

Differentiation of *entC1* from *entC2/entC3* with a single primer pair using simple and rapid SYBR Green-based RT-PCR melt curve analysis

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Abstract In spite of their involvement in foodborne illness, the epidemiological relevance of staphylococcal enterotoxin C (SEC) subtypes is poorly documented may be due to high sequence similarity. Among subtypes, SEC1, SEC2, and SEC3 exhibit more than 97 % homology because of which specific detection tools are seldom available to identify and differentiate them. In this study, a SYBR Green-based RT-PCR followed by melt curve analysis was developed for differentiation of *entC1* from *entC2/entC3* using a single primer pair. Nucleotide sequences of all three subtypes were analyzed using Clustal Omega program and the region with significant sequence variation/heterogeneity (where utmost SNPs were closely located and accessible for RT-PCR) was selected for amplification by designing a single primer pair that could amplify all three subtypes. In spite of same amplicon size, *entC1* showed distinct melt peak at 76 °C. However, due to high similarity between *entC2* and *entC3*, the developed format was deficient to discriminate between them and both showed melt peak at 82 °C. Reliability of developed RT-PCR was evaluated using various naturally contaminated samples and 91 food and clinical *Staphylococcus aureus* isolates where satisfactory results were obtained in comparison with commercial immunoassay kit and conventional PCRs using validated primers. To the best of our knowledge, this is the first method being reported to differentiate *entC1* from

entC2/entC3 using single primer pair which is unachievable by conventional PCR due to same amplicon size. As benefits, the method is sensitive, rapid, and inexpensive with no requirement of fluorescent probes, multiple primers, and post-PCR procedures. Thus, the assay might find its utility as a detection tool in epidemiological survey of foodborne outbreaks for simultaneous identification and differentiation of *entC1* from *entC2/entC3*.

Keywords SEC subtypes · SYBR Green · RT-PCR · Melt curve analysis · Melt peak · SNP

Introduction

Heat-labile *Staphylococcus aureus* is known to produce heat-stable enterotoxins, the ingestion of which leads to awful food poisoning consequences (Balaban and Rasooly 2000). More than 21 types of SEs have been identified including SEA-SEE, which are designated as classical enterotoxins; SEG-SEU, which are known as recently described enterotoxins and staphylococcal enterotoxin-like proteins (SEIs). Among the group, SEC exhibits more heat tolerance followed by SEB and SEA (Tibana et al. 1987). In addition, the member SEC is divided into molecular variants such as SEC1, SEC2, and SEC3 as well as SEC_{bovine}, SEC_{canine}, and SEC_{ovine} which are highly conserved proteins. The subtypes SEC1, SEC2, and SEC3 are encoded by *entC1*, *entC2*, and *entC3*, respectively, and are most immunologically related. More than 97 % homology has been encountered among three subtypes both in nucleotide and protein sequences (Betley et al. 1992). The C-terminal domain is highly conserved and variation is mainly confined to N-terminal region. The gene *entC1* shares 97.9 % homology with *entC2* and 97.4 % homology with *entC3*, whereas *entC2* and *entC3* share 98.0 % homology. As mature

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toxins, SEC2 and SEC3 share the highest homology with only four amino acid difference (98.3 % homology). SEC1 differs from SEC2 by seven amino acid residues (97.1 % homology) and from SEC3 by nine amino acids (96.2 % homology) (Hovde et al. 1990; Marrack and Kappler 1990).

In spite of high degree of sequence homology, each subtype differ biologically and physicochemically demonstrating unique properties. They exhibit distinct epitopes, different isoelectric points (8.6, 7.8, and 8.2 respectively for SEC1, SEC2, and SEC3), and ability to stimulate divergent T cell populations (Reiser et al. 1984; Deringer et al. 1996). Enterotoxin C has been proven for its superantigenic activity that induces enormous immune response leading to toxic shock (Bohach et al. 1990, 1997; Marrack and Kappler 1990). In fact, the ability of SEC to induce massive cytokine release has been studied for its effect to inhibit tumor formation. Consequently, the subtype SEC2 and its mutants have been reported for its antitumor activity (Ming-Kai and Cheng-Gang 2006; Wang et al. 2009; Zhou et al. 2013) and have been used for tumor therapy in China for decades (Chen 2001). On the other hand, the presence of other subtype SEC1 has been demonstrated in synovial fluid of arthritis patients (Ataee et al. 2014) and it was also found to be involved in sudden infant death syndrome (Zorgani et al. 1999). Thus, these structurally related toxins differ in their mode of action/biological properties also.

