IMPROVED MULTIPLEX POLYMERASE CHAIN REACTION FOR RAPID *Staphylococcus aureus* DETECTION IN MEAT AND MILK MATRICES

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Abstract: Staphylococcal food poisoning represents one of the most frequently occurring intoxications, caused by staphylococcal enterotoxins (SE-s) and staphylococcal enterotoxin-like proteins (SEl-s). Therefore, there is a need for rapid, sensitive and specific detection method for this human pathogen and its toxin genes in food matrices. The present work is focused on *Staphylococcus aureus* detection by a nonaplex polymerase chain reaction, which targets the 23S rRNA gene for identification of *S. aureus* at the species level, genes for classical SE-s (SEA, SEC, SED), new SE-s (SEH, SEI), SEl-s (SEK, SEL) and *tsst-1* gene (toxic shock syndrome toxin). Primers were properly designed to avoid undesirable interactions and to create a reliably identifiable profile of amplicons when visualized in agarose gel. According to obtained results, this approach is able to reach the detection sensitivity of 12 colony forming units from milk and meat matrices without prior culturing and DNA extraction.

Key words: *Staphylococcus aureus*, enterotoxin, multiplex PCR, rapid detection

1. Introduction

Foodborne pathogens causing toxins-associated diseases are important health problems throughout the world in both developed and developing countries (GUSTAFSON *et al.,* 2014). *S. aureus* is one of the major pathogens causing foodborne intoxications. Staphylococcal food poisoning (SFP) is an intoxication that results from the consumption of foods containing sufficient amount of one or more types of enterotoxin. Clinical characteristics of SFP comprise acute and rapid onset of symptoms (from 30 min to 8 hours after the consumption of contaminated food), with the most common nausea, vomiting, prostration, sometimes diarrhea and lowered blood pressure (LE LOIR *et al.,* 2003).

To date, the known repertoire of *S. aureus* enterotoxins comprises of the classical enterotoxins (SEs) SEA – SEE and the new SEs with demonstrated emetic activity in a primate model (SEG - SEJ, SER - SET) and staphylococcal enterotoxin-like (SEls) proteins, whose emetic properties remain unconfirmed (SelK-SElQ, SElU-SElX). TSST-1, the toxic shock-syndrome staphylococcal toxin, initially designated as SEF, lacks emetic activity (ARGUDÍN *et al.*, 2010; GRUMANN *et al.*, 2014). All of these toxins possess superantigenic activity and are encoded by mobile genetic elements, including plasmids, prophages, pathogenicity islands, genomic islands, or by genes located next to the staphylococcal cassette chromosome implicated in methicillin resistance (ARGUDÍN *et al.*, 2010; GRUMANN *et al.*, 2014; OTTO, 2014). Most

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commonly, *S. aureus* strains carry not only one *se*/*sel* gene and according to the results of epidemiology studies, the frequency of SE types and their combinations vary considerably (ROSEC and GIGAUD, 2002; ZSCHÖCK *et al*., 2005; BANIA *et al.*, 2006; HWANG *et al*., 2007; CHIANG *et al.*, 2008; RALL *et al.*, 2008; AYDIN *et al*., 2011; CREMONESI *et al*., 2015; ZEINHOM *et al.*, 2015; SHIN *et al.*, 2016; ROLA *et al*., 2016).

Unless heat processes are applied, staphylococci are expected to exist in any and all foods that are handled directly by humans or are of animal origin, as animals and humans are considered to be the main reservoir of *S. aureus* (LEROY *et al.*, 2016; ORTEGA MORENTE *et al.*, 2016). Once produced, staphylococcal enterotoxins survive heat processing during food manufacture in biologically active form because of their heat stability. Foods frequently implicated in staphylococcal food poisoning include meat and meat products; poultry and egg products; salads, such as egg, tuna, chicken, potato, and macaroni; bakery products, such as cream-filled pastries, cream pies, and chocolate éclairs; sandwich fillings; and milk and dairy products (REED, 1993). Strict application of good hygienic practices and good manufacturing practices are the key factors, which can prevent products being contaminated by *S. aureus* and its toxins. According to regulations of European Union (Commision regulation (EC) No. 1441/2007), milk and diary products must be tested for enterotoxins if coagulase-positive staphylococci are detected at levels higher than 10^5 CFU/g (http://eur-lex.europa.eu/eli/reg/2007/1441/oj).

