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PHENOTYPICAL AND MOLECULAR CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* STRAINS

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Staphylococcus aureus can be a normal part of human microflora; however in certain conditions it can cause serious diseases. Main treatment method against S. aureus is antimicrobial therapy. Comprehensive and unsubstantiated use of antibacterial agents causes selection of resistant bacterial strains. Methicillin-resistant S. aureus (MRSA) is one of the most common hospital acquired (HA-) and society acquired (SA-) antibiotic resistant microorganisms.

SA-MRSA strains are characterized by special factor of pathogenicity – PVL toxin. 51 S. aureus isolates obtained from Riga Eastern Clinical University Hospital (RECUH) "Gailezers" were used in this study. After antimicrobial sensitivity test and molecular verification of all isolates, using Multiplex PCR method, 21 MRSA isolates identified. spa typing, SCCmec type identification and MLST performed for selected isolates.

Study results shows, that analysed MRSA strains belong to well characterized ST 368-MRSA-II (t3563 and t4571) – hospital type of methicillin-resistant S. aureus. Also, toxin producing genes PVL, TST, hlg, and hlg-v identified. PVL toxin, which is typical for SA-MRSA strains, was not detected. Genes encoding γ-toxins or haemolysin genes (hlg and hlgv) were identified in all S. aureus isolates, but toxic shock toxin encoding gene (TST) was identified in 2 S. aureus isolates.

Key words: *Staphylococcus aureus*, mecA, MRSA, MSSA.

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INTRODUCTION

Staphylococcus aureus is main human pathogen, causing blood, respiratory tract and skin bacteriological infections. Due to the wide use of antibiotics against *S. aureus*, bacteria develop resistance mechanisms against many types of antimicrobial agents which were effective before some decades (DeLeo & Chambers 2009). One of the most known pathogen, rapidly developing resistance, is methicillin-resistant *S. aureus* (MRSA), which became a persistent problem in many health care institutions and society (Frank et al. 2009). It is important to identify hospital acquired (HA-) and society acquired (SA-) MRSA strains, since their virulence factors and control strategies significantly differs. Antimicrobial sensitivity, chromosomal background, presence of Panton-Valentine leukocidin encoding genes and typical SCC*mec* types helps to differentiate SA-MRSA and HI-MRSA strains (Malachowa & DeLeo 2010).

Timely and correct identification of SA- and HA-MRSA strains is an important factor for termination of disease distribution and prevention. Following hypothesis was built: a part of hospital MRSA strains are SA-MRSA. In order to examine it, *Staphylococcus aureus* isolates obtained from RECUH "Gailezers" patient clinical samples were studied.

MATERIAL AND METHODS

S. aureus strains collected from RECUH "Gailezers" stationed patient clinical samples for the time period of July 2010 till February 2011 were used. In total 51 isolate were tested using phenotypical and molecular methods. Strains were isolated from bronchi, urine, wounds, blood, nasal cavity and ulcers.

Staphylococcus aureus cultures and phenotypical analysis

All isolates were grown on 5% CNA agar (BD™), mannitol salt agar (BD™) and cromID agar (bioMerieux) and incubated 24-48 hours in 36 ^{0}C ± 1 ^{0}C.

Smears for non-typical *S. aureus* colonies were analysed according to Gram method. Belonging to *S. aureus* species is tested using plasma coagulation test (BD™).

In order to identify antimicrobial sensitivity of isolates, from 24 h fresh *S. aureus* colonies grown on 5% CAN agar suspensions in physiological liquid were prepared (bacterial cloudiness corresponding to 0.5 McFarland standard) and plated on Mueller-Hinton agar (BD™). Selected antibiotic discs (Oxacillin (OX), Cefoxitin (FOX), Gentamicin (GN), Ciprofloxacin (CIP), Sulphamethoprim (SXT), Clindamycin (CC), Erythromycin (E), Nitrofurantion (NIT), Vancomycin (VA), FusidinAcid (FUC), Oxoid. Kirby-Bauer disc diffucion method according to CLSI standart was used. Antimicrobial sensitivity by dilution were identified using CLSIM 100-S16 supplement (M100-A17, 2007) and commercial system according to CLSI M7-A7 standard tables. Diameters of poor bacterial growth zone were measured including disc diameters. Results were interpreted according to instructions of manufacturer (Oxoid).

MRSA molecular verification

Bacterial suspensions for all isolates (51) was prepared diluting one pure *S. aureus* colony in 200 µl of physiological liquid. PCR total volume was 25 µl and contained 1x Taq polymerase buffer, 3 mM $MgCl_2$, dNTP mix (0.4 mM each), em 81 and em 82 primers (0.02 µM each), 1 U/µl Taq polymerase (Fermentas) and 1 µl of bacterial suspension. PCR performed using following conditions: initial denaturation 94° C 5 min and following 40 cycles of 94° C 30 s, 55° C 30 s and 72°C for 1 min. Final elongation step – 72° C 10 min. Amplification products were analysed in 1.5% TBE-agarose gel (120 V, 90 mA, 40 min). Primer sequences and result interpretation according to (Doebbeling, 1994).

