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Modified Multiplex PCR Method for Detection of Pyrogenic Exotoxin Genes in Staphylococcal Isolates

Astrid Løvseth,* Semir Loncarevic, and Knut G. Berdal

Section of Feed and Food Microbiology, National Veterinary Institute, Ullevålsveien 68, 0033 Oslo, Norway

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A modified multiplex PCR method for detection of nine *Staphylococcus aureus* enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*) and one form of immunoreactive toxic shock syndrome toxin based on a previously published method (S. R. Monday and G. A. Bohach, J. Clin. Microbiol. 37:3411–3414, 1999) has been developed. The modified PCR protocol seems robust and gives reliable results.

Staphylococcal enterotoxins (SE), toxic shock syndrome toxin (TSST), and exfoliative toxins A and B belong to a family of related pyrogenic toxins produced by *Staphylococcus aureus* (4, 10). By virtue of its variety of enterotoxins *S. aureus* is an important food-borne pathogen. In addition to the well-characterized SEA, SEB, SEC, SED, and SEE, new serological types of SEs (SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, and SEU) have been identified in recent years (5, 6, 7, 11, 13, 15, 16, 18). Five of the SE genes (*seg*, *sei*, *sem*, *sen*, and *seo*) belong to the same enterotoxin gene cluster (*egc*), and detection of one of these genes usually indicates the presence of all five enterotoxin genes (5, 11). For routine detection of SEs, commercially produced kits, such as reverse passive latex agglutination assays and enzyme-linked immunosorbent assays, are most commonly used. However, these methods are, to date, designed only to detect SEA, SEB, SEC, SED, and SEE. As an alternative to these more traditional methods, the PCR approach can provide detection of toxin genes and is presently designed to detect at least nine SE genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*) (1, 9, 12, 14).

The objective of this study was initially to establish the multiplex PCR method for detection of the toxin genes in staphylococcal isolates published by Monday and Bohach (12). According to their article, any of the nine SE genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*), the TSST gene, and the 16S rRNA gene can be amplified in a single multiplex reaction (results were not shown). However, while establishing the described method on two different types of thermocyclers, no detection of *seb*, *sec*, and *tsst* could be observed, and the amplification of *sec* and *sei* gave various results. Some modifications and adaptations were therefore considered necessary.

The following modifications were made to Monday and Bohach's method (12): (i) using another DNA isolation method; (ii) redesigning four primers (*seb-sec* forward, *seb* reverse, *sei* forward, and *tsst* reverse); (iii) splitting the multiplex into two PCRs; (iv) significantly decreasing the amount of 16S rRNA primers; and (v) adding four cycles to the PCR program.

* Corresponding author. Mailing address: National Veterinary Institute, Section of Feed and Food Microbiology, P.O. Box 8156 Dep., 0033 Oslo, Norway. Phone: 47 23 21 62 41. Fax: 47 23 21 62 02. E-mail: astrid.lovseth@vetinst.no.

Bacterial isolates and DNA isolation. The *S. aureus* isolates used in this study are listed in Table 1. One colony of each isolate was incubated overnight in 3 ml of brain heart infusion broth medium at 37°C with agitation. Overnight culture (1.5 ml) was transferred to an Eppendorf tube and was centrifuged for 2 min at 17,500 × *g*. The pellet was resuspended in 1× Tris-EDTA buffer, and the cells were then lysed with 10 μl of 10-mg/ml lysostaphin (Sigma-Aldrich). This solution was incubated for 60 min at 37°C. The DNA was extracted by a standard cetyltrimethylammonium bromide procedure using chloroform followed by ethanol precipitation (17). DNA concentration was measured by the SYBR Green I method (2).

Amplification of selected staphylococcal genes. Monday and Bohach (12) compared and evaluated the sequences of the SE genes. Unique primer sequences were identified for each gene with the exception of *seb* and *sec*. The *seb-sec* primer pair produces an identical product for both *seb* and *sec*. As a solution to the lack of specificity of the *seb-sec* primers, Monday and Bohach (12) designed a separate *sec* forward primer that works in combination with the *seb-sec* primers and that produces an amplification product unique to *sec*. The *sec* primers detect all three subclasses of enterotoxin SEC (C1, C2, and C3) (3, 8). All samples were tested for the presence of the 16S rRNA gene in order to ensure correct interpretation of toxin-negative isolates (12). However, due to the existence of multiple copies of the 16S rRNA gene, the concentration of the corresponding primers was reduced in the modified method to avoid 16S rRNA amplification products outcompeting other amplification products.