Since the incidence of staphylococcal enterotoxicosis is associated with food safety and the conventional methods used to detect foodborne pathogen are time consuming and laborious, the effort of many research teams worldwide put their mind to development of methods that would allow food manufacturers quick, efficient and highly specific detection of pathogenic and potentially pathogenic microorganisms directly from samples either in the raw materials used in manufacturing or in final products. Rapid detection methods can be categorized into nucleic acid-based, biosensor-based and immunological-based methods (LAW *et al.*, 2015). PCR (polymerase chain reaction) and in particular multiplex PCR (mPCR) were proven as one of the most suitable way of approaching the issue of bacteria detection (SUN *et al.*, 2011; BRIZZIO *et al.*, 2013; HUMMERJOHANN *et al.*, 2014; CREMONESI *et al.*, 2015; ZEINHOM *et al.*, 2015; SHAWISH *et al.*, 2016). The PCR assay can be made within hours with high sensitivity and method accuracy, allowing for the detection of very low amounts of microorganisms. The aim of this study was to develop a rapid and highly specific method allowing routine proof of *S. aureus* and its toxigenic strains in meat and milk based on improved mPCR protocol, without prior culturing and DNA extraction.

2. Materials and methods

2.1 Bacterial strains and media

Staphylococcus aureus subsp. *aureus* CCM885, donated from EL spol. s.r.o. company, Slovakia, with all genes tested in this work was used for multiplex PCR of

S. aureus toxin genes. The number of colony-forming units (CFU) needed for detection of PCR product was determined by cultivation. Lysogeny broth (LB medium, 1 mL) was inoculated with a single colony and incubated overnight at 37°C, and serial 10–fold dilutions in LB were prepared, giving counts in the range of $10–10^{10}$ CFU/ml. Viable counts were obtained by culturing each dilution (50 µl) on LB-agar plates with overnight incubation at 37°C.

2.2 Primers

All primers used in the study are shown in Table 1. Primers were designed using the Primer 3 program for reliable use in nonaplex PCR, to yield a reliably identifiable profile of amplification products with defined molecular weight when visualized in agarose gel.

Table 1. Oligonucleotide primers used in this study.

Product size (bp)	**Primer**	**Sequence (5'-3')**	**Target gene**	**Genbank accession number**
1300	SAU1 (F) SAU2 (R)	GGACGACATTAGACGAATCA CGGGCACCTATTTTCTATCT	gene for 23S rRNA	CP009681.1
856	SEA1(F) SEA2 (R)	AGCGAGAAAAGCGAAGAAAT CCATAGGCACCACCTCCTTA	*sea*	L22566.1 (BORST and BETLEY, 1993)
603	SEC1 (F) SEC2 (R)	AGACCCTACGCCAGATGAGT CCTGGTGCAGGCATCATA	*Sec*	KF729631.1 (HUNT *et al.*, 2014)
530	SED1 (F) SED2 (R)	TCGGGAAAATCACCCTTAAC GCAGATAAAAATCCAATAATAGGAGA	*sed*	DQ630751.1 (NEMA *et al.*, 2007)
463	SEH1 (F) SEH2 (R)	TCACATCATATGCGAAAGCA TCGGACAATATTTTTCTGATCTTT	*seh*	AJ937548.1 (JØRGENSEN *et al.*, 2005)
385	SEI1 (F) SEI2 (R)	AAACTGGATATTTTTGGCATTG CAGGCAGTCCATCTCCTGTA	*sei*	AB060537.1 (OMOE *et al.*, 2002)
271	SEK1 (F) SEK2 (R)	CTAATAATGCCAGCGCTCAA GTAGCTGTGACTCCACCATA	*sek*	GQ358928.1
322	SEL1 (F) SEL2 (R)	GCGATGTAGGTCCAGGAAAT ACTGTTTGATGCTTGCCATT	*sel*	JN689383.1
196	SATS1 (F) SATS2 R)	GCGACAATCGCTACAGGTTT TGATGCTGCCATCTGTGTTT	*tsst-1*	EF531614.1 (MONECKE *et al.*, 2007)

Specificity of oligonucleotide sequences of primers for *S. aureus* was analysed by Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast). All oligonucleotide primers were synthesized by Microsynth AG (Switzerland). Primer mix containing all primers at equimolar concentrations was prepared for easy and reproducible handling of the numerous primers used in multiplex PCR. All primer stock solutions were normalized to a concentration of 100 µM using PCR water, thus the resulting 10× primer mix contained each primer at 2 µM.