Identification of toxin genes of S. aureus isolates

All isolates were tested for toxin encoding genes *luk-PV*, *tst*, *hlg* and *hlg-v*, using PCR.

Microorganism suspenion was prepared as described earlier. PCR total volume was 25 µl and contained 1x Taq polymerase buffer, 1.5 mM $MgCl_2$, dNTP mix (0.4 mM each), primers specific for each toxin gene (0.02 µM each), 1 U/µl Taq polymerase (Fermentas) and 1 µl of bacterial suspension. PCR performed using following conditions: initial denaturation 94° C 5 min and following 40 cycles of 94° C 30 s, 50° C 30 s and 72°C for 30 s. Final elongation step – 72° C 10 min. Amplification products were analysed in 1.5% TBE-agarose gel (120 V, 90 mA, 40 min). Primer sequences and result interpretation according to (Dinges et al. 2000)

Identification of SCCmec type

SCC*mec* type was identified by amplification of eight different SCC*mec* locuses and *mecA* gene for all verified MRSA isolates (21). Microorganism suspenion was prepared as described earlier. PCR total volume was 50 µl and contained 1x Taq polymerase buffer, 3 mM $MgCl_2$, dNTP mix (0.2 mM each), em95/em96; em99/em100; em105/em106; em108/em109 praimers (0.04 µM each), em101/em102, em107; em 110/em 111 praimers (0.08 µM each), 1.25 U/µl Taq polymerase (Fermentas) and 1 µl of bacterial suspension. PCR performed using following conditions: initial denaturation 94° C 5 min and following 40 cycles of 94° C 30 s, 50° C 30 s and 72°C for 3 min. Final elongation step – 72° C 10 min. Amplification products were analysed in 1.5% TBE-agarose gel (120 V, 90 mA, 40 min). Primer sequences and result interpretation according to (Doebbeling 1994)

Spa typing

For 5 MRSA isolates PCR amplification of *spa* gene was performed. PCR products obtained were purified and sequenced.

Microorganism suspenion was prepared as described earlier. PCR total volume was 50 µl and contained 1x Taq polymerase buffer containing 25 mM $MgCl_2$, dNTP mix (0.4 mM each), em163/em164 primers (0.02 µM each), 1.25 U/µl Taq polymerase (Fermentas) and 2 µl of bacterial suspension. PCR performed using following conditions: initial denaturation 94° C 5 min and following 40 cycles of 94° C 45 s, 57° C 45 s and 72°C for 90 s. Final elongation step – 72° C 10 min. Amplification products were analysed in 1.5% TBE-agarose gel (120 V, 90 mA, 40 min). Primer sequences and result interpretation according to (Shopsin et al. 1999).

Multilocus sequence typing MLST

MLST was performed for 2 MRSA isolates. Initially seven *S. aureus* housekeeping genes – *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL* were amplified. PCR products were purified and sequenced. Obtained sequences were compared with sequences available in SeqNet database, sequence types (ST) were identified.

Microorganism suspenion was prepared as described earlier. PCR total volume was 50 µl and contained 1x Taq polymerase buffer, 3 mM $MgCl_2$, dNTP mix (0.2 mM each), primers, specific for housekeeping genes (0.02 µM each), 1.25 U/µl Taq polymerase (Fermentas) and 2 µl of bacterial suspension. PCR performed using following conditions: initial denaturation 94° C 5 min and following 40 cycles of 94° C 30 s, 53° C 1 min and 72°C for 1 min. Final elongation step – 72° C 10 min. Amplification products were analysed in 1.5% TBE-agarose gel (120 V, 90 mA, 40 min). Primer sequences according to (Weller 2000).

Obtained housekeeping genes sequences were compared with data available in mlst.net database, MRSA sequences types were identified.

RESULTS AND DISCUSSION

One of the aims of this study was to identify resistance profile for *S. aureus* isolates in order to differentiate methicillin-resistant and methicillin-sensitive (MSSA) strains. According to sensitivity to different anibiotics of particular MRSA or MSSA strain it is possible to choose the most effective antibacterial treatment for the patient.

Table 1. Number of isolates in a range of antimicrobial sensitivity. S-sensitive, I-intermediate, R-resistant

Number of replicates	OX	FOX	GN	CIP	SXT	CC	E	NIT	VAN	FUC
1	R	R	S	S	R	S	S		S	S
1	R	S	R	I	S	S	S		S	S
1	R	S	R	R	R	R	R	S	S	S
1	R	R	R	R	S	R	R	S	S	S
1	R	R	S	R	R	R	R	S	S	S
1	R	R	R	R	R	R	R		S	S
1	R		R	R	R	R			S	S
9	R	R	R	R	R	R	R	S	S	S
2	R	R	R	R	R	R	R	S	S	S
3	R	R	R	R	R	R	R		S	S
24	S	S	S	S	S	S	S		S	S
2	S	S	S	S	S	S	S		S	R
1	S	S	S	S	S	S	S		S	
1	S	S	S	S	S	S	S			S
1	S	S	S	R	S	S	S		S	S
1	S	S	S	S	S	S	S		S	S

High resistance to penicillinum observed in MRSA and MSSA cases (data not shown). It is related to wide use of penicillinum in treatment after its discovery in previous century (DeLeo & Chambers 2009).