Monday and Bohach used 22 primers in the same PCR (12). Many primer sets in a single reaction may reduce robustness. Furthermore, some of the PCR products are similar in size and the bands were therefore found to be difficult to differentiate, particularly when analyzing unknown isolates. To avoid these problems we investigated several alternative primer combinations before the 11 primer sets were divided into two halves (reaction mixtures 1 and 2). Primers for *sed*, *see*, *seg*, *sei*, and *tsst* (Table 2) were combined in reaction mixture 1, and primers for *sea*, *seb-sec*, *sec*, *seh*, *sej*, and 16S rRNA (Table 2) were combined in reaction mixture 2. Monday and Bohach (12) used Gibco BRL *Taq* DNA polymerase (Life Technologies, Inc., Rockville, Md.) and an Amplitron II thermocycler (Barnstead Thermolyne Co., Dubuque, Iowa) for PCR amplification,

TABLE 1. Isolates used in this study and their expected toxin genotype

Isolate	Expected toxin genotype	Reference or source	Obtained toxin genotype in the present study by[a]:	
			Unmodified method	Modified method
R5371/00	*sea*, *seg*, *seh*, *sei*, and *tsst*	FSML,[b] 9	*sea*, *seg*, and *seh*	*sea*, *seg*, *seh*, *sei*, and *tsst*
R5460/00	*seb*, *seg*, *seh*, *sei*, and *tsst*	FSML, 9	*seg* and *seh*	*seb*, *seg*, *seh*, *sei*, and *tsst*
R5010/00	*sed*, *seg*, *sei*, and *sej*	FSML, 9	*sed*, *seg*, *sei*, and *sej*	*sed*, *seg*, *sei*, and *sej*
R4774/00	None	FSML, 9	None	None
R4571/00	*sec* and *tsst*	FSML, 9	*sec*	*sec*[c] and *tsst*
R4071/00	*seb*	FSML, 9	None	*seb*
R2102/00	*sec*, *seg*, and *sei*	FSML, 9	*seg* and *sei*	*sec*,[c] *seg*, and *sei*
R963/00	*sed*, *seg*, *sei*, and *sej*	FSML, 9	*sed*, *seg*, *sei*, and *sej*	*sed*, *seg*, *sei*, and *sej*
FRI472	*sed*, *seg*, *sei*, and *sej*	12	*sed*, *seg*, *sei*, and *sej*	*sed*, *seg*, *sei*, and *sej*
FRI913	*sea*, *sec*, *see*, and *tsst*	12	*sea*, *sec*, and *see*	*sea*, *sec*,[c] *see*, and *tsst*
FRI572	*seg* and *sei*	12	*seg* and *sei*	*seg* and *sei*
FRI445	*seg* and *sei*	12	*seg* and *sei*	*seg* and *sei*
3169	sec_{bovine}, *sed*, *sej*, and *tsst*	12	*sec*, *sed*, and *sej*	sec_{bovine}, *sed*, *sej*, and *tsst*

[a] Our results using MJ research PTC 225 and Perkin Elmer 9700 thermocyclers.
[b] FSML, Jim McLauchlin, Food Safety Microbiology Laboratory, Public Health Laboratory Service, London, United Kingdom.
[c] This isolate generates both the *seb-sec* and the *sec* amplification products and should therefore be analyzed further with unique primers for *seb* to ensure that there is no *seb* hidden in the *seb-sec* product.

whereas we used the following protocol: 5 μl of DNA (10 ng/μl) was added to 45 μl of reaction mixture containing final concentrations of 1× AmpliTaq buffer, 4 mM $MgCl_2$, 2 U of AmpliTaq Gold polymerase (all from Applied Biosystems), 400 μM each deoxynucleoside triphosphate (ABgene, Epsom, United Kingdom), 300 nM each SE primer, and 60 nM 16S rRNA primers. DNA was amplified on an MJ Research thermocycler PTC 225 by initial denaturation for 10 min at 95°C followed by 15 cycles of 95°C for 1 min, 68°C for 45 s, 72° for 1 min, 20 cycles of 95°C for 1 min, 64°C for 45 s, 72° for 1 min, and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis of 10 μl of reaction product in a 2.5% agarose gel (0.5× Tris-borate-EDTA buffer at 100 V for 100 min) and visualized on a UV transilluminator (Gel Documentation System; Bio-Rad, Hercules, Calif.). Product size was determined by comparison with a pUC-mix molecular weight ladder (Fermentas, Vilnius, Lithuania). Both the original and the modified protocol were tested on two different thermocyclers: MJ Research PTC 225 and Perkin-Elmer GeneAmp PCR System 9700.

Analysis of *S. aureus* isolates using both the unmodified and modified multiplex PCR methods. Figure 1 shows PCR results with the unmodified multiplex PCR protocol as described by Monday and Bohach (12) performed on the MJ Research PTC 225 thermocycler. No detection of *seb-sec* and *tsst* was observed, and the amplification of *sec* and *sei* gave various results.

