Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2009 Sep, 153(3):215–218.

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COMPARISON OF THE PREVALENCE OF GENES CODING FOR ENTEROTOXINS, EXFOLIATINS, PANTON-VALENTINE LEUKOCIDIN AND TSST-1 BETWEEN METHICILLIN-RESISTANT AND METHICILLIN-SUSCEPTIBLE ISOLATES OF *STAPHYLOCOCCUS AUREUS* AT THE UNIVERSITY HOSPITAL IN OLOMOUC

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Received: October 1, 2009; Accepted: July 1, 2009

Key words: Staphylococcus aureus/MRSA/Virulence factors/Real-time PCR

Aims: *Staphylococcus aureus* is an important pathogen characterised by its potential to express many virulence factors. Currently, special attention is being paid to methicillin-resistant strains of *S. aureus* (MRSA). The aim of this study was to compare the prevalence of 13 selected virulence factor genes in methicillin-resistant versus methicillin-susceptible *S. aureus* (MSSA) isolates and to investigate their accumulation in the same isolate.

Methods: Real-time PCR was used to detect the presence of genes in 200 isolates of *S. aureus* (100 MRSA and 100 MSSA) from the University Hospital Olomouc collected in 2005-2006.

Results: Six out of the 13 monitored genes were detected more frequently in MRSA isolates: *sea, seb, seg, sei, sej* and *eta*, coding for the production of the enterotoxins A, B, G, I, J and the exfoliative toxin A. On the other hand, the *pvl* and *tst* genes coding for Panton-Valentine leukocidin and TSST-1 were more frequent in MSSA. Statistical analysis (chi-squared test) of the prevalence of virulence factors in the two groups showed a significant difference ($P<0.05$) in two cases (*seg*, *sei*).

Conclusions: A higher prevalence of selected virulence genes was not confirmed in the methicillin-resistant *S. aureus* group. This indicates no further increase in their threat.

INTRODUCTION

Staphylococcus aureus is a much feared nosocomial pathogen. For infections caused by this species, the drugs of choice are beta-lactam antibiotics (in particular methicillin and oxacillin). However, increasing resistance to these drugs has recently raised the concerns of both microbiologists and clinicians, especially in the case of methicillin-resistant strains (MRSA). Methicillin resistance is characterized by the presence of the *mecA* gene coding for modified transpeptidases (penicillin-binding proteins 2a, PBP2a) with very low affinity to beta-lactam antibiotics[1].

The ability of *S. aureus* to cause various infections and intoxication, results from the production of different extracellular and surface virulence factors with adhesive properties to a range of molecules (MSCRAMMs)[2, 3]. The extracellular products include especially toxins with superantigenic properties, namely enterotoxins A-E, G-K, M-O and Q (*sea-seq* genes), exfoliative toxins A and B (*eta*, *etb*), toxic shock syndrome toxin-1 (TSST-1, *tst*) as well as, for example, Panton-Valentine leukocidin (*pvl*)[4-7].

The most common staphylococcal cell wall proteins have affinity to fibrinogen (clumping factors A and B encoded by the *clf*A, *clf*B genes), fibronectin (*fnb*A), collagen (*cna*), sialoprotein (*bbp*) and elastin (*ebp*S); in the other proteins, the function is as yet unknown (*sdr*C, *sdr*E)[8-14]. Experimental models have shown that expression of receptors for fibrinogen and fibronectin is usually associated with staphylococcal endocarditis whereas the presence of adhesins for sialoprotein, collagen and fibronectin is more common in staphylococcal arthritis and osteomyelitis[12, 15, 16].

The aim of this study was to compare the presence of genes of selected virulence factors in a group of methicillin-resistant and methicillin-susceptible isolates of *S. aureus*.

MATERIALS AND METHODS

Staphylococcus isolation

A total of 200 isolates of *S. aureus* were collected. Of these, 100 cases were MRSA, the other half were methicillin-susceptible (MSSA). All were obtained from clinical samples of patients in the University Hospital in Olomouc, collected in 2005-2006. Clinical samples were mostly skin and wound swabs, samples from respiratory tract, blood cultures, catheters, urine, tissue and others. Species identification of staphylococci was carried out by standard microbiological methods using the commercially available STAPHYtest 16 set (Pliva-Lachema Diagnostika). Susceptibility to methicillin was determined by the microdilution method (CLSI). In resistant isolates,

Table 1. Primers used for detection of virulence factors.