As a potential gastrointestinal toxin, SEC is involved in foremost foodborne outbreaks and the gene *sec* is very frequently encountered in myriad disease cases (Mizukane et al. 1998; Normanno et al. 2005). Particularly, *S. aureus* strains isolated from animals affected with mastitis produced SEC as a major toxin (Da Silva et al. 2005; Chu et al. 2012). However, information about the involvement of particular subtype is not available in majority of suspected cases. Although these subtypes and their respective genes were described in the middle of 1980s, their incidence in any foodborne outbreak is rarely reported may be due to lack of reliable detection systems which can detect and discriminate subtypes. Furthermore, SEC is commonly isolated from human and food sources and sequence heterogeneity among subtypes is associated with their survival in respective host (Bohach and Schlievert 1989; Marr et al. 1993). This suggests that, investigation of a specific subtype coupled with differentiation enables one to anticipate host specificity and possible consequences of particular toxin.

Commercial immunoassay kits such as RIDASCREEN, SET-RPLA, SET-EIA, VIDAS, and TECRA, though available for detection of SEA-SEE toxins, are unable to detect SEC subtypes. Further, immunoassay-based detection approaches have been reported for the detection of SEC1 and SEC2 (Gao et al. 2000; Dong et al. 2001; Hun and Zhujun 2007; Huang et al. 2015). But the methods usually identify only single subtype at a time. Previous attempts for molecular based detection of subtype-specific SEC include application of PCR and PCR-RFLP (Chen et al. 2001; Hsiao et al. 2003; Wang et al. 2003)

which necessitates post-PCR methods such as gel electrophoresis that involve carcinogenic reagents for result interpretation, time-consuming and may often lead to carry over contamination. Further in available methods, differentiation is not possible in a single reaction.

In this article, we report our study on the development of an SYBR Green-based RT-PCR followed by melt curve analysis to differentiate *entC1* from *entC2/entC3* using a single set of unlabelled primers. N-terminal sequence showing significant sequence variation/heterogeneity was opted, and a suitable primer pair was designed which can amplify all three subtypes. Application of the developed RT-PCR aided simple, rapid, and real-time detection and differentiation of *entC1* from *entC2/entC3* in a single, closed tube format.

Materials and methods

Bacterial strains and cultivation

S. aureus and other non-staphylococcal bacterial reference strains used in this study are listed in Table 1. In addition, 91 *S. aureus* strains isolated from various food and clinical sources were also used in the study (Table 3). These isolates were biochemically confirmed using tests that are specific to *S. aureus* in our previous study (Nagaraj et al. 2014). The bacterial strains were grown in Brain Heart Infusion (BHI) broth (HiMedia, Mumbai, India) for DNA extraction. Briefly, one loopful of bacterial culture was inoculated into 5 ml BHI broth and incubated in 37 °C shaking incubator for 18–24 h. For enterotoxin production, *S. aureus* strains were grown in Tryptic Soy Broth (TSB) (HiMedia, Mumbai, India) for 24 h. The cultures were stored as 15 % glycerol stocks at –80 °C for future use.

DNA extraction

Genomic DNA of bacterial strains was extracted by phenol:chloroform liquid-liquid extraction method (Sambrook and Russell 1989) with slight modification. Briefly, cells harvested from an overnight culture in BHI broth were pelleted and resuspended in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 20 µL lysostaphin (Sigma). After incubation at 37 °C for 1 h, 20 µL proteinase K (20 mg/mL) (Sigma) was added followed by incubation at 55 °C to get a clear lysate. This was followed by phenol:chloroform:isoamylalcohol in the ratio of 25:24:1. The aqueous phase collected in the previous step was precipitated by two volumes of absolute alcohol and washed by ice-cold 70 % ethanol. The residual ethanol was dried and DNA pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5). The purity as well as concentration of purified DNA was analyzed using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA).

