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Original Article

Comparison of biofilm production and virulence genes distribution among human and canine isolates of *Staphylococcus aureus*

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Abstract

Background: *Staphylococcus aureus* is an important human and animal pathogen that can cause a wide range of infections due to numerous virulence factors. **Aims:** The aim of this study was to compare biofilm formation ability with different virulence factors such as bacterial motility, genes encoding biofilm associated proteins, and Panton-Valentine leukocidin (PVL) among human and canine isolates of *S. aureus.* **Methods:** A total of 60 human (30 methicillin sensitive *S. aureus* (MSSA) and 30 methicillin resistant *S. aureus* (MRSA)) and 17 canine (all MSSA) isolates of *S. aureus* were tested for the capability of biofilm production, motility assay, and presence of genes encoding virulence factors: *ica* (encoding intercellular adhesion), *bap* (encoding biofilm-associated protein), *fnbA* (encoding fibronectin-binding protein A), *cna* (encoding collagen-binding protein), and *pvl* (encoding PVL). **Results:** Animal isolates of *S. aureus* performed better biofilm production than the human strains (P=0.042), as well as human MSSA compared to the MRSA isolates (P=0.013). Our results showed that *cna*, *fnbA*, and *ica* genes (67.5%, 66.2%, and 42.9%, respectively) were more prevalent than *bap* and *pvl* genes (0%, and 7.8%, respectively). The *ica* gene was significantly more prevalent in human isolates compared to animal isolates (n=31/60 vs. n=2/17, P=0.008), whereas the *cna* gene was more frequent in animal isolates than in human ones (n=15/17 vs. n=37/60, P=0.0201). Significant correlations were found between the biofilm formation of animal isolates, and the presence of *fnbA* (P=0.029) and *ica* genes (P=0.001). **Conclusion:** This study showed a correlation between biofilm production and the presence of certain biofilm-related genes in animal isolates, as well as stronger biofilm production among MSSA human and animal isolates.

Key words: Biofilm, Biofilm-associated genes, *pvl* gene, *Staphylococcus aureus*

Introduction

Staphylococcus aureus is an important human and animal pathogen. It is a well-known commensal bacterium and the causative agent of different infections, ranging from mild skin and soft tissue infection (SSTI) to life-threatening diseases such as septicemia and meningitis (Peton and Le Loir, 2014). Factors modulating *S. aureus* colonization and infection in humans remained unclear, including the role of a greater microbial community and environmental factors such as contact with companion animals (Ko *et al*., 2021).

Such a wide range of infections in warm-blooded animals and humans can be triggered due to the exceptionally high number of different virulence factors, including exotoxins, adhesins, and biofilm formation ability. A biofilm is a sessile microbial community, consisting of bacteria irreversibly attached to a substratum and to each other by virtue of the extracellular polymeric matrix. Bacteria embedded in the biofilm matrix exhibit an altered phenotype regarding growth rate, antibiotic resistance, and gene transcription

(Donlan and Costerton, 2002). The process of biofilm formation consists of several steps, which require the involvement of different bacterial proteins and enzymes, as well as precise coordination of their expression. Adhesion is the first step in the process of biofilm formation, mediated by different surface proteins. Those facilitating staphylococcal adherence to components of the extracellular matrix are therefore designated as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Collagen-binding adhesin (cna) and fibronectin-binding protein A (fnbA) are two important MSCRAMMs for biofilm initiation and establishment (Ghasemian *et al*., 2015). Besides, staphylococcal biofilm-associated protein (bap) promotes primary attachment of bacteria during biofilm formation, and has a tremendous impact on the biosynthesis of polysaccharide intercellular adhesin (PIA), encoded by the *ica* gene cluster. Notably, PIA contains N-acetylglucosamine which is the main constituent of the biofilm matrix (Namvar *et al*., 2013).