Primer	Sequence	Product size (bp)	Reference
*mec*A-F	5´-TCCAGATTACAACTTCACCAGG-3´	162	18
*mec*A-R	5´-CCACTTCATATCTTGTAACG-3´		
tst-F	5´-GCTTGCGACAACTGCTACAG-3´	559	19
tst-R	5´-TGGATCCGTCATTCATTGTTAT-3´		
pvl-F	5´-ATCATTAGGTAAAATGTCTGGACATGATCC-3´	433	20
pvl-R	5´-GCATCAASTGTATTGGATAGCAAAAGC-3´		
sea-F	5´-GCAGGGAACAGCTTTAGGC-3´	521	19
sea-R	5´-GTTCTGTAGAAGTATGAAACACG-3´		
seb-F	5´-ACATGTAATTTTGATATTCGCACTG-3´	667	21
seb-R	5´-TGCAGGCATCATGTCATACCA-3´		
sec-F	5´-CTT GTA TGT ATG GAG GAA TAA CAA-3´	284	19
sec-R	5´-TGCAGGCATCATATCATACCA-3´		
sed-F	5´-GTGGTGAAATAGATAGGACTGC-3´	385	19
sed-R	5´-ATATGAAGGTGCTCTGTGG-3´		
see-F	5´-TACCAATTAACTTGTGGATAGAC-3´	171	19
see-R	5´-CTCTTTGCACCTTACCGC-3´		
seg-F	5´-CGTCTCCACCTGTTGAAGG-3´	328	19
seg-R	5´-CCAAGTGATTGTCTATTGTCG-3´		
sei-F	5´-CAACTCGAATTTTCAACAGGTACC-3´	466	19
sei-R	5´-CAGGCAGTCCATCTCCTG-3´		
seh-F	5´-CAACTGCTGATTTAGCTCAG-3´	360	19
seh-R	5´-GTCGAATGAGTAATCTCTAGG-3´		
sej-F	5´-CATCAGAACTGTTGTTCCGCTAG-3´	142	19
sej-R	5´-CTGAATTTTACCATCAAAGGTAC-3´		
eta-F	5´-GCAGGTGTTGATTTAGCATT-3´	93	22
eta-R	5´-AGATGTCCCTATTTTTGCTG-3´		
etb-F	5´-ACAAGCAAAAGAATACAGCG-3´	226	23
etb-R	5´-GTTTTTGGCTGCTTCTCTTG-3´		

Table 2. Frequency of selected virulent factors in percent and the P-values (chi-square test or Yates' chi-square test*).

	pvl	*eta*	*etb*	*sea*	*seb*	*sec*	*sed*	*see*	*seg*	*seh*	*sei*	*sej*	*tst*
MRSA	0	10	0	12	3	2	17	0	77	0	77	17	2
MSSA	3	3	0	7	1	9	8	0	49	0	49	8	6
P	0.244*	0.085*	1.000	0.227	0.312*	0.062*	0.054	1.000	0.00007	1.000	0.00007	0.054	0.127*

PBP2a was detected by latex agglutination (Denka Seiken, Japan) and the *mec*A gene by PCR[17, 18].

In PCR detection of genes responsible for the ability to produce selected virulence factors, positivity was verified using reference strains from the Czech Collection of Microorganisms in Brno (*S. aureus* CCM 5756 – for enterotoxin A, CCM 5757 – enterotoxin B, CCM 5971 – enterotoxin C, CCM 5973 – enterotoxin D, CCM 5972 – enterotoxin E, CCM 7058 – exfoliatins A and B) and strains provided by Dr Petráš from the National Institute of Public Health in Prague (strains for enterotoxins E, G, H, I and J, TSST-1 and Panton-Valentine leukocidin).

DNA isolation

The isolated staphylococci were cultured on blood agar (Becton-Dickinson) for 24 hours. Subsequently, one colony was removed and resuspended in 100 µl of sterile deionized water and incubated at 99°C for 15 mins with gentle shaking in the Thermomixer comfort (Eppendorf) device. Centrifugation followed (1006 g, 5 mins) and the supernatant containing extract of staphylococcal DNA was transferred into new test tubes and frozen for later PCR amplification.

PCR amplification

To detect the presence of 13 genes coding for the presence of virulence factors (*tst*, *pvl*, *eta*, *etb*, *sea*, *sec*, *sed*, *seg*, *sei*, *seh*, *sej*, *seb* and *see*), real-time PCR was selected. Sequences of the primers used as shown in Table 1 have been published by other authors[19-23]. The 25-µl reaction mix contained 5 µl of 10x reaction buffer (100 mM of Tris-HCl, pH 8.8, 500 mM of KCl, 1 % Triton X-100, 15 mM of $MgCl_2$) 0.4 µl of dNTPs (10 mM); 50 pmol of primers, 2.5 U of *Taq* polymerase, 2.5 µl of LC Green and 1 µl of

bacterial DNA (dNTPs produced by Promega, USA; LC Green by Idaho Technologies, USA; other components by Top-Bio, Czech Republic). Amplification was carried out in the Rotor-Gene 6000 thermocycler (Corbett Research, Australia). Initial denaturation of DNA at 94°C for 5 mins was followed by another 40 cycles at 94°C for 10 s, annealing (56°C for *tst, pvl, sea*, *seg, sei*, *seh, sej* or 58°C, 20 s) and elongation (72°C, 40 s) and finally 5 mins at 72°C.