2.3 Cell suspensions for PCR

The tested *S. aureus* overnight culture prepared from single colony grown on LB medium was treated in two ways: by boiling and with NaClO solution. In case of boiling preparation, 1 ml of culture was centrifuged and cell pellet was suspended in 500 µl of sterile distilled water in a tube. The tube was vortexed and incubated at 96°C for 10, 20, 30 and 60 min, respectively, then chilled on ice for 5 min, and centrifuged at 10,000 × g for 2 min to remove the cell debris. The supernatant (5 µl) was added directly to the PCR mixture. In the case of NaClO samples, prepared according to our previous study (VIDOVÁ *et al.*, 2011), the 1 ml of the *S. aureus* overnight culture was treated with NaClO solution in resulting concentration of 5×10^{-4} % for 10 min. In both cases, the viability of the cells was verified by counting the number of CFU on LB-agar plate after overnight incubation at 37°C. With the aim to prepare the sample for PCR assay with milk matrix, the NaClO-treated cell pellet was first resuspended in 1 ml of sterile UHT-treated cow's milk, incubated at room temperature for 30 min, and then washed with 1 ml of water and phosphate buffered saline (PBS pH 8). Simultaneously, with the aim to prepare the sample with meat matrix, the washed cell sample was first mixed and incubated at room temperature for 30 min with 1 g of pork meat sample, and then washed with water and PBS. Cell suspension (5 µl) was used as a template in a final volume of 20 µl of PCR mixture containing: 1× optimized PCR Buffer 10× (with 1.5mM $MgCl_2$) (Applichem), dNTP Mix (10 mM) (Applichem), Taq DNA Polymerase (DNA-free, 5U/µl) (Applichem) and primer mix.

2.4 PCR conditions

PCR assay was carried out directly from *S. aureus* cells prepared according to procedure described previously by VIDOVÁ *et al.* (2011). The amplification programme consisted of denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 60s, annealing at 55°C for 90s and extension at 72°C for 2 min, followed by a final extension at 72°C for 8 min. If monoplex PCR was used, the concentration of each primer in PCR reaction was 0.625 µM and quantity of Taq DNA Polymerase was 0.2 U. The whole PCR reactions were analyzed by electrophoresis in 0.8% agarose gel stained with 0.5 µg/ml ethidium bromide, which was carried out in 1× BBE buffer (0.65 M boric acid, 0.029 M sodium tetraborate, 0.25 M EDTA, pH 7.8). UV transillumination of the bands on the agarose gel ought to show different sizes of nine desired amplification products. DNA molecular size marker was included in each gel (100 bp DNA Ladder; Invitrogen™). All obtained PCR products were sequenced by Microsynth AG (Switzerland) to verify their identity.

3. Results

3.1 Exclusion of DNA extraction by direct use of S. aureus cell suspensions as template for PCR

Extraction of chromosomal DNA suitable for PCR is often the most limiting step, in particular of time consuming, DNA quality, purity and integrity, which lead to

nonspecific results and reduction of the sensitivity of the method. Therefore, in this work was DNA extraction step excluded and replaced by direct use of washed bacterial cells into polymerase chain reactions. According to our previous experience (CHOTÁR *et al*., 2006; VIDOVÁ *et al*., 2011), bacterial cells prepared by washing method with NaClO pretreatment were used. Briefly, two ways to prepare cell suspensions from overnight culture before washing steps were used; and samples (5 µL) after washing with sterile water were used directly to PCR. The rest of cell preparations were subsequently stored at 4°C for a week. Both preparations, freshly prepared and stored ones, were tested for viability. In the case of boiling preparation, the 10 min of incubation at 96°C was enough to achieve PCR products, but only with fresh preparation. There were no PCR products observed after one week of storage, although boiling treatment was enough to kill all viable cells in sample as well. The NaClO treated samples exhibited satisfactory results with both freshly prepared and stored ones, with the same results observed after more than three months of storage. Therefore, the NaClO samples of overnight culture were used in further experiments. The additional advantage of this treatment is that there was no need to prepare other overnight cultures, which reduce the handling with infective living cells. Therefore, laboratory testing of such bacterial pathogen samples is more safety, and further testing proceeded with one standardized batch of cell suspension. In addition, NaClO samples of characterized bacterial cell suspensions are able to be used as positive controls due to the possibility of their long term storage without affecting PCR sensitivity. Bypassing of DNA extraction still remains beneficial in such cases as well, resulting in time and labor-savings.