Although *S. aureus* has been considered a non-motile bacterium, two forms of motility were detected; spreading, and gliding (Kaito and Sekimizu, 2007). Spreading is a type of sliding motility and results in broadly circular colonies or colonies with large broad lobes extending radially, while gliding involves the formation of comets (Pollitt *et al*., 2015). Both types of movement are passive motility and not depending on flagella or type IV pili (T4P). Although there is a well-established correlation between the presence of flagella or type IV pili with the bacterial capacity to form a biofilm, there is scarce data about the association of passive motility with biofilm production of *S. aureus* isolates (Conrad, 2012; Otton *et al*., 2017).

Panton-Valentine leukocidin (PVL), is a two-component (LukS-PV and LukF-PV), pore-forming toxin with high affinity for human neutrophils and macrophages, and with weak activity on bovine macrophages (Castellazzi *et al*., 2021). Although PVL is not directly related to biofilm production, the immense importance of this toxin in the pathogenesis of staphylococcal infection has already been confirmed. *S. aureus* isolates carrying PVL-encoding genes are epidemiologically linked to severe human staphylococcal infections, such as necrotizing pneumonia and staphylococcal toxic shock syndrome (Bakthavatchalam *et al*., 2017).

The aims of this study were i) to assess the biofilm production ability and motility, ii) to determine the presence of genes encoding biofilm-related proteins and gene encoding PVL, and iii) to correlate biofilm production with genetic determinants underlying different stages of biofilm formation among human and animal isolates of *S. aureus*.

Materials and Methods

Ethical approval

The written informed consent was obtained from all human patients and verbal informed consent was obtained from the dog owners. This study was approved by Ethical Committee of Faculty of Medicine University of Banjaluka, according to national legislation (18/4.218/22).

Sample collection

In the study, a total of 543 samples were collected during 2019 and 2020 from humans (363) and animals (180). In total, 363 human swabs, *S. aureus* isolates were detected in 90 samples, out of which 60 isolates (30 MSSA and 30 MRSA) were randomly selected. During the same period, among 180 nasopharyngeal swabs obtained from dogs, *S. aureus* isolates were detected in 17 swabs. *S. aureus* recovered from humans was isolated from patients with infections and asymptomatic colonizers. A total of 39 isolates of *S. aureus* were recovered from randomly selected patients admitted to hospitals and outpatient units in Banja Luka city due to the SSTI and acute respiratory tract infections (15 wound swabs and 24 nasopharyngeal swabs). Additionally, 21 isolates of *S. aureus* were recovered from nasal-swab samples obtained from healthy carriers in routine medical examinations of food handlers working in Banja Luka. All human *S. aureus* were isolated in the microbiological laboratory of the Public Health Institute of the Republic of Srpska (ethical approval No.: 18/4.218/22). Canine *S. aureus* were isolated from nasopharyngeal swabs obtained from dogs with clinical signs and symptoms of respiratory tract infections admitted to a PI Veterinary Institute of Republic of Srpska “Dr. Vaso Butozan” in Banja Luka.

Bacterial isolation and identification

All collected samples were processed through standard operating procedures (Isenberg, 2004). Isolation and identification of *S. aureus* were done during the routine work in the regional microbiological laboratories by VITEK® 2 system (bioMérieux, Marcy-l’Étoile, France) and conventional bacteriological techniques, such as colony appearance, gram staining, catalase, coagulase, and DNase tests. Susceptibility of *S. aureus* to methicillin was performed by disk diffusion test using 30-μg cefoxitin disk (BioRad, USA) according to the EUCAST recommendations (2021). All isolated *S. aureus* were sent to the coordinating laboratory at the Faculty of Medicine, the University of Banja Luka for further investigation. All patients and animals were de-identified and recoded in regional laboratories before the bacterial strains were sent to the coordinating laboratory where the confirmation of species identification and methicillin resistance was done. All isolates of *S. aureus* were screened for the presence of *nuc* and *mecA* genes, using PCR, as previously reported (Yousefi *et al*., 2016). The primers used for the amplification are shown in Table 1. As positive control we used clinical strain containing *mecA* gene. All isolated *S. aureus* strains were suspended in skim milk broth and stored at -80°C until further analysis.

