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EVALUATION OF THE EXPRESSION OF *LUXS* GENE IN CLINICAL ISOLATES OF *STAPHYLOCOCCUS EPIDERMIDIS* FROM BLOODSTREAM INFECTIONS

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ABSTRACT

Staphylococcus epidermidis is a common, nosocomial bacteria causing bloodstream infections. There is an urgent need to develop techniques which assist for differentiating between invasive and contaminating strains. Phenol-soluble modulin (PSM) peptides important for biofilm formation of *S.epidermidis* are regulated by the *luxS*/Autoinducer-2. To evaluate the expression of *luxS* gene for diagnostic purpose, we analysed changes in gene expression *in vitro* in *S.epidermidis* specimens isolated from bloodstream infections (n = 43) and in those from a control group (n = 35) produced by quantitative PCR. The mean expression of *luxS* gene was not significantly different between the clinical and control groups. We also studied correlation of the expression of *luxS* gene with previously described *icaADBC* operon. The presence of *icaADBC* operon regulating polysaccharide intercellular adhesin (PIA) production was detected by amplification of *icaA* gene fragment in all isolates. *S.epidermidis* isolates from patients with bloodstream infections were not different in presence of *icaA* gene from the nasal flora of healthy volunteers. In the clinical group, the mean expression of *luxS* gene was significantly higher (p = 0,0026) in isolates positive for *icaA* (*icaA*$^+$) then in isolates negative for *icaA* (*icaA*$^-$) gene (21,3% and 8,3%, respectively). The results of our study show that the capacity to form biofilm *in vivo* is strongly influenced by many environmental factors independent of *luxS* expression and the *icaADBC* products.

INTRODUCTION

Coagulase negative *Staphylococci* (CoNS), particularly, S*taphylococcus epidermidis*, have emerged as important cause of nosocomial infections in recent twenty years[1]. *S.epidermidis* is the opportunistic pathogen which colonises and infects both hospitalised patients with decreased host response and healthy, immuno-competent people in the community. Given the epidermal origin of CoNS, it is often difficult to differentiate *S.epidermidis* strain isolated from a single blood culture as a contaminating or an infecting strain.

The pathogenity of *S.epidermidis* is mainly due to the ability to form biofilms on indwelling medical devices[2, 3]. Many candidate genes have been explored as potential virulence markers[4, 5, 6, 7], however, the molecular basis of virulence in *S.epidermidis* is not completely understood.

Biofilm formation in *S.epidermidis* involves a bacterial intercommunication system called quorum sensing. *S.epidermidis* has a well known quorum-sensing (QS) system termed *agr* for accessory gene regulator[8]. A recent novel QS system described *luxS* in staphylococci[9]. Gene *luxS* was shown *in vitro* as playing a major role in biofilm formation. QS system controls the expression of several genes using small signalling molecules called autoinducers (AIs). A gene called *luxS* is required for the synthesis of autoinducers, particularly, for AI-2[10]. It is known that AI-2 has a signalling function in *S.epidermidis*, among other genes (e.g. from *agr* QS system) involving regulation of virulence-associated genes coding for the proinflammatory phenol-soluble modulin (PSM) peptides[11]. It was shown, that the deletion of *luxS* in *S.epidermidis* increased pathogen success during a biofilm–associated infection in a rat model by causing more severe bacteraemia[9]. However, there are no clinical studies indicating the role of *luxS* in biofilm formation during bloodstream infections.

The goal of the present study was to evaluate the expression level *in vitro* of *luxS* gene in clinical isolates. The expression of *luxS* was measured by real-time PCR and compared between *S.epidermidis* isolates recovered from patients in cases of life-threatening infections such as bacteraemia and sepsis, and those from healthy people. This is the first study determining the expression of *luxS* gene in *S.epidermidis* isolates from patients with bloodstream infections. Also, we evaluated the functional correlation of *luxS* and the presence of well-known *icaADBC* operon, which is important for biofilm formation in

catheter-related infections due to production of polysaccharide intercellular adhesin (PIA)[12]. It is hypothetically known, that the autoinducer-2 molecule (AI-2) downregulates biofilm formation through *icaADBC* operon at transcriptional level[9]. The conveyance of *icaADBC* operon was characterised by the detection of *icaA* gene in both groups of *S.epidermidis* as a factor involved in pathogenesis. The correlation of *icaA* with the expression of *luxS* was estimated.