3.2 Monoplex PCRs

All primer pairs designed for identification of *S. aureus* toxin genes generated single PCR products of expected sizes when washed cell suspension of this pathogen was directly used in PCR reactions (Fig. 1). No PCR products were generated with these primers by using negative control strain. Amplification of 1300 bp PCR product was observed when species-specific primer pair (SAU1 and SAU2; CHOTÁR *et al*., 2006) was used. Detection limits, i.e. number of cells needed to obtain a visible PCR product, for every single primer pair were determined by cultivation. The amount of 4 cells was enough for positive amplification of appropriate PCR products with primer pairs SEA1/SEA2, SEC1/SEC2 and SED1/SED2. Two cells were enough for the amplification of PCR products with primer pairs SEH1/SEH2 and SEI1/SEI2, and even one single cell with primer pairs SEK1/SEK2, SEL1/SEL2 and SATS1/SATS2. The observation of detection limit for species-specific SAU primer pair was the same as stated previously by CHOTÁR *et al*. (2006).

3.3 Multiplex PCRs

Optimization of multiplex PCR conditions revealed crucial steps that should not be neglected. The first one is the length of initial denaturation step, which is required to be at least 5 minutes. This step is important due to presence of whole bacterial cells in reactions and allowed adequate destruction of cells, DNA release and denaturation of

contaminating cell contents that could have an inhibitory effect on Taq-polymerase during PCR. The second important step is using the equal concentrations (0.2 μM) of all primers prepared in one mix. And finally, keeping the annealing time for 90 seconds showed to be essential as well.

Testing of multiplex PCR in this work comprised three experimental models depending on the type of matrix, in which the presence of *S. aureus* cells was tested. The prepared cells suspensions were then directly used in the multiplex PCR. In the first model overnight culture was treated for 10 min with NaClO solution (resulting concentration of 5×10^{-4} %), washed with water and PBS, and then directly used as template in PCR. In the second model the overnight culture was treated with NaClO mixed with a sample of UHT-treated cow's milk, followed by washing with water and PBS, and then directly used as template in PCR. Finally, in the third model the NaClO-treated overnight culture was mixed with a sample of meat, followed by washing with water and PBS, and then directly used as template in PCR.

Because the sensitivity of multiplex PCR is linked with visibility of all amplicons, the results of testing suitability of cell suspension prepared only from the overnight culture for multiplex PCR were comparable with that of monoplex PCR. The visibility of the upper PCR product for 23S rRNA gene was detectable for at least 8 cells in the multiplex PCR. The presence of milk background and meat matrix decreased the sensitivity by 0.5-times (to 12 cells).

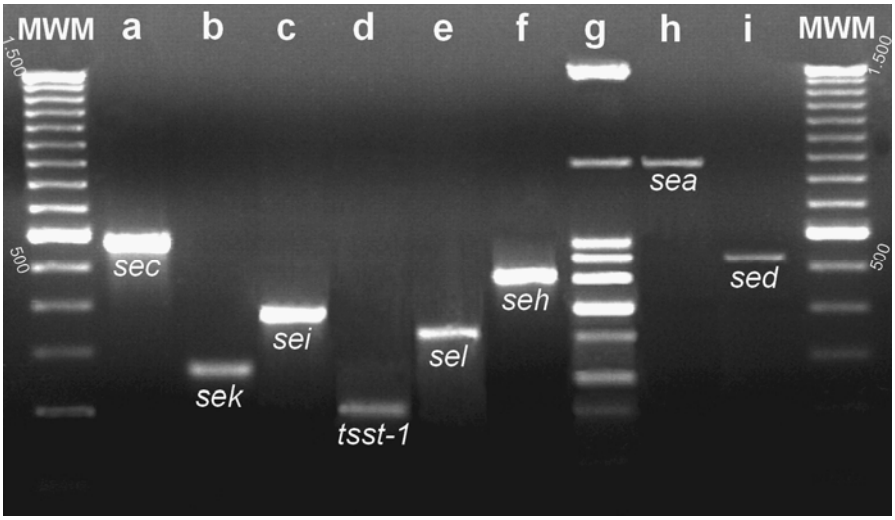

Fig. 1. Amplicons of targeted genes visualized in agarose gel (monoplex and multiplex PCRs). **a**: *sec* gene (603 bp), **b**: *sek* gene (271 bp), **c**: *sei* gene (385 bp), **d**: *tsst-1*gene (196 bp), **e**: *sel* gene (322 bp), **f**: *seh* gene (463 bp), **g**: nonaplex PCR with 23S rRNA gene (1500 bp) **h**: *sea* gene (856 bp), **i**: *sed* gene (530 bp); **MWM**: standard of molecular weight (100 bp DNA ladder).