Human isolates were divided into two groups according to the susceptibility of *S. aureus* to cefoxitin:

Table 1: Primers, annealing temperature, and references used in this study

Primers	Nucleotide sequence (5´-3´)	Length (bp)	Annealing temperature	Reference
nucF	ATGGCTATCAGTAATGTTTCG	318	55°C for 1 min	Yousefi *et al*. (2016)
nucR	TTTAGGATGCTTTGTTTCAGG			
mecAF	AAAATCGATGGTAAAGGTTGGC	533	55°C for 30 s	Yousefi *et al*. (2016)
mecAR	AGTTCTGCAGTACCGGATTTGC			
bapF	CCCTATATCGAAGGTGTAGAATTGCAC	971	55°C for 30 s	Cucarella *et al*. (2001)
bapR	GCTGTTGAAGTTAATACTGTACCTGC			
icaF	TATACCTTTCTTCGATGTCG	700	42°C for 20 s	Cucarella *et al*. (2004)
icaR	CTTTCGTTATAACAGGCAAG			
cnaF	AAAGCGTTGCCTAGTGGAGA	192	52°C for 30 s	Tangchaisuriya *et al*. (2014)
cnaR	AGTGCCTTCCCAAACCTTTT			
fnbAF	GATACAAACCCAGGTGGTGG	191	52°C for 30 s	Tangchaisuriya *et al*. (2014)
fnbAR	TGTGCTTGACCATGCTCTTC			
pvlF	ATCATTAGGTAAAATGTCTGGACATGATCCA	430	55°C for 30 s	Lina *et al*. (1999)
pvlR	GCATCAASTGTATTGGATAGCAAAAGC			

methicillin-susceptible *S. aureus* (MSSA, n=30), and methicillin-resistant *S. aureus* (MRSA, n=30). All canine isolates were MSSA.

Assessment of biofilm production

Biofilm production assay and classification of isolates based on the strength of biofilm formation (weak, moderate, strong) were performed using the protocol described by Stepanovic *et al*. (2000). Briefly, the aliquots of bacterial suspension were cultured in brain heart infusion (BHI) broth (300 µL) with a final concentration of 10^6 CFU/ml, transferred to each well of the 96-well microtiter plate, and incubated for 24 h. Adherent cells were stained with 300 µL of 2% crystal violet. The optical density (OD) of each well was measured at 570 nm using an automated microtiter plate reader (ICN Flow Titertek Multiscan Plus reader). The negative control wells contained only BHI broth. *Staphylococcus epidermidis* (ATCC 14990) was used as positive control. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. The results were interpreted as follows: OD ≤ ODc = non-biofilm producers; ODc < OD ≤ (2 × ODc) = weak biofilm producers; (2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producers; and OD > (4 × ODc) = strong biofilm producers. All analyses were performed in triplicate and repeated at least two times.

Motility assay

Motility assay was performed on the modified spreading motility media (0.34% agar) according to Pollitt *et al*. (2015). A bacterial culture (2 µL) was spotted in the center of a plate and incubated upright overnight at 37°C. The diameter of motility was then measured (in mm). All isolates with diameter >5 mm (diameter of the spotted area before incubation) were labelled as motile.

DNA extraction

The bacterial DNA was extracted from an overnight culture on Columbia blood agar using a QIAamp DNA Mini kit (Qiagen, Germany), according to the manufacturer's instructions.

Detection of biofilm-associated genes, MSCRAMMs genes, and PVL encoding gene by PCR

All strains were screened for the presence of genes associated with biofilm formation. Simplex PCRs were used to detect the following genes: *ica*, *bap*, *fnbA*, *cna*, and *pvl*. All PCR assays were performed using the Qiagen Taq PCR Master Mix Kit-250U (Qiagen, Germany) according to the manufacturer's instructions. Briefly, each PCR mixture had a final volume of 25 µL containing 12.5 µL of PCR master mix, 1 pmol of each primer, and 5 µL of isolated DNA. The specific primers used for amplification, annealing temperature, and the corresponding size of amplicons are summarized in Table 1. Amplification was carried out in the thermocycler of Master Cycler Gradient (Eppendorf, Germany) under the following conditions: firstly, 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, different annealing time-temperature (Table 1), and 72°C for 30 s, and finally, one cycle of 72°C for 5 min. The visualization of PCR amplicons was performed by electrophoresis in 2% agarose gel stained with Midori Green (NIPPON Genetics Europe). As positive control for *bap* gene, reference strain *S. aureus* V329 was used. *S. aureus* ATCC 49775 was used as positive control for *pvl* gene, and for other genes *S. aureus* ATCC 29213 and 12598 were used. 100 bp ladder (Invitrogen, ThermoFisher Scientific, USA) was used to determine the size of the amplified DNA fragments.