MATERIAL AND METHODS

Strain collection

In total, 78 *S.epidermidis* cultures were isolated from clinical cases and healthy people. 43 clinical specimens of *S.epidermidis,* were isolated from blood (n = 37) or intravenous catheters (n = 6) in cases of clinically and laboratory confirmed bloodstream infections (bacteraemia and sepsis) from the four hospitals of Riga, Latvia. Patients were ranging in age from 35 to 85 with the mean age of 61. The 43 patients were 24 males (55%) and 19 females (45%). They had different approved diagnoses, including coronary heart disease, bacterial endocarditis, sepsis, catheter infection, and systemic inflammatory response syndrome. All patients with at least two positive blood cultures, which were drawn at different times or simultaneously from the central line and the other through a peripheral puncture site, were considered as having true bacteraemia, provided that the isolates had the same antibiogram and phenotypic appearance. In order to minimise the influence of clonal spread, only one culture from each patient or healthy person was included in this study.

A control group of 35 *S.epidermidis* isolates from normal nose epithelium flora was included. It was represented by 20-22 year old healthy volunteers not involved in public health care. The initial samples were cultured on mannitol-salt agar plates. After 24 hours, one colony of each donor was selected.

Identification and susceptibility testing

S.epidermidis cultures were isolated from the clinical specimens and were identified microbiologically up to a species level with the halfautomated BBL Crystal system (Becton, Dickinson). Susceptibility to a panel of antimicrobials was tested according to CLSI standards by the agar disk diffusion test (Becton, Dickinson) and the E-test using Mueller-Hinton agar (MHA, Oxoid, UK) against the following panel of antibacterials: penicillin, gentamicin, cefazolin, erythromycin, clindamycin, vancomycin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Methicillin resistance was tested by cefoxitin disk on an agar screen plate. In parallel, resistance was confirmed by the presence of *mecA* by PCR, as described previously[13]. Plates with *S. epidermidis* cultures were incubated for 20-24 hours. *S.epidermidis* ATCC 12228 was used as a biofilm-nonforming reference strain and *S.epidermidis* RP62A was used as a biofilm-forming strain. Biofilm formation using microtitre plate was carried out as described by Christensen et al[14].

RNA isolation

RNA isolation and subsequent experiments were performed in Laboratory of Bioanalytical Methods, University of Latvia. RNA was isolated from fresh overnight *S.epidermidis* cultures with Nuclisens Lysis buffer and magnetic extraction reagents on the MiniMag system (BioMérieux, France) according to manufacturers' instructions. Measuring of RNA concentration was done using NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA). The RNA quality was controlled by agarose gel electrophoresis. After reverse-transcription reaction with 250ng RNA, random hexamer primers, and reverse-transcription reaction kit (Fermentas, Lithuania), the cDNA was stored at -20°.

Quantitative PCR

Oligonucleotide primers were synthesised by Invitrogen (USA). The sequences for the *luxS* primers were as those primers used in the study of *Xu et al*[9]. The sequences used for the housekeeping gene 16S rRNA were as follows: the forward primer 5'-CCGGATTGGAGTCTGCAACT-3' and the reverse primer 5'-GTGGCATTCTGATCCACGATTAC-3'. The expression of *luxS* was measured on cDNA in quantitative PCR. All PCR experiments were performed in triplication, with 16S RNA used as a reference gene control. The quantitative PCR was performed using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas, Lithuania) in a 96-well plate of ABI Prism 7300 Fast Real-Time PCR system (Applied Biosystems, USA). The amplification product and the negative control samples were afterwards visualised by the 1.5% agarose gel electrophoresis.

Detection of the *icaA* gene

The genomic DNA was isolated using the Genomic DNA Purification Kit, (Fermentas, Lithuania). The 502bp long gene fragment of the *icaA* gene was detected by amplification of the DNA region using the primers described previously[15]. Amplification of DNA was carried out by using the ready PCR Master Mix 2× (Fermentas, Lithuania) in a Veriti 96 Well thermal cycler (Applied Biosystems, USA) with the following cyclic profile: an initial denaturation at 94° for 2 min followed by 35 cycles of amplification (denaturation at 94° for 30 s, annealing at 55° for 30 s and extension at 72° for 30 s) and a final extension of 5 min at 72°.