4. Discussion

In our work, we initially designed primer pairs for 23S rRNA gene and for *se*/*sel* genes *sea, sec, sed, seh, sei, sek, sel* and *tsst-1*. Monoplex PCRs for individual genes

targeted were standardized to determine the optimum amplification conditions on collection strain of *S. aureus*. After successful optimization of PCR conditions, amplified products were sequenced and the obtained data confirmed identity with expected sequences (according to the GenBank). We used intact cells of *S. aureus* as a template for PCR. In our laboratory, this advanced procedure without prior DNA extraction from bacterial cells proved to be more sensitive in comparison to protocol with extracted DNA. We were able to reach the detection sensitivity of 12 CFU in milk (ml^{-1}) and meat (g^{-1}). NAGARAJ *et al.* (2014) reported much lower detection sensitivity of 10^6 CFU/ml or CFU/g in food when using extracted DNA as a template for mPCR assay. DNA extraction is the commonly used step in PCR protocols published in articles dealing with similar issue (AYDIN *et al*., 2011; QUIGLEY *et al.*, 2011; ALI *et al.*, 2014; LU *et al.*, 2015). There are some studies about optimization of DNA extraction as an essential step for successful PCR (RAMESH *et al*., 2002; CREMONESI *et al.*, 2006; BINET *et al.*, 2014). However, we found out that this step can be completely dropped, thus shortening the time and reducing the costs for analysis. TILSALA-TIMISJARVI and ALATOSSAVA (2004) used a membrane-based method for DNA preparation from diary samples, avoiding the contact with volatile solvents or other toxic reagents used during conventional DNA isolation but this approach was i) more expensive, ii) required more time than procedure described in our work and iii) the sensitivity was comparable with conventional method.

Subsequently, the conditions for multiplex PCR were optimized. Primer pairs generated a set of amplicons with molecular sizes expected from the gene sequences (Fig. 1): 1300 bp (sequence coding 23S rRNA), 856 bp (*sea*), 603 bp (*sec*), 530 bp (*sed*), 463 bp (*seh*), 385 bp (*sei*), 271 bp (*sek*), 322 bp (*sel*), 196 bp (*tsst-1*). These products were reliably separated in 0.8 % agarose gel. The resulting profile represents a *S. aureus* reference DNA marker. We chose this set of primers for multiplex PCR because it covers i) the most frequently occurring classical enterotoxins (A, C, D), ii) some new types of SE (H, I), iii) SE-like superantigens (K, L) and iv) toxic shock syndrome toxin 1 (TSST-1). The superantigenic toxin TSST-1 is found to be associated with enterotoxins (JOHLER *et al.*, 2013). There are reports regarding the production of TSST-1 from *S. aureus* strains isolated from food handlers (SOSPEDRA *et al.*, 2012). Analysis of 147 isolates of *S. aureus* from patients associated with staphylococcal food poisoning outbreaks in Taiwan confirmed 91.8 % of strains to be positive for at least one SE or TSST-1 (CHIANG *et al.*, 2008). TSST-1 was found also in raw bulk tank milk samples from Switzerland (SCHERRER *et al.*, 2004). Distribution of classical and new SE and SAgs is variable and depends on country of origin and sample used for isolation of *S. aureus* (BECKER *et al.*, 2004; OMOE *et al.*, 2005; MORANDI *et al.*, 2007; TRNČÍKOVÁ *et al.*, 2010). Classical enterotoxins are known as common cause of the food poisoning outbreaks. Type A is the mostly occurring one in various food samples (CHEN *et al.*, 2004; HWANG *et al.*, 2007; RALL *et al.*, 2008; MEDVEĎOVÁ *et al.*, 2014; RALL *et al.*, 2014), while type E is not frequently detected. However, it was identified and quantified in the cheese responsible for food poisoning, which was the first evidence of food poisoning in France caused by this enterotoxin (OSTYN *et al.*, 2010). As there are still many cases with unknown cause, the newly described SEs and SEl proteins are also of importance

because they can be the reason explaining some of these enterotoxicosis with unknown causative agent.

5. Conclusions

Highly specific methodology utilizing PCR and multiplex PCR for routine detection of isolates of *S. aureus* in dairy and meat products has been developed. Method described in this study enables to identify the presence of coagulase-positive *S. aureus* and selected staphylococcal toxins using specifically designed primers directly in one sample at a time without prior culturing and DNA extraction, avoiding the contamination of people and the environment. This methodology gives a clear overview of the presence of pathogenic *S. aureus* and various toxic genes in food matrices. It is potentially applicable in the agricultural and food industry in the control of animal health and food quality, which enables fast and reliable food quality control of starting materials and finished products.

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