The Expression of Selected Genes Encoding Enterotoxins in *Staphylococcus aureus* Strains

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Abstract: *Staphylococcus aureus* is an important food-borne pathogen, which produces many toxic substances that cause a variety of illnesses. Some strains of *S. aureus* produce thermostable enterotoxins that can be responsible for alimentary intoxication. The aim of this work was to establish a protocol for the study of 9 enterotoxin genes expression (*sea-sej*). First, a method for the detection of genes encoding enterotoxins was established and then a method for the determination of the expression of these genes was optimised, using a range of the PCR techniques (multiplex, touchdown and real-time). The expression of staphylococcal enterotoxin genes was evaluated both qualitatively and quantitatively. In present study were used *S. aureus* strains from culture collections as well as those newly-isolated from raw milk samples. The obtained results indicate the various expression of the different genes for enterotoxin. However the main benefit of this work is the established protocol for the study of enterotoxin gene expression, which can provide a better understanding of the conditions for the enterotoxins production.

Keywords: staphylococcal enterotoxin; gene expression; multiplex PCR; real-time PCR; mRNA analysis

Staphylococcus aureus is a Gram-positive bacterium producing a wide variety of toxins that can cause various illnesses (Dinges *et al.* 2000). From the perspective of food microbiology, the most important characteristic of *S. aureus* is the production of thermostable enterotoxins (SEs) leading to food-borne intoxication. These alimentary diseases are characterised by such symptoms as nausea, vomiting, abdominal cramps, and diarrhea lasting from 24 to 48 h, where complete recovery usually requires 1–3 days (Lee *et al.* 2007). The ability to produce enterotoxins is generally possessed by approximately half of *S. aureus* strains (Alouf & Müller-Alouf 2003; Letertre *et al.* 2003).

To date, 21 different SE-types have been identified (types A–E and G–V), which share similar structure and characteristics (Becker *et al.* 2007; Ono *et al.* 2008). They are highly stable, being resistant to the majority of proteolytic enzymes and to high temperature. Owing to these properties, they are able to retain their activity during food processing and cooking, and then in the gastro-intestinal tract after ingestion (Loir *et al.* 2003).

From this standpoint, it is very important to be able to detect contaminating *S. aureus* in food and especially their enterotoxins, which are the real reason for the food poisoning caused by *S. aureus*. However, due to the relatively large number of SE-types, it is difficult to establish a method for the routine detection of all SE-types. At the present time there are available several kits, which are able to determine so-called classical enterotoxins (types A–E), but not the other so-called new enterotoxins (types G–V; Becker *et al.* 2007).

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Another possible approach, which has also been used in this study, is the detection of genes for enterotoxins. For this purpose the methods based on the PCR principle are almost entirely used. Only a few studies focused on the detection of enterotoxin genes have been published yet (e.g. Monday & Bohach 1999; Rosec & Gigaud 2002; Løvseth *et al.* 2004; Omoe *et al.* 2005; Hwang *et al.* 2007). In this context, it should be pointed out that the detection of SE-genes using the PCR does not provide information on the gene expression and thus on the presence of toxins in the food (Becker *et al.* 2007). For the study of the gene expression there are presently available several methods based on mRNA analyses (Northern blotting, real-time PCR, microarrays, Serial Analysis of Gene Expression etc.). These methods have their individual benefits and disadvantages. In the case this study was used both real-time PCR (RTi-PCR) and a conventional variant of the PCR.

In the past few years there has not been published too many studies examining the expression of genes encoding enterotoxins (Omoe *et al*. 2002; Lee *et al*. 2007; Akineden *et al*. 2008; Chiang *et al*. 2008). Available data can provide much new information about the regulation of these genes expression and about the influence of external factors on the expression. Here are presented the results of the study that has lead to the protocol developed for the assignment of the expression of selected genes encoding enterotoxins.