Statistical analysis

Statistical analysis was performed using SPSS ver. 21.0 (SPSS Inc., Chicago, IL, USA). The Chi-squared and Fisher's exact tests were used, as appropriate, to compare data from different groups and to detect a correlation between biofilm production, motility, and gene presence. The differences were considered significant if $P<0.05$, and highly significant in the case of $P<0.01$.

Table 2: Biofilm production of canine (n=17) and human (n=60) isolates of *Staphylococcus aureus*

Bacterial isolates	Capacity of biofilm production N (%)			
	Non-producers	Weak producers	Moderate producers	Strong producers
Human MSSA	1 (3.3)	14 (46.7)	15 (50)	0 (0)
Human MRSA	6 (20)	18 (60)	5 (16.7)	1 (3.3)
Canine MSSA	0 (0)	10 (58.9)	3 (17.6)	4 (23.5)
Total	7 (9.1)	42 (54.5)	23 (29.9)	5 (6.5)

Results

Biofilm production

As quantified by the crystal violet assay, biofilm formation capacity among the 77 isolates was distributed as follows: 6.5% (n=5) were strong biofilm producers, 29.9% (n=23) were moderate biofilm producers, 54.5% (n=42) were weak biofilm producers, and 9.1% (n=7) isolates were classified as non-biofilm producers (Table 2). Thus, comparative analysis of the biofilm assay demonstrated that the majority of strains (n=70, 90.9%) were biofilm producers. Out of 5 strong biofilm producers, 4 isolates (80%) and 1 isolate (20%) were canine MSSA and human MRSA, respectively. Non-producers were noticed only in human isolates, while all canine *S. aureus* formed biofilm at different levels (Table 2). MSSA isolates had a significantly higher capacity to form biofilm than MRSA (P=0.013), as well as a group of canine isolates compared to *S. aureus* isolated from human specimens (P=0.042).

Motility assay

Movement across semisolid agar around the inoculation point was detected in 6/30 (20%) of MSSA, in 3/30 (10%) of MRSA, and in 9/17 (53%) of animal isolates. The diameter of motility ranged from 6 to 25 mm. An average diameter was 6.5 mm in the human MSSA group, 22.6 mm in the human MRSA group, and 10.3 mm in the group of animal MSSA isolates. Indeed, the movement capacity of animal isolates was significantly higher compared to the human MRSA group (P=0.00215). All motile isolates were biofilm producers. However, there was no significant correlation between motility and biofilm production (P>0.05).

Presence of biofilm-associated genes and Panton-Valentine leukocidin encoding gene

Overall, *cna*, *fnbA*, and *ica* genes were found in 67.5%, 66.2%, and 42.9% of tested isolates, respectively. The gel electrophoresis of the *ica* and *fnbA* genes are shown in Figs. 1 and 2. Nevertheless, *pvl* gene was detected in 7.8% of *S. aureus* and only in human isolates, whereas *bap* gene was not detected in any tested isolate.

Furthermore, the *cna* gene was present in 15/17 (88%) of animal and 37/60 (62%) of human isolates (MSSA: n=22; MRSA: n=15), as shown in Fig. 3. The *cna* gene was significantly more frequent in animal isolates (88%) than in human ones (62%) (P=0.02). The *fnbA* gene was also detected in all three groups of isolates, being found in 13/17 (76%) of animal isolates and in 38/60 (63%) of human isolates (MSSA: n=20; MRSA: n=18). Contrary to the *cna* gene distribution, the occurrence of *ica* gene was significantly higher in human isolates (51%) than in animal ones (12%) (P=0.008) (MSSA: n=17; MRSA: n=14). The prevalence of all tested biofilm-associated genes, MSCRAMMs genes, and *pvl* gene in all three groups of isolates are shown in Fig. 3. The difference of the prevalence of the biofilm-related genes in the group of human MSSA and human MRSA isolates was not statistically significant.