Table 1 List of staphylococcal and non-staphylococcal reference strains used in the study

Bacterial strains	Source	RT-PCR with <i>entC</i> primers	
		(T_m 76 °C) <i>entC1</i>	(T_m 82 °C) <i>entC2/entC3</i>
<i>S. aureus</i>	ATCC 700699	–	+
<i>S. aureus</i>	ATCC 19095	+	–
<i>S. aureus</i>	FRI 361	–	+
<i>Bacillus cereus</i>	ATCC 10876	–	–
<i>Burkholderia pseudomallei</i>	NCTC 10274	–	–
<i>Clostridium perfringens</i>	Isolate	–	–
<i>E. coli</i>	ATCC 10536	–	–
<i>Enterococcus</i> spp.	Isolate	–	–
<i>Klebsiella pneumoniae</i>	ATCC 13883	–	–
<i>Lactobacillus</i> spp.	Isolate	–	–
<i>Listeria monocytogenes</i>	ATCC 19114	–	–
<i>Proteus vulgaris</i>	ATCC 33420	–	–
<i>Pseudomonas</i> spp.	Isolate	–	–
<i>Salmonella paratyphi A</i>	ATCC 9150	–	–
<i>Shigella flexneri</i>	ATCC 9199	–	–
<i>Streptococcus</i> spp.	Isolate	–	–
<i>Yersinia enterocolitica</i>	ATCC 23715	–	–
<i>Vibrio parahaemolyticus</i>	ATCC 17802	–	–

ATCC American Type Collection Centre, Rockville, USA; FRI Food Research Institute, University of Wisconsin, USA; NCTC National Collection of Type Cultures, Salisbury, UK

In silico analysis of *entC1*, *entC2*, and *entC3* gene sequence

Clustal Omega, a multiple sequence alignment tool available on EMBL-EBI website (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) was used for the conservation and variation analysis of *entC1* (Accession No. X05815.1), *entC2* (Accession No. GQ461752.1), and *entC3* (Accession No. X51661.1) genes. N-terminal sequence where the subtypes show many SNPs was considered to differentiate the subtypes.

RT-PCR primer design

Based on in silico analysis, a small sequence of 125-bp length was selected to facilitate effectual amplification in RT-PCR. A single set of primer (*entC*) was designed for the amplification of selected region using the conserved region of all three variants, so that the primer could amplify *entC1*, *entC2*, and *entC3* (Table 2). Gene Runner version 3.01 software (Hastings Software Inc. Hastings on Hudson, NY, USA) was employed for primer designing.

Conventional PCR and sequencing analysis

A conventional singleplex PCR was performed to ensure the ability of *entC* primers to amplify all three subtypes. DNA from the standard strains ATCC 19095 (SEC1), FRI 361 (SEC2), and ATCC 700699 (SEC3) were used as reference

templates for respective subtype specific gene amplification. The PCR was achieved in 20 μ L reaction mixture with 1 \times PCR buffer, 2.0 mM MgCl₂, 0.2 mM dNTPs (Fermentas GMBH, Saint Leon-Rot, Germany), 0.6 μ M primers, 50 ng of template DNA, and 1.25 U *Taq* polymerase (Sigma). The amplification conditions included an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 10 s, and extension at 72 °C for 10 s. This was followed by a final extension at 72 °C for 5 min. Thermal cycling was carried out in Master Cycler-Pro thermal cycler (Eppendorf, Hamburg, Germany). The PCR product was resolved on 1.5 % agarose (Seakem LE) gel in 0.5 \times Tris-borate EDTA buffer containing 0.5 μ g/mL ethidium bromide at 150 V for 15 min. The amplicons were examined and documented using GelDoc system (Syngene, UK). The amplicons were purified by NucleoSpin, PCR clean-up, and gel extraction kit (Macherey-Nagel, Duren, Germany) and sequenced at Pentavalent Biosciences Pvt. Ltd., Bangalore. The sequencing result was analyzed by BLAST program.