MATERIALS AND METHODS

Bacterial strains. Strains of *S. aureus* with different enterotoxin genes were obtained from National Institute of Public Health (Brno, Czech Republic) and from milk samples provided by dairy plants in the Czech Republic.

Isolation and identification of** S. aureus **from milk samples. Isolation and identification of *S. aureus* from milk samples was performed according to the standard EN ISO 6888-1 (1999). In general, tested samples were diluted with sterile peptone water, and spread onto the Baird-Parker agar (BPA) supplemented with egg-yolk tellurite emulsion. Thereafter, the BPA was incubated at 37°C for 48 ± 2 h under the aerobic conditions, presumptive colonies were picked, placed on new BPA and after the next incubation period identified by the Gram staining and the coagulase test.

The coagulase-positive staphylococci were further tested with help of the species-specific duplex PCR. For the confirmation of *S. aureus* species and for the control of right progress of the PCR reaction were used specific primers according to literature (Table 1; SA 442 and 16S rRNA). PCR reactions were performed in a total volume of 25 µl, containing 15.06 µl of nuclease-free water, 0.625 µl of species-specific primers solution (20 µmol/l), 0.156 µl of primers for 16S rRNA gene solution (20 µmol/l), 2.5 µl of reaction buffer (supplied with used polymerase), 2.5 µl of $MgCl_2$ solution (50 mmol/l), 1.25 µl of solution of dNTP (4mM), 0.125 µl of a *Taq* DNA polymerase (Promega Corporation, USA) and finally 2.0 µl of the template (method of the DNA isolation is described later). The following thermal cycling program was used: one denaturation cycle at 95°C for 5 min and 35 amplification cycles for at 95°C 1 min, annealing at 55°C for 30 s, extension for at 72°C 1 min, and last termination at 72°C for 5 minutes. The PCR products were determined by gel electrophoresis in 2% standard agarose (Bio-Rad Laboratories, Inc., USA) in tris-borate-EDTA (TBE) buffer (45 mmol/l Tris-Borate, 1 mmol/l EDTA, pH 8.3) with a 100 bp DNA ladder (New England BioLabs Inc., USA) as molecular marker.

DNA isolation. One milliliter of an overnight culture in tryptic soy broth (TSB) or one loopful of S. aureus strain grown on tryptic soy agar (37°C, stationary cultivation) and washed with 1 ml of sterile physiological solution was centrifuged at 14 000 × g at 4°C for 10 minutes. The supernatant was removed and the pellet was resuspended in 0.2 ml of sterile distilled water. The sample was incubated at 100°C for 20 min and then centrifuged at 17 000 × g at 4°C for 6 min to give a supernatant that was used either immediately in a PCR reaction or stored frozen at –20°C for future use.

PCR detection of genes encoding enterotoxins. The detection of the genes encoding enterotoxins SEA to SEJ was performed using the method already described by Løvseth *et al.* (2004). There were two alterations compared to the original protocol: first the use of different specific primers that served as internal controls of the correct progression of the PCR reaction and second the absence of the detection of the gene for toxic shock syndrome toxin (*tsst*), which was detected in the original published method. The primers used in this study to detect the *sea, seb, sec, sed, see, seg, seh, sei, sej*

genes and the gene for 16S rRNA were based on published sequences (Table 1). The PCR products were determined by gel electrophoresis in 2% standard agarose (Bio-Rad Laboratories, Inc., USA) in TBE buffer (45 mmol/l Tris-Borate, 1 mmol/l EDTA, pH 8,3) with a 100 bp DNA ladder (New England BioLabs Inc., USA) as molecular marker.