Statistical analysis

The results of *luxS* expression were analysed using the relative quantification (Livak) method with the 16S RNA gene as the reference gene. Program GraphPad Prism 5.0 version was applied to analyse the results of *luxS* expression profiles and *icaA* gene detection in clinical and control groups. All p-values are two-tailed and calculated with
the t-test.

RESULTS

In this study, 43 clinical isolates from bacteraemia and sepsis and a control group of 35 of *S.epidermidis* isolates were studied for the *luxS* expression. The mean expression level for *luxS* among 43 clinical samples was 13.46%, which was lower in comparison to the mean value of 19.02% in the control group (t = 1.146, the actual p value = 0.2560). The results are graphically shown at Figure 1.

Our data are showing that mean expression of the *luxS* gene in *S.epidermidis* isolates from bacteraemia and sepsis is variable *in vitro* and is slightly decreased. However, the mean expression of the *luxS* gene was not significantly different between the clinical and control groups. Taking this fact into consideration, we cannot recommend the *luxS* gene as a molecular marker for *S.epidermidis* strains in case of bacteraemia.

Analysis of biofilm formation by inoculation on the microtitre plate was available for some cultures. The

microtitre plate analysis was done for 28 cultures isolated from bloodstream infections and for 26 cultures from the control group. In those samples, the percentage of biofilm forming cultures was quite similar in both clinical and control groups, 28.57% and 30.76% ($p > 0.05$), respectively. The mean expression level for *luxS* was not significantly different for the biofilm forming and non-forming culture isolates in both groups (data not shown).

The presence of polysaccharide intercellular adhesin (PIA) production regulating the *icaADBC* operon is important for biofilm production. To determine whether the *icaADBC* operon has an effect on *luxS* gene expression, the presence of the *icaA* gene was detected by amplification of 502bp long gene fragment in all isolates. Among 43 clinical samples, there were 18 (44.2%) positive for the *icaA* gene (*icaA*$^+$) and 25 (55.8%) negative for the *icaA* (*icaA*$^-$) (Table 1). This data show that the detection of the *icaA* gene alone is not useful as a virulence molecular marker in bloodstream infections. However, in the 35 healthy volunteers, there were 10 (25.7%) *icaA*$^+$ and 25 (74.3%) *icaA*$^-$ results, with a significant number of *icaA*$^-$ isolates. In the clinical group, the mean expression of the *luxS* gene was significantly higher (21.3%, $p = 0.0026$) in the *icaA*$^+$ samples then in the *icaA*$^-$ samples (8.3%), showing some correlation between *icaA*$^+$ and the expression level of *luxS* gene (Table 1). The mean expression of the *luxS* gene was similar among the *icaA*$^+$ in the *icaA*$^-$ samples in control group ($p = 0.87$).

DISCUSSION

There is an urgent need for the development of the virulence markers, which could be useful for clinical decision-making in cases of *S.epidermidis* infections. In this study, we aimed to estimate a level of the *luxS* gene expression for distinguishing blood and catheter infecting bacteria from the contaminants. The molecular basis of *S.epidermidis* virulence has been extensively studied for the last decades. The role of *luxS*/AI-2 in virulence of *S.epidermidis* was explored by Min Li and colleagues in mutated *S.epidermidis* strains *in vitro*[11]. It was shown that the expression of the *luxS* gene has an impact *in vitro* for biofilm formation. Therefore, we proposed that strains with low expression of the *luxS* gene should be more effective in biofilm production. Hypothetically, the most virulent are the *S.epidermidis* strains that are more effective in biofilm production due to the expression of the *luxS* gene which make them different from those in healthy people. However, despite of the variation in the mean expression level of *luxS* in the analysed groups there was no statistically significant difference.