TABLE 2. Primers used in this study for detection of SE genes, TSST gene (*tsst*), and 16S rRNA gene

Primer[a]	Primer sequence (5′-3′)	Amplified product size (bp)	GeneBank accession no.	Reference or source	Multiplex PCR reaction mixture no.
sea forw.	GCA GGG AAC AGC TTT AGG C	521	M18970	12	2
sea rev.	GTT CTG TAG AAG TAT GAA ACA CG				
seb-sec forw.	ACA TGT AAT TTT GAT ATT CGC ACT G	667	M11118 (*seb*)	This study	2
seb rev.	TGC AGG CAT CAT GTC ATA CCA				
sec forw.	CTT GTA TGT ATG GAG GAA TAA CAA	284	X05815 (*sec1*)	12	2
sec rev.	TGC AGG CAT CAT ATC ATA CCA		AY450554 (*sec2*) X51661 (*sec3*)		
sed forw.	GTG GTG AAA TAG ATA GGA CTG C	385	M28521	12	1
sed rev.	ATA TGA AGG TGC TCT GTG G				
see forw.	TAC CAA TTA ACT TGT GGA TAG AC	171	M21319	12	1
see rev.	CTC TTT GCA CCT TAC CGC				
seg forw.	CGT CTC CAC CTG TTG AAG G	328	AF064773	12	1
seg rev.	CCA AGT GAT TGT CTA TTG TCG				
seh forw.	CAA CTG CTG ATT TAG CTC AG	359	U11702	12	2
seh rev.	GTC GAA TGA GTA ATC TCT AGG				
sei forw.	CAA CTC GAA TTT TCA ACA GGT ACC	466	AF064774	This study, 12	1
sei rev.	CAG GCA GTC CAT CTC CTG				
sej forw.	CAT CAG AAC TGT TGT TCC GCT AG	142	AF053140	12	2
sej rev.	CTG AAT TTT ACC ATC AAA GGT AC				
tsst forw.	GCT TGC GAC AAC TGC TAC AG	559	J02615	This study, 12	1
tsst rev.	TGG ATC CGT CAT TCA TTG TTA T				
16S rRNA forw.	GTA GGT GGC AAG CGT TAT CC	228	X68417	12	2
16S rRNA rev.	CGC ACA TCA GCG TCA G				

[a] forw., forward; rev., reverse.

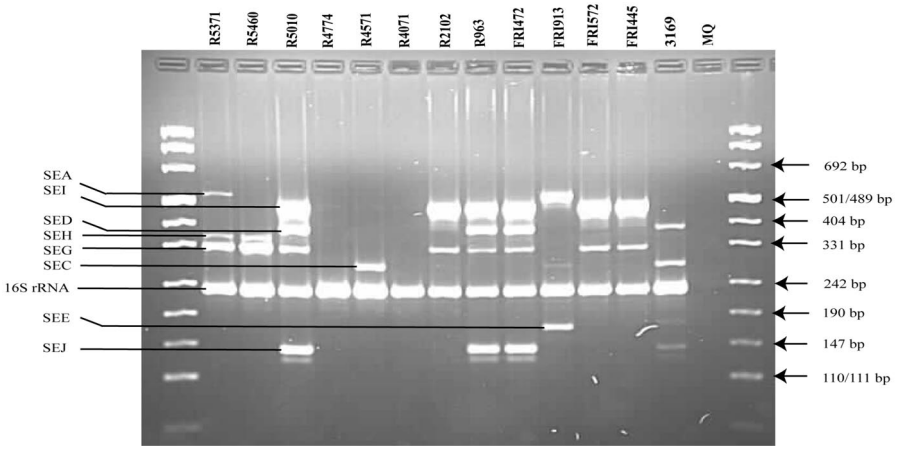

FIG. 1. Agarose gel electrophoresis of PCR products amplified with the multiplex PCR method described by Monday and Bohach (12). Primers detecting genes encoding all the nine SEs, the TSST, and the 16S rRNA are represented in this PCR mixture. *S. aureus* isolates, MQ (MilliQ water) negative control, amplification products, and DNA fragment sizes are indicated.

