Characterization of *Staphylococcus aureus* strains isolated from food produced in Slovakia and Slovenia with regard to the presence of genes encoding for enterotoxins

TEREZA TRNČÍKOVÁ – SAŠA PISKERNIK – EVA KACLÍKOVÁ – SONJA SMOLE MOŽINA – TOMÁŠ KUCHTA – BARBARA JERŠEK

Summary

Staphylococcus aureus strains containing genes encoding for enterotoxins are a problem from a food safety point of view as it may lead to the staphylococcal food poisoning in humans. In this study, 300 food samples were analysed for the presence of *Staph. aureus*, 38 *Staph. aureus* strains were isolated and analysed for the presence of genes encoding for SEA, SEB, SEC and SED using real-time polymerase chain reaction (PCR). Fourteen strains (37%) contained at least one gene encoding for SEA–SED, when 1 isolate (3%) was found positive for SEA, none was positive for SEB, 10 isolates (26%) were positive for SEC and 4 isolates (11%) were positive for SED. These rates are not very high, but the strains containing the respective genes represent a threat for food safety. The real-time PCR detection systems employed in our study proved to be a proper tool to fulfill the requirement for rapid and reliable screening of *Staph. aureus* strains isolated from food for genes encoding for the most frequent enterotoxins.

Keywords

Staphylococcus aureus; enterotoxin; food poisoning; real-time PCR

Food-borne diseases are of great public concern, bacteria being the causative agents of more than two thirds of outbreaks. *Staphylococcus aureus* is beside non-typhoid *Salmonella*, *Campylobacter* spp. and *E. coli* one of a major cause of gastroenteritis, being able not only to contaminate food products during processing, but also because a portion of strains is able to produce enterotoxins that are thermostable and cannot be eliminated by heat processing [1–4]. Consumption of food contaminated with staphylococcal enterotoxins is the fifth most reported cause of food-borne illness [5]. Food is also important source of methicillin resistance genes for human population and some of the strains could be resistant (MRSA) [6, 7].

Staphylococcal enterotoxins are produced in food mainly by coagulase-positive *Staph. aureus* strains, but also by certain non-*Staph. aureus* species, e. g. coagulase-positive *Staph. intermedius*. Up to date, 18 enterotoxins of *Staph. aureus* were identified. They possess a great degree of homology but differ in structure, biological activity, amounts produced and mechanisms of gene regulation. The main groups are represented by SEA through SEE that may be further divided into two groups according to their amino acid sequence. Food poisoning is most related with SEA, the second most involved being SED [8, 9]. The production of staphylococcal enterotoxins usually started in late exponential growth phase when the *Staphylococcus aureus* counts exceeded 10^6 CFU·ml^{-1} [10]. The expression of staphylococcal enterotoxin-encoding genes in food depends also on various environmental factors, being highest at neutral pH and in the presence of certain amino acids, e. g. arginine and cystine for SEA–SEC. Low pH and salt concentration above 12% inhibit the production of staphylococcal enterotoxins [2, 11].

The methods currently used for the identification of staphylococcal enterotoxins in food products involve microbial culture with subsequent immunochemical detection [12, 13]. As an

Tereza Trnčíková, Eva Kaclíková, Tomáš Kuchta, Department of Microbiology and Molecular Biology, VÚP Food Research Institute, Priemyselná 4, SK – 824 75 Bratislava, Slovakia.

Saša Piskernik, Sonja Smole Možina, Barbara Jeršek, Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia.

Correspondence author:
Barbara Jeršek, e-mail: barbka.jersek@bf.uni-lj.si

Tab. 1. Oligonucleotides used.

Target gene	Oligonucleotide designation	Sequence	Reference
acr	aurF	CTAGCTTTATTTCGCAGGTGACGAT	[20]
	aurR	TCAACATCTTTCGCATGATTCAACAC	
	aurP	FAM-CTTGCTCCGTTTCACCAGGCTTCGGTG-TAMRA	
femB	femB-fw5'	AATTAACGAAATGGGCAGAAACA	[17]
	femB-rv5'	TGCGCAACACCCTGAACTT	
	femB-pb	FAM-AGAAATTAACTGGATGGTACGCGCGAAGA-TAMRA	
entA	SEA-fw5'	AAAATACAGTACCTTTGGAAACGGTT	
	SEA-rv5'	TTTCCTGTAAATAACGTCTTGCTTA	
	SEA-pb	FAM-AACGAATAAGAAAAATGTAACTGTTCAGGAGTTGGATC-TAMRA	
entB	SEB-fw5'	ACACCCAACGTTTTAGCAGAGAG	
	SEB-rv5'	CCATCAAACCAGTGAATTTACTCG	
	SEB-pb	FAM-CAACCAGATCCTAAACCAGATGAGTTGCACA-TAMRA	
entC	SEC-fw5'	AATAAAACGGTTGATTCTAAAAGTGTGAA	
	SEC-rv5'	ATCAAAATCGGATTAACATTATCCATTC	
	SEC-pb	FAM-TAGAAGTCCACCTTACAACAA-TAMRA	
entD	SED-fw5'	TGATTCTTCTGATGGGTCTAAAGTCTC	
	SED-rv5'	GAAGGTGCTCTGTGGATAATGTTTT	
	SED-pb	FAM-TATGATTTATTTGATGTTAAGGGTGATTTTCCCGAA-TAMRA	