RT-PCR conditions

Genomic DNA from ATCC 19095, FRI 361, and ATCC 700699 were used as reference template to optimize RT-PCR for *entC1*, *entC2*, and *entC3*, respectively. Each PCR assay was performed in 20 μ L final volumes containing 1 \times

Table 2 List of primers used in the study

Target gene	Primer sequence (5'–3')	Accession no.	Source	Amplicon size (bp)
<i>sec</i>	F - CCACCTTGATAATGGGAACTTAC R - GATTGGTCAAACCTTATCGCCTGG	AB084256.1	Schmitz et al. 1998	270
<i>entC</i>	F - GAGAGTCAACCAGACCCTA R - TCTACAGACATAACTTTA	GQ461752.1	This study	125
ENTC1	F - ACAGAGTTATTAATGAAGG R - ATCATAACCAAAAAGTATTGC	–	Chen et al. 2001	402
ENTC2	F - GTATCAGCAACTAAAGTTAT R - ATCATAACCAAAAAGTATTGC	–	Chen et al. 2001	501
ENTC3	F - AAGAGATTATTTATTTACAGT R - ATCATAACCAAAAAGTATTGC	–	Chen et al. 2001	672

SYBR Green PCR master mix (TaKaRa), 600 nM of *entC* primers, and 10 ng of respective reference DNA templates. Cycling was performed as follows: initial denaturation step at 98 °C for 2 min, 40 cycles of 98 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s. The fluorescence acquisition was done at the end of each extension step. Finally, melt curve analysis was performed by cooling the PCR products to 70 °C followed by heating at 95 °C at a rate of 0.5 °C per second with data acquisition every 1 s. Reaction containing all components except DNA was used as no template control (NTC). RT-PCR assays were performed on a fluorometric thermal cycler CFX 96™ Real-Time PCR Detection System. The data were collected and processed using the software Bio-Rad CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc., Hercules, CA). The threshold cycle (C_T) was defined as the cycle number at which fluorescence of the sample crossed threshold point. Melt curves were later converted into melt peaks (T_m) based on initial fluorescence curves (RFU/T) by plotting negative derivative of fluorescence versus temperature ($-d(\text{RFU})/dT$ versus T). The T_m value was defined as the peak of the melt curve. The assay was performed in duplicates for reproducibility.

Sensitivity

The sensitivity of the developed assay was determined using purified genomic DNA as well as culture from bacterial strain ATCC 19095. In case of purified genomic DNA, 10-fold serially diluted template DNA ranging from 10 ng to 100 fg was subjected to RT-PCR and analyzed for melt curve peaks. In case of bacterial cell load, target bacteria were grown overnight at 37 °C in BHI broth, turbidity was adjusted to 0.5 McFarland standard followed by 10-fold serial dilutions from 10^6 to 10^0 CFU/mL. Template was prepared from each dilution by boil-lysate technique (Theron et al. 2000) and 1 μ L of obtained template was used to perform RT-PCR as mentioned earlier. Parallely, 100 μ L aliquot from each dilution was spread on plate count agar (HiMedia, Mumbai, India) plate and incubated

at 37 °C for overnight and the grown bacterial colonies were counted in order to determine the bacterial load. In both cases, an NTC was also included in the reaction. Based on the C_T values obtained in RT-PCR, limit of detection was determined which is defined as the least concentration of DNA where detectable fluorescence was achieved. The experiment was performed in duplicates for reproducibility.