Differences in sensitivity level against Ox and Fox, in antibiotic profile observed. Using these two antibiotics it is possible to differentiate MRSA and MSSA strains, since MRSA is often resistant to various antibiotics (Also Gen, Cip, Sct, Cli and Ery).

High sensitivity level in both MRSA and MSSA cases against Nit is observed. Nit is drug of choice for treatment of urinary tract diseases and Van, which is main treatment of choice agaist different types of infectios caused by MRSA.

Also resistance agaist Fuc, which is local action drug, is observed (Gemmel et al. 2006).

To make sure that all analysed *S. aureus* strais are methicillin-resistant, all isolates were tested, implicating molecular twchniques. Using PCR, screening for *mecA* gene was performed. Molecular verification confirmed results obtained by phenotypical methods – 21 isolate were identified as MRSA and contained *mecA* gene sequence. In turn, other 30 isolates were identified as MSSA. Molecular and phenotypic methods fulfil each other. It is possible to achieve more accurate and reliable results implementing bot of them in MRSA screening practice.

S. aureus are characterized by toxins they produce, depending on the source of bacterial strains. Therefore by identifying groups of toxing coding genes it is possible to distinguish HA- and SA-MRSA strains (Dinges et al.2000:21-28). Within the framework of this study all *S. aureus* isolates were tested for presence of Toxic shock toxin (TST) and γ-toxins coding genes. For 21 MRSA isolate PVL encoding gene (*luk*-PV) presence. TST producing gene *tst* was identified in 1 of 30 MSSA isolates. It can be explained by the fact, that TST and other enterotixin genes of *S. aureus* are connected mainly with Staphylococcal strains related to food infections and are not common for HA- Staphylococci. γ-toxins coding genes *hlg* and *hlg-v* were identified in all isolates.

In 8 samples both *hlg* and *hlg-v* genes were detected. In 5 isolates only *hlg* gene was found, but in 36 isolates – only *hlg-v.* Gamma toxins in a combination with PVL enhance virulence of strains, however without the presence of PVL, *S. aureus* are not considered as virulent.

Implicating PCR technique screening for PVL toxin gene *luk*-PV was performed. Presence of the Panton-Valentine leukocidin coding gene is one of the discriminating markers for SA-MRSA strains (Lina et al. 1999). Comparing to other screening methods, PCR is simple, less time consuming and inexpensive. For these reasons it was chosen for the primary testing of MRSA isolates in order to identify their origin. *Luk*-PV gene was not detected in any of MRSA isolates, implying that all isolates tested are HA-MRSA.

For verified MRSA isolates SCC*mec* type was identified. SCC*mec* is mobile genetic element, conatining antibiotic resistance coding genes (including *mecA*) and their regulatory elements. Eight different SCC*mec* types are known (Katamaya et al. 2000). In all MRSA strains, analysed in this study, SCC*mec* type III is identified. SCC*mec* III, comparing to type IV and V, is bigger mobile element, conatining not only methicillin resistance regulating sequence, but also genes, responsible for the resistance to other antibiotics in medical practice (http://saureus.mlst.net/).

According to these data SCC*mec* type III is considered to be a typical HA-MRSA strain.
Typing of pathogens is essential tool in characterization of outbrakes and control of local strains. Historically PFGE is considered as a „golden" typing method for *S. aureus* strains. However typing of X region of protein A (*spa*), a method introduced by Frenay et all. in 1996 (Frenay et al.1996) now is a method of choice, since it allows easier data exchange among laboratories (Aires-de-Sousa et al. 2006) For 5 MRSA isolates *spa* typing was performed. In 4 of them type 3563 was identified. Also type 4571 identified in 1 sample. Both types identified are well characterized in literature and belongs to already discribed HA-MRSA strains in Latvia (Balode 2011).

In order to investigate RECUH „Gailezers" MRSA strains, MLST was used. Method is based on amplification and further sequence analysis of seven *S. aureus* housekeeping genes (Maiden et al. 1998). Each nucleotide polymorphism detected is considered as a new allele. These alleles are highly polymorphyc and are called as sequence types (ST). S. aureus strais showing differences in few nucleotides in their housekeeping gene sequences are consideret to be closely related and belonging to one clonal complex (Chambres & DeLo 2009) For the characterization of RECUH „Gailezers"strains, 2 random MRSA isolates were chosen. In both samples ST368 was identified.

According to SCC*mec* type, sequence and *spa* type all isolates were genetically similar. ST368-MRSA-III isolated from Latvian patients belongs to clonal complex CC8. Genetically related strains belonging to same clonal complex were also isolated in Great Britain, Germany and Poland (Balode 2011).

We can conclude that all MRSA isolates analysed in this study are HA-MRSA, previously isolated in other Latvian hospitals. However it is necessary to investigate larger data amounts in order to get more accurate insight in MRSA strains in Latvian healthcare institutions.

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