alternative, polymerase chain reaction (PCR)-based methods for the detection of genes encoding for enterotoxins in *Staph. aureus* strains have become widely used [14–16]. Recently, a collection of highly effective real-time PCR-based methods for the detection of genes encoding for individual staphylococcal enterotoxins has become available [17]. In this study, we applied the real-time PCR methods to the detection of genes encoding for SEA, SEB, SEC and SED in *Staph. aureus* strains isolated from food products on the market in Slovakia and Slovenia.

MATERIALS AND METHODS

Food samples, detection and isolation of *Staph. aureus*

Food samples were obtained from shops in Bratislava, Slovakia and Ljubljana, Slovenia. The types of food samples were selected on the basis of known frequent contamination with *Staph. aureus*. Food samples were analysed by the standard method according to EN ISO 6888-1 [18] or EN ISO 6888-3 [19], using selective enrichment in modified Giolitti and Cantoni broth (Merck, Darmstadt, Germany) with potassium tellurite under anaerobic conditions. Presumptive *Staph. aureus* colonies were isolated on Baird-Parker agar (Merck). Selected colonies were analysed by the coagulase activity test using rabbit plasma (Bio-Rad, Hercules, California, USA). Identification was confirmed using API Staph system (bioMérieux, Marcy l'Etoile, France).

DNA extraction

Cultures of individual strains were grown in Brain Heart Infusion broth (Merck) for 18–22 h at 37 °C at shaking of 2 Hz, centrifuged at 10 000 × *g* for 5 min, and the sedimented cells were lysed by boiling in PCR buffer (Biotium, Hayward, California, USA) supplemented with 1% Triton X-100 (Serva, Heidelberg, Germany) at 95 °C for 20 min as described previously [15]. DNA from cultures of reference strains was extracted by chaotropic solid phase extraction using QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany).

Real-time PCR

Each reaction sample of 25 ml contained 12.5 ml Power SybrGreen PCR master mix 2X (Applied Biosystems, Foster City, California, USA), 300 nmol·l^{-1} of each primer and 2 ml of the cell lysate containing template DNA. Amplifica-

tion was performed in a ABI Prism 7500 real-time PCR cycler (Applied Biosystems) using a programme of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The dissociation step to measure temperature of melting was appended. Alternatively, real-time PCR with TaqMan probes was performed in reaction samples of 25 ml, each containing 2.5 ml of 10× concentrated reaction buffer (Biotium), 4.5 mmol·l^{-1} $MgCl_2$, 500 mmol·l^{-1} of each dNTP, 1.5 U Cheetah Hot Start Taq polymerase, 300 nmol·l^{-1} of individual primers, 200 nmol·l^{-1} of the probe and 2.5 ml of the cell lysate containing template DNA. Amplification was performed in an Opticon 2 real-time PCR cycler (MJ Research, Waltham, Massachusetts, USA) using a programme of 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Oligonucleotides were synthesized by Applied Biosystems or by Eurofins MWG Operon (Ebersberg, Germany) and are listed in Tab. 1.

RESULTS AND DISCUSSION

At first, real-time PCR systems targeting individual enterotoxin-encoding genes were tested with reference *Staph. aureus* strains. The results confirmed their good performance (Tab. 2). We found the reference strain *Staph. aureus* NCTC 10652, which was originally declared to be positive only for SEA, to be positive also for SED. This result is in agreement with previously published results [21].

Tab. 2. Results of real-time PCR regarding the presence of individual genes in reference strains.

Strain	Real-time PCR result				
	femB	SEA	SEB	SEC	SED
Staph. aureus NCTC 10652*	+	+	–	–	+
Staph. aureus NCTC 10654	+	–	+	–	–
Staph. aureus NCTC 10655	+	–	–	+	–
Staph. aureus NCTC 10656	+	–	–	–	+

* – reference strain positive for SEA, but found positive for SED, too.