RNA isolation and cDNA synthesis. The RNA was extracted from 1 ml of a stationary-phase culture grown in TSB at 37°C for 4 h from a starting optical density $OD_{(590\ nm)}$ of 0.1 (culture in the transition from exponential to stationary growth phase). Total RNA was isolated using a RiboPure^{TM}-Bacteria Kit (Ambion Inc., USA) according to the manufacturer's recommendations. The RiboPure^{TM}-Bacteria Kit was provided with DNase I to remove contaminating DNA. Concentration and purity of the isolated total RNA was determined by the spectrophotometric method, according to the recommendations of the RNA isolation kit manufacturer. The isolated RNA was then diluted to the required concentration (usually 50–200 ng/µl) with DEPC treated water. This extract was used immediately for the reverse transcription PCR. cDNA was synthesised using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's recommendations. Synthesised cDNA was used in the next reaction or stored frozen at –20°C for future use.

Expression of** S. aureus **enterotoxin genes. The expression of selected genes for enterotoxins was

Table 1. Primers used in this study

Gene	Primer sequence (5'→3')[a]	PCR product size (bp)	References
sea	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	520	Monday and Bohach (1999)
seb-sec	ACA TGT AAT TTT GAT ATT CGC ACT G TGC AGG CAT CAT GTC ATA CCA	667	Løvseth *et al.* (2004)
sec	CTT GTA TGT ATG GAG GAA TAA CAA TGC AGG CAT CAT ATC ATA CCA	283	Monday and Bohach (1999)
sed	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	384	Monday and Bohach (1999)
see	TAC CAA TTA ACT TGT GGA TAG AC CTC TTT GCA CCT TAC CGC	170	Monday and Bohach (1999)
seg	CGT CTC CAC CTG TTG AAG G CCA AGT GAT TGT CTA TTG TCG	327	Monday and Bohach (1999)
seh	CAA CTG CTG ATT TAG CTC AG GTC GAA TGA GTA ATC TCT AGG	360	Monday and Bohach (1999)
sei	CAA CTC GAA TTT TCA ACA GGT AC CAG GCA GTC CAT CTC CTG	465	Monday and Bohach (1999)
sej	CAT CAG AAC TGT TGT TCC GCT AG CTG AAT TTT ACC ATC AAA GGT AC	142	Monday and Bohach (1999)
16S rRNA	GGA GGA AGG TGG GGA TGA CG ATG GTG TGA CGG GCG GTG TG	241	Martineau *et al.* (1996)
SA 442	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	108	Martineau *et al.* (1998)

[a]The 5' primer is provided first (above), followed by that of the 3' primer (below)

observed using both conventional and real-time PCR (RTi-PCR). For the conventional PCR the protocol for the detection of SE-encoding genes (see above) was modified by using a cDNA-like template in the PCR reaction. For the RTi-PCR a protocol was designed and optimised and, in comparison with the conventional PCR, only the expression of 4 selected enterotoxin genes (*sed, see, seg* and *sei* gene) were detected. Successful optimisation of the RTi-PCR protocol was verified by determination of the reaction specificity (established by melting curve analysis) and the reaction efficiency, which was performed using the method described by Wong and Medrano (2005). Optimised results were transferred to the following RTi-PCR protocol.

The RTi-PCR analysis was performed using an ABI Prism 7000 SDS system (Applied Biosystems, USA). The primers (shown in Table 1) targeted the enterotoxin genes *sed, see, seg*, *sei* and the gene for the 16s rRNA (used the same primers as in conventional PCR). PCR reactions were performed in a total volume of 25 µl, containing 12.5 µl of SYBR® Green Master MIX (Applied Biosystems, USA), 1.5 µl of each primer solution (10 µmol/l of primers for enterotoxins; 5 µmol/l primers for 16S rRNA gene), 4.5 µl of nuclease-free water and finally 5 µl of the relevant cDNA (10–0.1 ng/µl). The following thermal cycling program was used: one denaturation cycle at 95°C for 10 min and 40 amplification cycles at 95°C for 1 min, annealing at 64°C for 45 s, extension at 72°C for 1 min, and last termination at 72°C for 5 min. Fluorescence readings were taken after each extension step. At the end of the real-time PCR analysis, a melting curve analysis was performed at 60–95°C using continuous fluorescence readings.