The *pvl* gene was detected exclusively in human isolates (6/60; 10%), in both MSSA (n=3) and MRSA (n=3) strains. Four out of the six *pvl* positive human strains were isolated from clinical wound specimens, while the other two strains were isolated from nasal swabs obtained during mandatory staphylococcal carriage screening of healthy food handlers.

Overall, *fnbA* and *cna* genes were more prevalent among the strong biofilm-forming isolates (80% and 100%) than in moderate biofilm producers (65.2% and 65.2%), respectively. Analysis of the correlation between biofilm production and the presence of genes encoding biofilm-associated proteins (*fnbA*, *ica*, and *cna*) indicated that human isolates did not show any association (P≥0.05), whereas statistical correlation was found

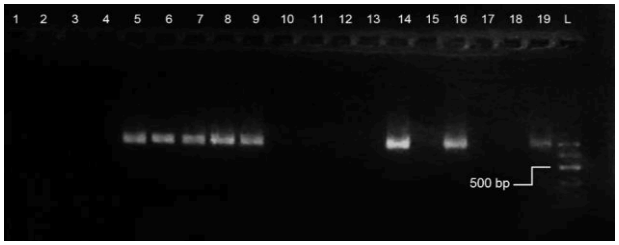

Fig. 1: Agarose gel electrophoresis of the PCR detection of the *ica* gene. Lanes 1-4, 10-13, 15, and 17: Negative samples, Lanes 5-9, 14, and 16: Positive samples, Lanes 18, and 19: Negative and positive controls (700 bp), and L: 100 bp Ladder

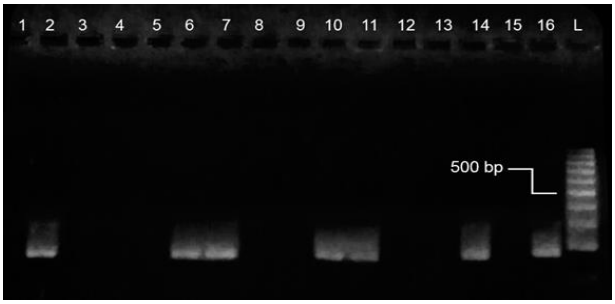

Fig. 2: Agarose gel electrophoresis of the PCR detection of the *fnbA* gene. Lanes 1, 3-4, 8-9, and 12-13: Negative samples, Lanes 2, 5-7, 10-11, and 14: Positive samples, Lane 15: Negative control, Lane 16: Positive control (191 bp), and L: Ladder 100 bp

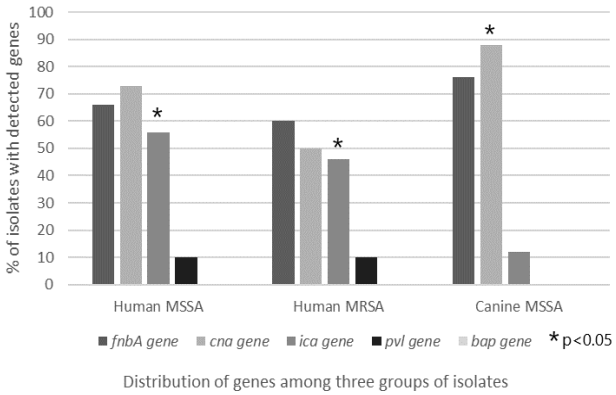

Fig. 3: Bar graph comparing the prevalence of biofilm-associated genes (*ica*, *bap*), MSCRAMMs genes (*cna*, *fnbA*), and *pvl* gene in human MSSA, human MRSA, and canine MSSA