Reliability

For determination of specificity of the developed assay, staphylococcal as well as non-staphylococcal bacterial strains listed in Tables 1 and 3 were used. We investigated 91 *S. aureus* strains isolated from various food and clinical samples by RT-PCR to detect the presence of subtype specific genes and differentiate among them based on melt curve analysis. The clinical isolates (CI) were obtained from diarrhea patients with suspected foodborne cases. Briefly, genomic DNA was extracted from bacterial strains and 10 ng of DNA was used as template to carry out RT-PCR as mentioned earlier. Parallely, the assay was compared with PCR tests for the presence of *sec* as well as subtype specific genes (*entC1*, *entC2*, and *entC3*) using reported primers (Schmitz et al. 1998; Chen et al. 2001) (Table 2). Additionally, immunoassay was also performed with a commercial kit for the production of SEC toxin. The reaction volume of conventional PCR was 20 μ L containing 1 \times PCR buffer (Sigma), 2.0 mM MgCl_2 , 0.2 mM dNTPs (Fermentas GMBH, Saint Leon-Rot, Germany), 1 μ M primer, 50 ng of template DNA, and 1.25 U *Taq* polymerase (Sigma). The amplification conditions included an initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 30 s. This was followed by a final extension at 72 °C for 5 min. Thermal cycling and gel analysis were performed as mentioned earlier. Tecra™ Staph Enterotoxin Visual Immunoassay kit (TECRA International Pvt. Ltd., Frenchs Forest, NSW, Australia) was used to detect the enterotoxin production and Tecra™ Staph Enterotoxins ID Test was used

Table 3 Detection of SEC-positive *S. aureus* strains by conventional PCR, RT-PCR, and immunoassays

Sl. no.	<i>S. aureus</i> strains	SEC strains identified by conventional PCR	SEC strains identified by Tecra™ kit	Subtyping by PCR using reported primers			Subtyping by RT-PCR using <i>entC</i> primers	
				ENTC1	ENTC2	ENTC3	(T_m 76 °C) <i>entC1</i>	(T_m 82 °C) <i>entC2/entC3</i>
1	ATCC 700699	+	+	-	+	-	-	+
2	ATCC 19095	+	+	+	-	-	+	-
3	FRI 361	+	+	-	-	+	-	+
4	DI-1	-	-	-	-	-	-	-
5	DI-2	+	+	-	-	+	-	+
6	DI-3	-	-	-	-	-	-	-
7	DI-4	+	+	+	-	-	+	-
8	DI-5	+	+	-	-	+	-	+
9	DI-6	+	+	-	-	-	-	-
10	DI-7	+	+	-	-	-	-	-
11	DI-8	+	+	+	-	-	+	-
12	DI-9	+	+	+	-	-	+	-
13	DI-10	+	+	-	-	-	-	-
14	DI-11	+	+	-	+	-	-	+
15	DI-12	+	+	-	-	+	-	+
16	DI-13	+	+	+	-	-	+	-
17	DI-14	+	+	-	-	-	-	-
18	DI-15	+	+	-	-	-	-	-
19	CI-1	+	+	-	-	+	-	+
20	CI-2	+	+	+	-	-	+	-
21	CI-3	-	-	-	-	-	-	-
22	CI-4	-	-	-	-	-	-	-
23	CI-5	+	+	+	-	-	+	-
24	CI-6	-	-	-	-	-	-	-
25	CI-7	+	+	-	-	+	-	+
26	CI-8	+	+	-	-	-	-	-
27	CI-9	+	+	-	-	-	-	-
28	CI-10	+	+	-	-	-	-	-
29	CI-11	+	+	-	-	+	-	+
30	CI-12	-	-	-	-	-	-	-
31	CI-13	+	+	-	+	-	-	+
32	CI-14	+	+	-	-	+	-	+
33	CI-15	+	+	-	-	-	-	-
34	CI-16	-	-	-	-	-	-	-
35	CI-17	-	-	-	-	-	-	-
36	CI-18	-	-	-	-	-	-	-
37	CI-19	-	-	-	-	-	-	-
38	CI-20	+	+	-	-	+	-	+
39	CI-21	-	-	-	-	-	-	-
40	CI-22	+	+	-	-	+	-	+
41	CI-23	-	-	-	-	-	-	-
42	CI-24	+	+	-	-	-	-	-
43	CI-25	+	+	-	-	-	-	-
44	CI-26	-	-	-	-	-	-	-
45	FI-1	+	+	+	-	-	+	-
46	FI-2	+	+	+	-	-	+	-
47	FI-3	+	+	+	-	-	+	-
48	FI-4	-	-	-	-	-	-	-
49	FI-5	+	+	-	-	-	-	-
50	FI-6	-	-	-	-	-	-	-
51	FI-7	+	+	-	-	+	-	+
52	FI-8	+	+	-	+	-	-	+
53	FI-9	+	+	-	+	-	-	+
54	FI-10	-	-	-	-	-	-	-
55	FI-11	-	-	-	-	-	-	-
56	FI-12	+	+	-	-	+	-	+
57	FI-13	-	-	-	-	-	-	-
58	FI-14	-	-	-	-	-	-	-
59	FI-15	-	-	-	-	-	-	-
60	FI-16	-	-	-	-	-	-	-
61	FI-17	-	-	-	-	-	-	-