The evaluation of the gene expression was performed by the method described by Pfaffl (2001). This technique gives a relative quantification of the target gene expression compared with the other samples carrying the same gene. The obtained value represents the extent of the target gene expression in the sample compared with the calibrator (a sample of the highest level of the expression – a relative expression level is 100%). The mathematical model for the calculation of the expression is given by:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}\,(\text{calibrator}-\text{sample})}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}}\,(\text{calibrator}-\text{sample})}} \quad (1)$$

where:

E_{target} – target gene PCR efficiency

E_{ref} – reference gene PCR efficiency (16S rRNA gene was used as a reference gene due to the assumption of its stable expression level; Scheu *et al.* 1998)

CT – number of cycles required for the fluorescent signal to cross the threshold, i.e. exceeds background level

ΔCT_{target} – difference between measured CT of the calibrator and the sample in the reaction detecting target gene

ΔCT_{ref} – difference between CT measured for the calibrator and the sample in the reaction detecting the reference gene

CT values were measured for the identical amount of the cDNA in reactions and by the identical values of the fluorescence (ΔRn).

RESULTS AND DISCUSSION

Isolation of *S. aureus* from milk samples

The milk samples were taken to isolate *S. aureus* in different dairy plants in the Czech Republic from different stages of milk processing (bulk-storage milk tanks, milk before and after pasteurisation). Positive results of isolation (in total 7 *S. aureus* strains) were obtained only from milk samples from bulk-storage milk tanks and milk samples before pasteurisation with counts of *S. aureus* colonies ranging from 5×10^2 to 2×10^3 CFU/ml. The confirmation of *S. aureus* species was done with help of species-specific PCR.

Detection of genes encoding staphylococcal enterotoxins

To confirm the presence of nine enterotoxin genes (*sea-sej*) two multiplex PCR reactions (Løvseth *et al.* 2004) were performed. The method was applied both to the strains known as the carriers of enterotoxin genes (*S. aureus* strains provided by National Institute of Public Health) and on the strains with unknown presence of enterotoxin genes (isolates from milk samples). The SE-genes were found in 2 isolates from the 7 tested *S. aureus* strains isolated from milk samples and in both cases there was determined a presence of genes

for enterotoxins G and I. This assumption is in accordance with the most frequent incidence of the gene combination *seg-sei* previously reported by Zouharova and Rysanek (2008).

Determination of the expression of SE-encoding genes by conventional PCR

For the determination of the expression of SE-encoding genes, conventional PCR was performed using the protocol of Løvseth *et al.* (2004). Overall 13 *S. aureus* strains from various sources were tested (Figures 1A and 1B). These results are compared with the presence of genes for enterotoxin in Table 2.

The data indicate that the expression levels of the different enterotoxin genes vary from strain to strain. The genes *sea, seb* and *see* are expressed in all strains, which carry these genes. On the other hand the gene *sei* is expressed only rarely (1 of 7 strains). It can be concluded that the expression level of the *seg* and *sei* genes measured here (during the transition from exponential to stationary phase of growth) is lower in comparison with the results of previously published studies (Omoe *et al.* 2002; Akineden *et al.* 2008), where the expression of these genes was measured in cultures at the late stationary phase of growth. The expression of the genes *seg* and *sei* in the studies of both Akineden *et al.* (2008) and Omoe *et al.* (2002) was almost 100% and the results of the present study show that the *seg* gene was expressed in 4 of 7 strains and the *sei* gene in 1 of 7 strains. However, the difference could be affected by the use of the different cultivation media or by the relatively small number of tested samples. Further studies will be necessary for the confirmation of the conclusion presented here.

Determination of the expression of SE-encoding genes by the RTi-PCR

On the basis of the results obtained by the conventional PCR, 8 *S. aureus* strains with the confirmed expression of the genes for enterotoxins D, E, G, I (Table 2) were chosen for the determination of the gene expression by RTi-PCR based on SYBR® Green I fluorescence.