In control strains, the size of PCR products of individual primer pairs was verified by agarose gel electrophoresis (2 % w/v), compared with a DNA marker (200-1500 bp, Top-Bio, Czech Republic). Additionally, high-resolution melting analysis (HRMA) of the PCR product was performed in each amplification of a control strain and products of the tested staphylococcal isolates were then compared with the melting curve.

RESULTS AND DISCUSSION

The frequency of the studied genes and the chi-squared (or Yates' chi-square) values for the two groups of isolates are summarised in Table 2. The most frequent genes were *seg* and *sei*, coding for enterotoxins G and I (MRSA 77 %, MSSA 49 %) and the difference in frequency in the two groups was statistically significant (P<0.05). In this study, they were always present together and this accords with their previously detected localisation together with other genes – *sem, sen* and *seo* – in the same *egc* cluster[24, 25]. The frequency agrees with the data of Peacock *et al*.[26] who reported detection of *seg* in 55 % and *sei* in 52 % of invasive strains and Becker *et al*.[27] who similarly found a prevalence in 55 %.

The *sea* gene was present in 12 % of MRSA and 7 % of MSSA, i.e. less frequently than earlier described prevalence of strains isolated from blood and nasal specimens (16 %)[27], and nasal specimens from healthy people (20 %)[19]. However, the findings are comparable to those reported by the University Hospital in Magdeburg, Germany (10.9 % of MSSA)[28].

Other genes for the studied extracellular virulence factors were present significantly less frequently. The *sej* gene for enterotoxin J was always detected together with the gene for enterotoxin D, in 17 % of MRSA and 8 % of MSSA. Becker *et al*.[27] reported the isolation of *S. aureus* with the *sej* gene from blood in 11 %, Layer *et al*.[28] in 15 %.

Enterotoxin B (*seb*) was detected less frequently: in 3 % of MRSA and in 1 % of MSSA. The results are comparable to detection of *seb* in 3 % of strains from blood[27], but lower than those published by Peacock *et al*.[26] (7 % of carrier strains, 9 % of invasive strains) as well as by Monday *et al*[19].

The *sec* gene for enterotoxin C was detected in 2 % of MRSA and 9 % of MSSA. Similar studies reported its detection in 9 % and 14 % of staphylococci isolated from blood and nasal specimens, respectively[27] or in 11 % and 10 % of carrier and invasive strains, respectively[26].

The *seh* gene was not detected in any group of isolates; however, it was reported in 15 % of invasive and in 18 % of carrier strains by Peacock *et al*.[26] and in 5 % of strains by Becker *et al*.[27].

The absence of the *see* gene in the studied groups corresponds with the findings of both Peacock *et al*.[26] and Becker *et al*[27].

The *eta* gene was detected more frequently – in 10 % of MRSA and 3 % of MSSA – than by Becker *et al*.[27] (1 %). Another type of staphylococcal exfoliative toxin, *etb*-encoded, was not detected at all and this agrees with Peacock *et al*.[26] showing that 22 % of invasive strains carried *eta* but no *etb*.

The *tst* gene was present less frequently (2 % of MRSA and 6 % of MSSA) than in the work by Becker *et al*.[27], who reported *tst* in 18 % of strains from blood and 22 % of strains from nasal specimens.

The gene coding for Panton-Valentine leukocidin (*pvl*) was not detected at all in the MRSA group and it was present in 3 % of MSSA. It was reported in 5 % by Holmes *et al*.[29]. Higher detection of *pvl* – 15 % of MRSA collected in 2002 – was described by Wannet *et al*. [30]. No MRSA isolate with *pvl* in studied group is delectable information, because of present spread of these dangerous MRSA clones in the Czech Republic.

CONCLUSION

The prevalence of virulence genes detected by real-time PCR was consistent with that assessed by end-point PCR[31]. Of the 13 studied genes, 7 were detected more frequently in MRSA isolates: *sea, seb, sed, seg, sei, sej* and *eta*, coding for the production of enterotoxins A, B, D, G, I, J and the exfoliative toxin A. On the other hand, the *pvl, tst* and *sec* genes for Panton-Valentine leukocidin, TSST-1 and enterotoxin C were more frequent in MSSA. Statistical analysis of the comparison of the prevalence of virulence factors in the two studied groups using the chi-square test showed a significant difference (P<0.05) in detection of the *seg* and *sei* genes. In the studied group of clinical isolates of *S. aureus*, none exhibited prominent accumulation of virulence factors that would increase its danger (especially in the case of MRSA). The proportion of different genetic elements (that means variaety of strains) detected in various patients was higher – it predicates good epidemiological situation (without massive clonal spread of dangerous strains) in the University Hospital in Olomouc. Finally, the overall prevalence of virulence factors corresponds with that seen in other European countries.

Abbreviations: MRSA, MSSA, TSST-1, MSCRAMMs

ACKNOWLEDGEMENTS

The study was supported by grants IGA NR/9065-3 and MSM6198959223.

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