results of phenotypic and genotypic tests. It is also important to remember that the conditions of all *in vitro* phenotypic methods proposed to date, *i.e.*: the Christensen method, the CRA plate assay and the spectrophotometric biofilm assay are much different from that *in vivo*, in the host tissue. Such difference may result in false negative or positive results. Thus it is also essential to search for new phenotypic tests, less sensitive to the influence of the *in vitro* test conditions. Recently, a promising modification of the CRA plate assay has been proposed (Atshan and Shamsudin, 2011), however it needs an additional verification in other laboratories. Findings of the present studies are in agreement with the generally accepted opinion that biofilm production is an important virulence factor of *Staphylococci* causing bovine mastitis. Its important role in pathogenesis makes enzymes involved in biofilm formation potential new targets for new antistaphylococcal agents for prevention and treatment of bovine mammary glands infections caused by bacteria of this genus.

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