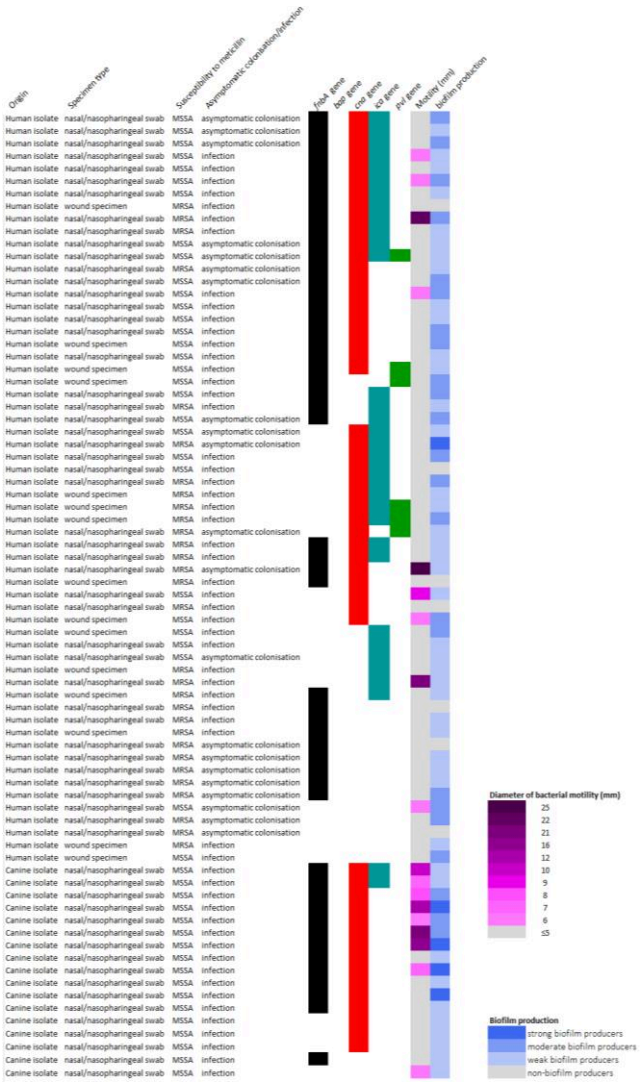

Fig. 4: Distribution of origin, specimen type, susceptibility to methicillin, biofilm-related genes, *pvl* gene, motility in semisolid medium, and biofilm production among isolates. Diameter of motility and biofilm formation are mapped according to the color codes shown in the right corner. All genes are represented here with equal lengths and color codes going from left to right: *fnb*A gene: Black, *cna* gene: Red, *ica* gene: Blue, and *pvl* gene: Green

between animal isolates and two detected genes, *fnbA* (P=0.029) and *ica* (P=0.001).

The genetic patterns of all tested isolates based on the distribution of the virulence genes, motility, and the ability of biofilm formation are summarized in Fig. 4.

Discussion

The ability of *S. aureus* to form biofilm has been identified as the most important means of a major defense mechanism against antimicrobial agents and host immune responses (Edwards *et al*., 2012). To understand the molecular mechanism of biofilm production, the frequency of selected genes encoding surface proteins involved in biofilm formation was evaluated in 77 *S. aureus* isolated from human and animal samples.

Although our research was conducted on a relatively small number of isolates, our results indicate that over 90% of the tested *S. aureus* were biofilm producers with variations in biofilm mass. The biofilm production of canine *S. aureus* was higher than the biofilm formation ability of human-derived isolates. Interestingly, in the present study, both human and animal MSSA isolates showed more efficient biofilm formation than human MRSA isolates. Accordingly, Qi *et al*. (2016) suggested that biofilm producers are less dependent on antibiotic resistance mechanisms than non-producers.

S. aureus is historically defined as a non-motile organism. However, the novel data discovered different types of motilities under certain conditions, such as spreading and gliding or dendrite formation (Pollitt *et al*., 2015). In this study, most human isolates, especially those among MRSA group showed a poor capacity to move across semisolid agar, contrary to the animal isolates which performed improved motility. Although motility is important in several bacterial behaviors, such as colonization and biofilm formation, the correlation between motility and biofilm formation was not detected in this study.