Table 3 (continued)

Sl. no.	<i>S. aureus</i> strains	SEC strains identified by conventional PCR	SEC strains identified by Tecra™ kit	Subtyping by PCR using reported primers			Subtyping by RT-PCR using <i>entC</i> primers	
				ENTC1	ENTC2	ENTC3	(<i>T_m</i> 76 °C) <i>entC1</i>	(<i>T_m</i> 82 °C) <i>entC2/entC3</i>
62	FI-18	–	–	–	–	–	–	–
63	FI-19	–	–	–	–	–	–	–
64	FI-20	–	–	–	–	–	–	–
65	FI-21	–	–	–	–	–	–	–
66	FI-22	–	–	–	–	–	–	–
67	FI-23	–	–	–	–	–	–	–
68	FI-24	+	+	–	–	–	–	–
69	FI-25	+	+	–	+	–	–	+
70	FI-26	–	–	–	–	–	–	–
71	FI-27	+	+	–	–	+	–	+
72	FI-28	–	–	–	–	–	–	–
73	FI-29	–	–	–	–	–	–	–
74	FI-30	–	–	–	–	–	–	–
75	FI-31	+	+	–	–	+	–	+
76	FI-32	+	+	–	–	–	–	–
77	FI-33	+	+	+	–	–	+	–
78	FI-34	+	+	–	–	–	–	–
79	FI-35	–	–	–	–	–	–	–
80	FI-36	+	+	–	–	–	–	–
81	FI-37	+	+	–	–	+	–	+
82	FI-38	+	+	–	–	+	–	+
83	FI-39	+	+	–	–	+	–	+
84	FI-40	+	+	–	–	+	–	+
85	FI-41	–	–	–	–	–	–	–
86	FI-42	+	+	+	–	–	+	–
87	FI-43	+	+	+	–	–	+	–
88	FI-44	+	+	–	–	–	–	–
89	FI-45	–	–	–	–	–	–	–
90	FI-46	+	+	–	–	+	–	+
91	FI-47	+	+	–	–	+	–	+
92	FI-48	+	+	–	+	–	–	+
93	FI-49	+	+	–	–	+	–	+
94	FI-50	+	+	–	+	–	–	+

ATCC American Type Collection Centre, Rockville, USA; FRI Food Research Institute, University of Wisconsin, USA; DI Dharwad isolates, SDM College, Dharwad, Karnataka; CI clinical isolates; FI food isolates

to identify specific type of the toxin (SEA, SEB, SEC, SED, and SEE) following the manufacturer's instructions.

Naturally contaminated samples

In order to evaluate the real-time application of RT-PCR to detect and differentiate target genes, numerous ($n = 89$) naturally contaminated samples were tested. Samples were processed and template was prepared as per Nagaraj et al. (2014) following pre-enrichment. Briefly, 10 g of each sample was homogenized and transferred to 90 mL modified MSB containing 0.4 % sodium pyruvate. Following an overnight enrichment, 1 mL aliquot of sample was processed by boil-lysate technique for conventional PCR to assess the presence of *sec* and RT-PCR for the detection of *entC1/entC2/entC3*. Concurrently, same samples were subjected to immunoassay using Tecra™ kit following the manufacturer's instructions.