The modified multiplex PCR protocol gave correct identification of all expected toxins (Table 1). Figures 2 and 3 show the PCR results with reaction mixtures 1 and 2 and detection of the SED, SEE, SEG, SEI, and TSST genes and the SEA, SEB-SEC, SEC, SEH, SEJ, and 16S rRNA genes, respectively. When DNA from several isolates was combined (R5460 plus R5010 plus FRI913 or FRI472 plus FRI913 plus R5460), the modified method was able to detect all enterotoxin genes simultaneously (results not shown). The modified protocol, including redesigned *seb-sec* forward and *seb* reverse primers (Table 2), resulted in the expected amplification of the SEB-SEC gene in known isolates (Table 1, Fig. 3). However, it has been suggested (9, 10, 14) that isolates that give both the *seb-sec* and *sec* products should be analyzed further with unique primers for *seb* to ensure that there is no *seb* hidden in the *seb-sec* product. It is well known that some isolates express SEC_{bovine} and thereby generate only the *sec* product instead of both the *seb-sec* and *sec* products (12). This was confirmed in this study by isolate 3169 (Table 1, Fig. 3). The TSST gene could not be detected in the isolates previously reported as TSST producers with the unmodified protocol (Table 1, Fig. 1). After redesigning the *tsst* reverse primer (Table 2), the expected *tsst* amplification product was detectable by the modified protocol (Table 1, Fig. 2). Similarly, after redesigning the *sei* forward primer (Table 2) we were able to detect the expected *sei* amplification product in two isolates (R5371/00 and R5460/00) that were negative by the unmodified protocol (Table 1, Fig. 1 and 2). In a personal communication, Monday and Bohach suggested we try different brands of thermocyclers, because they also observed that their original protocol did not work on an Eppendorf thermocycler while it worked on a Perkin-Elmer GeneAmp model 2400 (Applied Biosystems), possibly due to differences in ramping time and block controls. In the present study we did not observe differences in performance between the MJ Research PTC 225 and Perkin Elmer GeneAmp 9700 in either the unmodified or modified method.

Splitting the multiplex PCR into two halves increases the workload. However, this may be justified by the observation

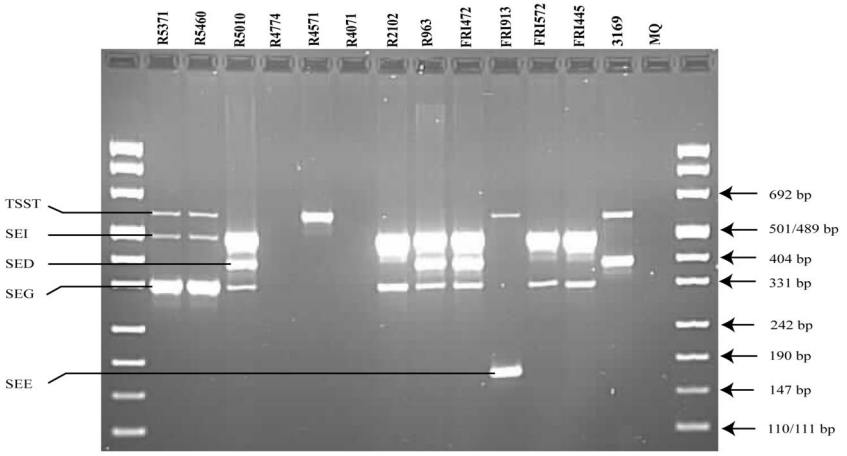

FIG. 2. Agarose gel electrophoresis of PCR products amplified with the modified multiplex PCR protocol—reaction mixture 1. Primers detecting genes encoding SED, SEE, SEG, SEI, and TSST are represented in this PCR mixture. *S. aureus* isolates, MQ (MilliQ water) negative control, amplification products, and DNA fragment sizes are indicated.

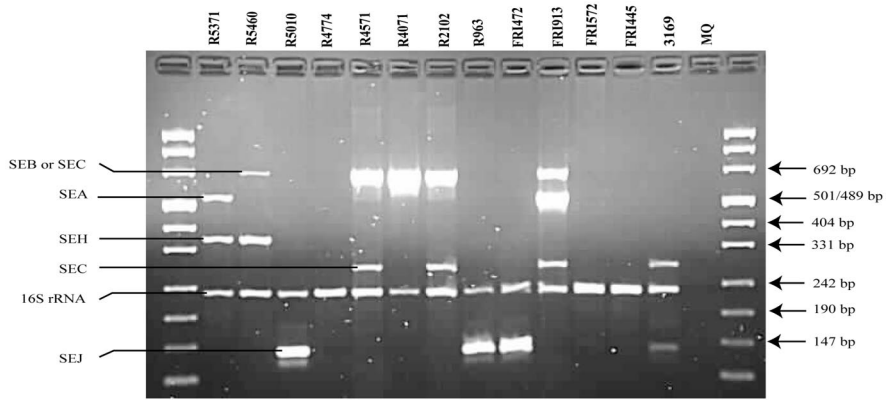

FIG. 3. Agarose gel electrophoresis of PCR products amplified with the modified multiplex PCR protocol—reaction mixture 2. Primers detecting genes encoding SEA, SEB, SEC, SEH, SEJ, and 16S rRNA are represented in this PCR mixture. *S. aureus* isolates, MQ (MilliQ water) negative control, amplification products, and DNA fragment sizes are indicated.

that this approach yielded more reliable results. Furthermore, the modified protocol appeared to be more robust, as observed by the absence of differences in performance on two different brands of thermocyclers.

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