Optimised RTi-PCR protocol was verified by the determination of the reaction specificity (Figure 2)

Table 2. Determination of the expression of SE-encoding genes using the conventional PCR

S. aureus strain[a]	Origin	Present genes for SEs	Determined expression of SE-genes
SA 673	confectionery	D, I, G	–
SA 719	chicken	E	E
SA 720	faeces	A, C, E	A, C, E
SA 921	raw milk	D, I, G, J	D, J
SA 1003	salami	B, D, J	B
SA 1041	raw milk	D, J	–
SA 1117	confectionery	C, I, G	C, G
SA 1173	camembert	A, B, D, J	A, B, D, J
SA 1176	minced meat	D, J	–
SA 1247	salami	A, C, I, G	A
SA 1249	raw milk	B, I, G	B, G
54	raw milk	I, G	I, G
16 A	raw milk	I, G	G

[a]In bold type are the strains selected for the determination of gene expression by the real-time PCR
Strains 54 and 16 A were obtained from milk samples provided by dairy plants in the Czech Republic, rest of tested strains were obtained from National Institute of Public Health (Brno, Czech Republic)

Table 3. The source data and the calculated expression rate of gene expression

Sample (target gene)[a]	Mean CT (n = 3)[b]	*CV* of CT (%)	ΔCT	Efficiency (E)	*CV* of E (%)	Relative expression ratio (R)	Expression rate (%)
1173 (*sed*)	22.271	0.92	–1.735	0.62	1.66	1.843	**54.3**
921 (*sed*)	20.536	2.07					**100**
1173 (16S rRNA)	15.911	1.03	–1.300	0.86	0.98		
921 (16S rRNA)	14.611	0.78					
720 (*see*)	30.486	1.01	–3.291	0.38	1.51	22.049	**4.5**
719 (*see*)	27.195	0.92					**100**
720 (16S rRNA)	17.751	0.59	–2.082	0.97	2.97		
719 (16S rRNA)	15.668	0.80					
1249 (*seg*)	29.432	0.37	–2.654	0.99	1.70	1.039; 1.013; 1.082	**96.3**
54 (*seg*)	26.778	0.23					**100**
16 A (*seg*)	28.159	0.45	–1.381				**98.7**
1117 (*seg*)	27.198	0.90	–0.420				**92.4**
1249 (16S rRNA)	14.084	0.47	–0.677	1.04	2.62		
54 (16S rRNA)	13.406	0.38					
16 A (16S rRNA)	13.574	1.41	–0.167				
1117 (16S rRNA)	15.526	0.22	–2.119				

[a]In bold type are the strains which served as the calibrator (sample of the highest level of the expression)

[b]CT values were measured for the identical amount of cDNA in reactions, cDNA(*se-*) = 2 ng/reaction and cDNA(16S rRNA) = 0.4 ng/reaction and by the identical values of the fluorescence (ΔRn = 0.15)