Then, 300 food samples were tested for the presence of *Staph. aureus* and 38 *Staph. aureus* strains were isolated. Besides *Staph. aureus*, also other species from the genus were isolated within a group of 32 food products from the market in Slovenia. Out of 8 presumptive *Staph. aureus* in this group, only 6 isolates were confirmed to be *Staph. aureus* by PCR targeting *femB* (Tab. 3). The 38 confirmed *Staph. aureus* isolates were tested by real-time PCR for the presence of genes encoding for SEA, SEB, SEC and SED. Fourteen strains (37%) contained at least one gene encoding for SEA–SED, when 1 isolate (3%) was found positive for SEA, none was positive for SEB, 10 isolates (26%) were positive for SEC and 4 isolates (11%) were positive for SED (Tab. 4). The determined rate of occurence of genes encoding for SEA–SED is not very high, but somewhat higher than reported for *Staph. aureus* isolated from food by other authors. Also the highest occurence frequency of SEC is in agreement with previously published data [2, 3, 22]. In a similar study carried out by RŮŽIČKOVÁ et al. [23] in Czech Republic, 60% of *Staph. aureus* strains isolated from various food samples were determined to be enterotoxigenic. The most frequently found were SEI and SEG (32% and 31%, respectively) followed by SEC (14%), SEH (13%), SEB (7%), SEA (3%), SED (2%) and SEJ (1%) genes. In another study done by PEREIRA et al. [24], 69% of the *Staph. aureus* food isolates were found to be enterotoxigenic. A percentage of 12% of these isolates possessed one kind of staphylococcal enterotoxin gene, and the remaining 88% possessed more than one staphylococcal enterotoxin gene. The most common were SEA, SEG, SEI and SEI genes. HUONG et al. [25] investigated the prevalence and genetic diversity of *Staph. aureus* and staphylococcal enterotoxins in ready-to-eat foods marketed in Hanoi, Vietnam. In that study, 45 out of 212 samples tested were found to be contaminated with *Staph. aureus* and 40% strains had enterotoxins SEA–SEE. Our results are in agreement with HUONG et al. [25], but some of our results are not entirely consistent with published data [23, 24] since we were detecting only SEA–SED genes.

Tab. 3. Results of the analysis of food samples from Slovenian market, isolation of *Staphylococcus* strains and species identification.

Sample number	Sample	Strain	Result of classical identification	Result of real-time PCR targeting *femB*
1	Minced meat	ŽMJ 243	*Staph. saprophyticus*	–
		ŽMJ 244	*Staph. epidermidis*	–
		ŽMJ 245	*Staph. hominis*	–
2	Pig liver	ŽMJ 324	*Staph. aureus*	–
		ŽMJ 246	*Staph. hominis*	–
		ŽMJ 247	*Staph. auriculari*	–
3	Chicken wing	ŽMJ 249	Staph. hominis	–
		ŽMJ 252	*Staph.sciuri*	–
4	Beef lung	ŽMJ 253	*Staphylococcus* spp.	–
		ŽMJ 254	*Staph. auricularis*	–
		ŽMJ 325	*Staph. aureus*	–
5	Chicken liver	ŽMJ 257	*Staph. auricularis*	–
6	Minced meat	ŽMJ 258	*Staphylococcus* spp.	–
		ŽMJ 326	*Staph. aureus*	–
7	Chicken liver	ŽMJ 259	*Staphylococcus* spp.	–
8	Chicken leg	ŽMJ 260	*Staphylococcus* spp.	–
9	Chicken wing	ŽMJ 262	*Staphylococcus* spp.	–
		ŽMJ 327	*Staph. aureus*	+
10	Sandwich “Mega”	ŽMJ 300	*Staphylococcus* spp.	–
11	Sandwich “Student”	ŽMJ 264	*Staphylococcus* spp.	–
12	Sandwich with tuna	ŽMJ 265	*Staphylococcus* spp.	–
13	Sandwich “mega”	ŽMJ 266	*Staphylococcus* spp.	–
14	Sandwich “Poli”	ŽMJ 267	*Staphylococcus* spp.	–
15	Sanwich “Frik”	ŽMJ 306	*Staphylococcus* spp.	–
16	Biscuits with cream “kremšnita”	ŽMJ 271	*Staphylococcus* spp.	–
17	Fruit (sour cherry) cake	ŽMJ 273	*Staph. sciuri*	–
		ŽMJ 309	*Staphylococcus* spp.	–
18	Minced meat	ŽMJ 274	*Staphylococcus* spp.	–
19	Chicken wing	ŽMJ 277	*Staphylococcus* spp.	–
20	Minced meat	ŽMJ 281	*Staphylococcus* spp.	–
21	Chicken wing	ŽMJ 282	*Staphylococcus* spp.	–
22	Chicken liver	ŽMJ 284	*Staphylococcus* spp.	–
23	Minced meat	ŽMJ 286	*Staphylococcus* spp.	–
		ŽMJ 328	*Staph. aureus*	+
24	Chicken wing	ŽMJ 289	*Staphylococcus* spp.	–
		ŽMJ 329	*Staph. aureus*	+
25	Chicken fillet	ŽMJ 290	*Staphylococcus* spp.	–
		ŽMJ 330	*Staph. aureus*	+
26	Beef liver	ŽMJ 291	*Staphylococcus* spp.	–
		ŽMJ 331	*Staph. aureus*	+
27	Chicken liver	ŽMJ 293	*Staphylococcus* spp.	–
28	Minced meat	ŽMJ 295	*Staph. sciuri*	–
		ŽMJ 317	*Staphylococcus* spp.	–
29	Minced meat	ŽMJ 318	*Staphylococcus* spp.	–
30	Biscuits with cream “kremšnita”	ŽMJ 323	*Staphylococcus* spp.	–
		ŽMJ 332	*Staph. aureus*	+
31	Minced meat	ŽMJ 319	*Staphylococcus* spp.	–
32	Minced meat	ŽMJ 321	*Staphylococcus* spp.	–