Many studies have shown the role of the intracellular adhesion locus (*ica*) in biofilm production (Qi *et al*., 2016; Bissong and Ateba, 2020). Apart from adherence as a critical step in the biofilm initiation, the production of polysaccharide matrix is essential in the biofilm's survival and growth. The biofilm matrix of staphylococci mostly consists of polysaccharides such as the PIA, which synthesis and modification are coded by *icaADBC* operon (Qi *et al*., 2016). Accordingly, obtained results showed that PIA-dependent biofilm formation was dominant among the tested human isolates. In addition, PIA-independent biofilm formation was present in the majority of animal isolates despite their high ability to form a biofilm, suggesting the presence of additional loci relevant to biofilm formation.

In line with other reports (Khoramian *et al*., 2015; El-Nagdy *et al*., 2020) results from this study showed that the *bap* gene was not detected among the tested isolates. Indeed, the *bap* gene is present in the pathogenicity island SPIbov2, which has been identified in only a small proportion of *S. aureus* isolates (Tang *et al*., 2013;

Kıvanç *et al*., 2018), even though *bap* is the first gene essential for the biofilm formation via *icaADBC* independent mode in *S. aureus* from bovine mastitis isolates (Cucarella *et al*., 2004). However, contrary to our reports, the study by Li *et al*. (2012) reported that the *bap* gene was detected in 43.9% of biofilm-positive *S. aureus* strains, confirming the importance of the *bap* gene in biofilm formation.

In the present study, the PCR amplification of biofilm-associated genes in the *S. aureus* isolates revealed a high prevalence of *fnbA* and *cna* genes in both human and canine isolates, being presented simultaneously in half of the *S. aureus* isolates. In agreement with other studies, the correlation between *fnbA* gene and biofilm production was found in animal isolates (Chen *et al*., 2020; Torres *et al*., 2020). Besides, in this study, the prevalence of the *cna* gene was significantly higher among canine isolates compared to those obtained from humans, as well as in biofilm-producing strains than in non-biofilm-producing *S. aureus*. Such a high proportion of the *cna* gene was already been described among various animal isolates (Monistero *et al*., 2018; Chen *et al*., 2020). Another study reported that *cna*-positive isolates were identified as moderate or strong biofilm producers (Pereyra *et al*., 2016). Furthermore, Lisowska-Łysiak *et al*. (2019) showed that *cna* gene was associated with certain *spa* clonal complexes. Further studies could elucidate the clonal composition of the tested animal isolates with a high frequency of this gene.

In the present study, none of the canine isolates harbored *pvl* gene, whereas the incidence among human isolates was pretty low (10%). Fortunately, only a few isolates simultaneously harbored *pvl* gene and formed biofilm. Such a combination of virulence factors might lead to hypervirulence. As expected, in the present study, the majority of the *pvl*-positive strains originated from wound specimens. Indeed, PVL role in severe forms of staphylococcal infections, as well as in recurrent skin infections has already been proven (Bakthavatchalam *et al*., 2017; Hanitsch *et al*., 2020). Similar findings concerning *pvl* gene prevalence among human and animal MRSA isolates were found in the neighboring countries and all over the world (Asanin *et al*., 2019; Oh *et al*., 2020). Thus, all MRSA isolates evaluated in Croatia were negative for the above-mentioned gene, whereas, in Serbia, its prevalence was only 6.7% among human isolates (Budimir *et al*., 2014; Rebic *et al*., 2019). On the contrary, the frequency of the *pvl* gene in another entity of our country was found to be threefold higher than in this study, although *pvl*-positive strains were isolated only from the wound and nasal swabs, which is in line with our findings (Rebic *et al*., 2019). We speculate that such differences in the frequencies of the *pvl* gene among *S. aureus* originated from two entities of Bosnia and Herzegovina might be due to the differences in the number of tested samples and the number of the tested MRSA strains. As the *pvl* gene could be used as a marker for community-acquired MRSA, our goal for further studies would be to carry out a broader investigation about both genetic and phenotypic correlation among human community acquired *S. aureus* isolates with animal strains isolated from companion animals and livestock.

Our study showed a slight correlation between biofilm production and the presence of certain biofilm-related genes among canine isolates, as well as stronger biofilm production among MSSA human and canine isolates.

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Conflict of interest

Authors have no conflict of interest to declare.

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