CV – coefficient of variation

Relative expression ratio (R) was calculated according to Eqution 1

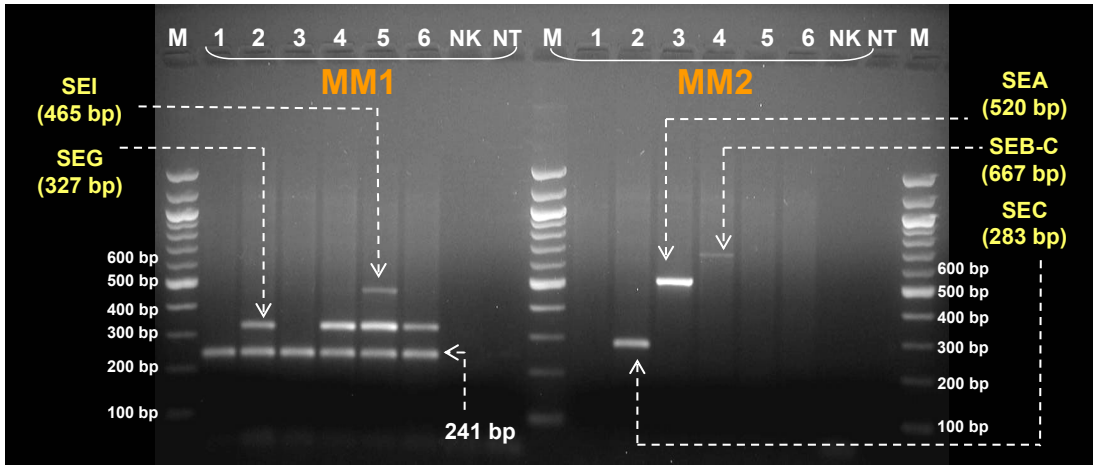
and efficiency (Table 3). The melting curve analysis for the reaction specificity checking resulted in single product specific melting points as follows: *seg* 73.0°C; *sed* 75.0°C; *see* 75.1°C; *sei* 73.8°C and product of 16S rRNA gene 80.9°C. PCR efficiency was calculated according to the equation (Wong & Medrano 2005):

$$E = 10^{[-1/slope]} - 1 \quad (2)$$

High PCR efficiency was achieved only in the case of the reaction used for the detection of the *seg* and 16S rRNA genes (almost 100%), whereas in the other cases the values were 38–71% (Table 3). These values may seem unsatisfactory, but for the purposes of this work and in view of the method of the evaluation of the gene expression they are fully sufficient.

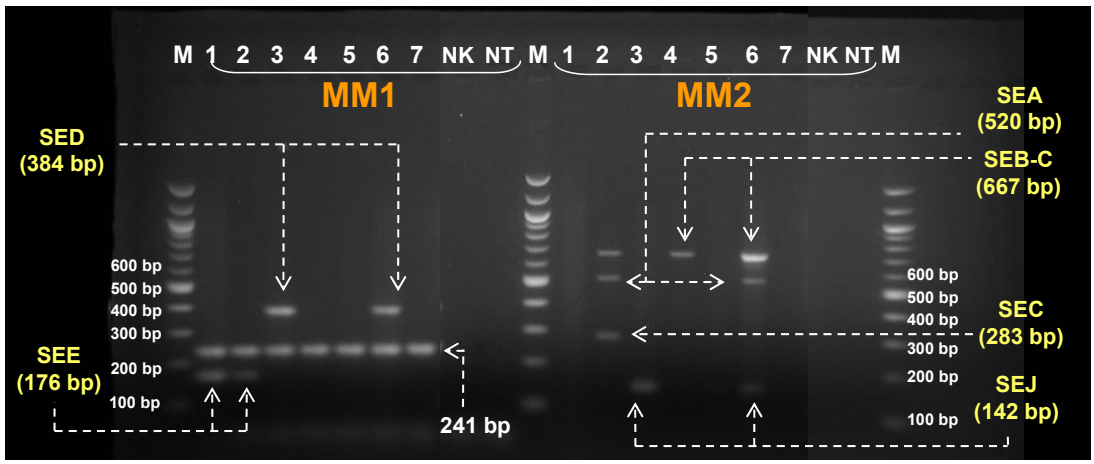
The relative expression of enterotoxin genes was calculated according to Equation 1. The source data and the calculated expression rate of the expression are shown in Table 3. Results are presented in a form used by Pfaffl (2001).

These results show that there is a relatively constant expression rate of the gene for enterotoxin G (100%, 96.3%, 98.7%, and 92.4%) in the 4 bacterial strains. In contrast, in those strains expressing the gene for enterotoxin D there was a difference in the level of the expression that was significant (100% and 54.3%), whilst the largest difference in the level of the expression was detected at those strains with the gene for enterotoxin E (100% and 4.5%). The relative expression of the gene for enterotoxin I was not established, because its expression was detected only in one strain (it could not be compared with another strain).