Tab. 4. Detection of genes encoding for SEA–SED in *Staph. aureus* isolates.

No.	Strain	Source	Result of real-time PCR			
			SEA	SEB	SEC	SED
1	170207/02	Sausage	+	–	–	–
2	070307/18	Raw meat product	–	–	–	–
3	280307/48	Chicken filets	–	–	–	–
4	280307/64	Pork meat	–	–	+	–
5	030407/06	Pork meat	–	–	+	–
6	240107/12	Pork meat	–	–	+	–
7	030407/17	Sliced bacon	–	–	–	–
8	140207/29	Cream dressing	–	–	–	–
9	040407/31	Mincemeat product	–	–	–	+
10	040407/37	Chicken sausage	–	–	+	–
11	040407/41	Eggs	–	–	–	+
12	280307/72	Sliced pork	–	–	–	–
13	280307/77	Mixed minced meat	–	–	–	–
14	280307/89	Fresh cheese	–	–	–	–
15	030407/12	Cream dressing	–	–	–	–
16	VUP 605	Ice cream	–	–	+	–
17	VUP 621	Salami	–	–	+	–
18	VUP 602	Soft steamed cheese	–	–	–	–
19	VUP 622	Mayonnaise salad	–	–	–	–
20	VUP 603	Bryndza cheese	–	–	–	–
21	VUP 604	Sausage	–	–	–	–
22	VUP 624	Bryndza cheese	–	–	–	–
23	VUP 625	Bryndza cheese	–	–	+	–
24	VUP 489	Bryndza cheese	–	–	–	–
25	VUP 490	Meat product	–	–	–	–
26	VUP 491	Pork meat	–	–	–	–
27	VUP 492	Mixed minced meat	–	–	–	–
28	VUP 493	Chicken liver	–	–	–	–
29	VUP 494	Meat product	–	–	–	–
30	VUP 495	Pork meat	–	–	+	–
31	VUP 496	Sliced pork meat	–	–	–	–
32	VUP 497	Pork meat	–	–	+	–
33	ŽMJ327	Chicken wing	–	–	–	–
34	ŽMJ 328	Minced meat	–	–	+	+
35	ŽMJ 329	Chicken wing		–	–	–
36	ŽMJ 330	Chicken fillet		–	–	–
37	ŽMJ 331	Beef liver	–	–	–	+
38	ŽMJ 332	Biscuits with cream “kremšnita”	–	–	–	–

CONCLUSIONS

Staph. aureus strains isolated from food produced in Slovakia and Slovenia were found at a rate of 37% to contain at least one of the genes encoding for enterotoxins SEA–SED. This rate is not very high, but the strains containing the respective genes represent a threat for food safety. The real-time PCR detection systems employed in our study proved to be a proper tool to fulfill the requirement for rapid and reliable screening of *Staph. aureus* strains isolated from food for genes encoding for the most frequent enterotoxins.

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