Latest data about the role of *luxS* quorum-sensing system confirm our previous knowledge that biofilm formation is a complex process influenced by many regulating factors described earlier[16, 17, 18]. Some findings show, that *luxS* can negatively regulate the *icaADBC* operon at transcriptional level[9]. In contrast, the study done by Li and colleagues did not confirm the impact of the *luxS/AI-2* quorum-sensing on the *icaADBC* operon[11]. In our study, we evaluated the stationary phase of *luxS* expression, which, unfortunately, does not always correlate to the production of the AI-2 signalmolecule. According to Li et al., *luxS/AI-2* can influence the expression of PSMs in an *agr* quorum-sensing system independent way[11].

To provide more data about correlation of *luxS* gene expression with the presence of the *icaADBC operon*, we detected the presence of the *icaA* gene in the clinical and control groups. Our data showed that detection of the *icaA* gene alone in a bloodstream infection is not discriminative, which confirmed some previous findings[15, 20]. The high prevalence of *aap* and *icaA* in skin isolates and their higher prevalence in colonising than in invasive isolates led to a low specificity, therefore, the presence of these genes cannot be used to differentiate between contamination, colonisation, and invasive infection[15]. In addition, Ziebuhr showed that about 30% of staphylococci are missing biofilm formation due to the inactivation of either the *icaA* or the *icaC* gene due to the insertion of insertion sequence element IS256[16]. To note, those *S.epidermidis* isolates from bloodstream infections in our study, which were tested on microtiter plate, were non-biofilm producers in similar proportion with the biofilm producers. This data indicates toward low activity in biofilm accumulation in isolates from bloodstream infections.

Interestingly, we observed a statistically significant difference ($P=0.0026$) in expression of the *luxS* gene between *icaA* positive and *icaA* negative *S.epidermidis* isolates among the clinical group with a higher *luxS* expression in *icaA* positive isolates. Hypothetically, those *icaA* positive isolates with higher *luxS* expression should be in the stage of biofilm dissemination rather than accumulation. The proposed pathway of *luxS*- dependent control of the *icaADBC* operon in late growth stages is still disputable[9, 11]. From the previous findings, when the *S.epidermidis* PSMs causes disaggregation of ready biofilm at later stages, it is triggered by the *agr* quorum sensing system of *S.epidermidis* in yet an undiscovered pathway[21]. Our findings support the new pathway of *luxS*-dependent upregulation of phenol soluble modulins and further biofilm dissemination in bloodstream infections, a hypothesis that remains to be confirmed on a molecular level. This study provides novel and clinically relevant information about the potential virulence factors in *S.epidermidis*, thus pointing towards a better understanding and treatment of bloodstream infections.

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STATEMENT OF CONFLICT OF INTEREST

The sponsors of the study had no role in the study design, data collection, analysis, and interpretation. None of the authors had any conflict of interest.

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Figure 1. The mean expression level of the *luxS* gene in the clinical group of *S.epidermidis* compared to that in the control group.

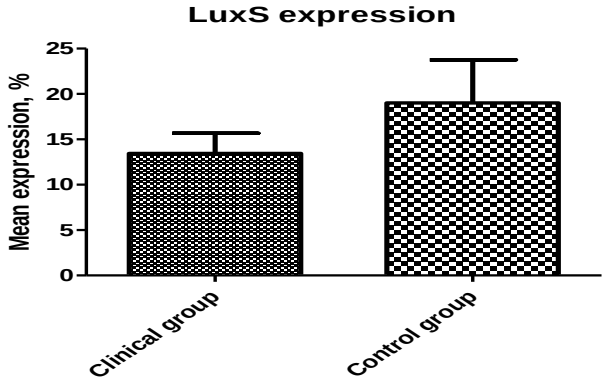

Table 1. Detection of *icaA* gene in clinical isolates and in those from healthy volunteers (control group).

Gene	Clinical group (n = 43)	Control group (n = 35)
icaA$^+$	18 (44.2%)	10 (25.7%)
icaA$^-$	25 (55.8%)	25 (74.3%)
luxS mean expression (%) in *icaA*$^+$	21.3%	14.8%
luxS mean expression (%) in *icaA*$^-$	8.3%, p = 0.0026	15.8%, p = 0.87

Notes: Results for the presence of icaA gene were expressed by both number and proportion (in parenthesis); icaA$^+$ were the isolates positive and icaA$^-$ were the isolates negative for icaA gene.