Lines 1–6 *S. aureus* strains in following order (in parenthesis are the present genes for enterotoxins/the detected gene expression): 673 (D, I, G/–), 1117 (C, I, G/G, C), 1247 (A, C, I, G/A), 1249 (B, I, G/G, B), 54 (I, G/I, G), 16A (I, G/G); SE-genes detected in two groups: master mix 1 (MM1) – *sed, see, seg, sei* and master mix 2 (MM2): *sea, seb-c, sec, seh, sej*; 241 bp product of 16S rRNA gene serves as an inner control; NK – negative control of the DNA isolation; NT – non-template, M – 100 bp marker

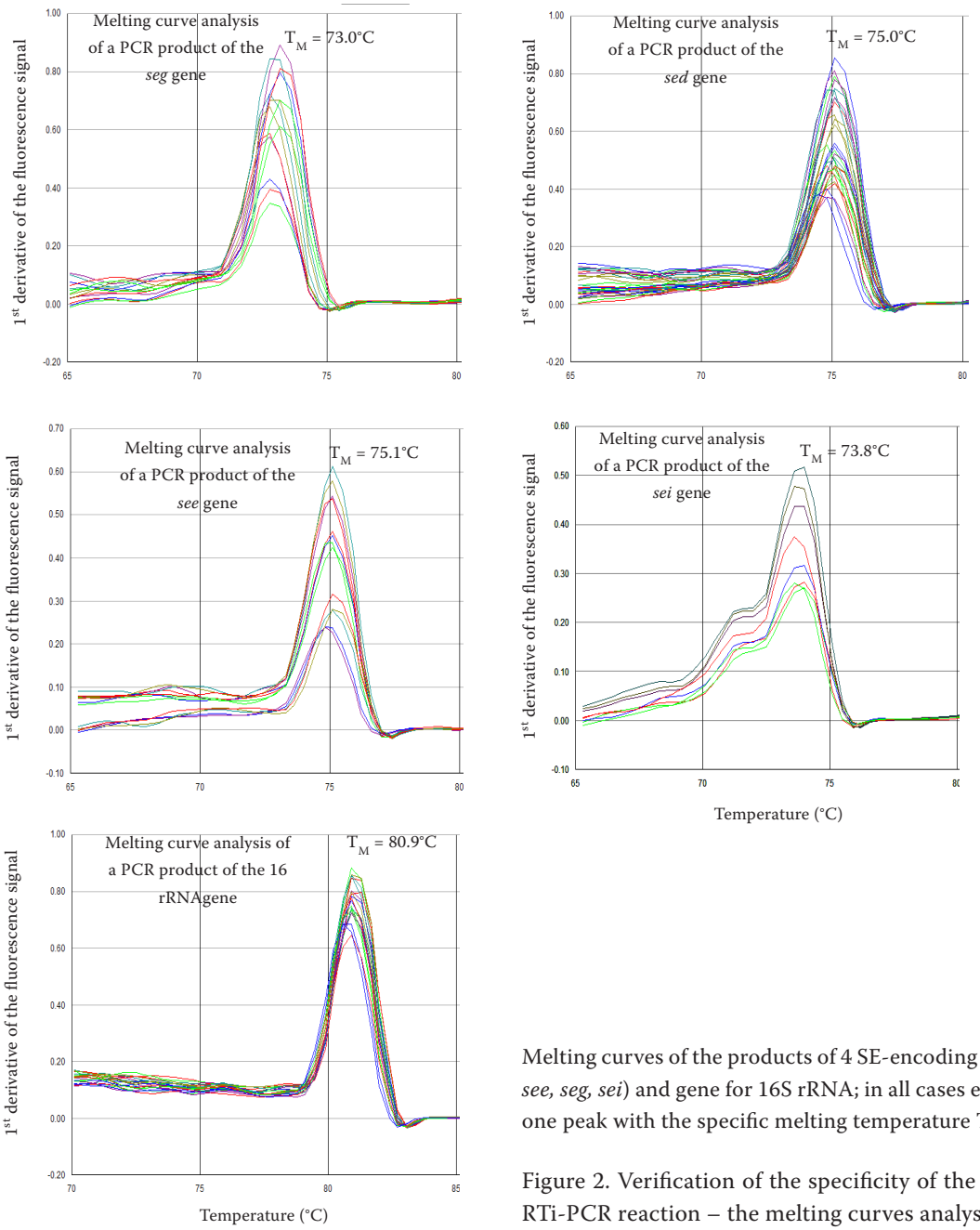
Figure 1A. Determination of the expression by conventional PCR – 6 tested strains



Lines 1–7 SA strains in following order (in parenthesis are the present genes for enterotoxins/the detected gene expression): 719 (E/E), 720 (A, C, E/A, C, E), 921 (D, I, G, J/D, J), 1003 (B, D, J/B), 1041 (D, J/–), 1173 (A, B, D, J/A, B, D, J), 1176 (D, J/–); SE-genes detected in two groups: master mix 1 (MM1) – *sed, see, seg, sei* and master mix 2 (MM2): *sea, seb-c, sec, seh, sej*; 241 bp product of 16S rRNA gene serves as an inner control; NK – negative control of the DNA isolation; NT – non-template, M – 100 bp marker. Note: In the event that in the master 2 are detected the products of genes *seb* and *sec* together (lines 2, MM2), this result is interpreted as positive only for the presence of the gene *sec* (Løvseth *et al.* 2004)

Figure 1B. Determination of the expression by conventional PCR – 7 tested strains

A general comparison of the present results with the other studies is relatively difficult. There are several reasons: (*i*) the small number of other published papers on the same topic (to the present was published only one paper with the similar object of the study; Lee *et al.* 2007), (*ii*) the total number of tested strains is relatively small, and (*iii*) the essential problem is the method of the evaluation, also the relative quantification, which enables to compare only the results obtained within the framework of one study.



Melting curves of the products of 4 SE-encoding genes (*sed, see, seg, sei*) and gene for 16S rRNA; in all cases ensued only one peak with the specific melting temperature T_M

Figure 2. Verification of the specificity of the individual RTi-PCR reaction – the melting curves analysis

CONCLUSION

The aim of this study was to establish effective tools for the investigation of the expression of staphylococcal enterotoxin genes.

In the first step there was detected the presence of nine enterotoxin genes (*sea-sej*) in tested *S. aureus* strains. The detection what has been performed by two multiplex PCR. The chosen PCR protocol was successfully used.

The protocol for the determination of the expression of SE-genes was projected involving the steps of RNA isolation, reverse transcription and transcripts detection. A qualitative evaluation of the expression has been performed by the PCR with the identical primers as in the case of the multiplex PCR mentioned above. Relative quantification of the expression of selected genes has been determined by RTi-PCR with SYBR® Green.

The expression of the 9 SE-encoding genes using conventional PCR was determined at 13 *S. aureus* strains from cultures at the transition from exponential to stationary phase of growth. Further 8 strains have been chosen for the determination

of a quantitative relative expression of 4 SE-genes by RTi-PCR.

The results of the study indicate both the various expression of the different genes for enterotoxin (Table 2) and the various relative quantitation of the expression of a concrete gene among sample carrying the same gene for an enterotoxin (Table 3). However, the present results are only the beginning of the following work. The main benefit of this work is the establishing of the methods for the study of the SE-gene expression, which would be helpful for better understanding of the conditions of the enterotoxins creation. A higher number of *S. aureus* strains should be analysed, and gene expression in various phases of growth or in different media should be investigated. It will be useful for further improvement of current methods, detection of more types of enterotoxins etc.

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