Result

Sequence analysis and primer designing

The major objective of the study was to identify polymorphic sequence and to design a single primer pair that could amplify and differentiate all three subtypes. Due to availability of partial gene sequence of *entC2* in NCBI database (81 bases are not available towards N-terminal with respect to *entC1* and *entC3* sequences) (Fig. S1), Clustal Omega multiple sequence alignment was considered from 82nd position so that the alignment contained sequence from all three subtypes. Thus, performed in silico comparative analysis of the partial nucleotide sequences of *entC1*, *entC2*, and *entC3* revealed that the bases are highly conserved in the C-terminal region and divergence in a short stretch was found near N-terminal region. To explain,

though at nucleotide level *entC1* shares 97.9 % homology with *entC2* and 97.4 % homology with *entC3*, Clustal Omega multiple sequence analysis revealed the presence of 19 SNPs among three variants (from 82nd position) (Table 4). Of the identified SNPs, 15 were closely located between 87 and 197 position of nucleotides, whereas four SNPs were distantly located at 255, 296, 402, and 478 positions on *entC1* (Table 4). On the other hand, overall, only five SNPs were documented between *entC2* and *entC3* variants and were found to be individually dispersed throughout the gene stretch (Fig. S1). Accordingly, a 125-bp region of *entC1*, *entC2*, and *entC3* was selected for primer designing (Fig. 1). Since the aim was to design a single set of primers to amplify all three subtypes, care was taken to include all possible SNPs within the selected 125-bp region. As a result, within the selected region, all 15 closely located SNPs were present in case of *entC1*, whereas only three of five SNPs in case of *entC2* and *entC3* (Fig. 1). Thus, designed pair of primer was comprised of a 19-mer forward primer spanning from 82 to 100 positions and an 18-mer reverse primer spanning from 189 to 206 positions with respect to *entC1* sequence. The forward primer was 100 % identical in case of *entC2* and *entC3*, but possessed an SNP at 6th position from 5' end in case of *entC1*. Similarly, in case of reverse primer, all three subtypes had an SNP at 9th position from 5' end. However, these SNPs were found to be highly conserved in case of each subtype.

Conventional PCR

To assess the efficiency of designed primer to hybridize and amplify all three subtypes, DNA from respective *S. aureus* reference strains was amplified by conventional PCR and the products were sequenced. The primer could amplify all three subtypes with a band length of 125 bp as interpreted by gel electrophoresis, irrespective of different templates (Fig. 2b). Further, BLAST analysis of sequencing results confirmed the presence of respective nucleotide sequences with conserved and polymorphic regions specific to each subtype (data not shown).

RT-PCR

RT-PCR was carried out using *entC* primers with three different subtype-specific DNA as templates. Post-PCR melt curve analysis revealed that two distinct melt peaks were obtained even though a single primer pair was used for amplification. *entC1* showed peak at 76 °C (Fig. 3a) whereas *entC2* and *entC3* showed peak at 82 °C (Fig. 3b). Also, a primer dimer was observed as a low melt peak at around 70 °C that could be distinguished from the intended product since they melt at a lower temperature (Fig. 3).

Detection limit

Various DNA concentrations and cell load were tested. The C_T values increased with decreasing DNA concentration and the limit of detection was obtained at a concentration of 100 fg of template (Fig. 4), and the result was found to be reproducible. Similarly the RT-PCR could detect 10 CFU/mL of bacterial cell load (data not shown).

Specificity of the assay

The assay was found to be specific to amplify DNA from staphylococcal strains only. There was no amplification plot or increased fluorescence observed in presence of non-staphylococcal DNA. Further, RT-PCR generated amplification plot only with SEC *S. aureus* strains which showed positive result for conventional PCR and Tecra™ immunoassay kit as well. Of the 91 isolates, 56 were found to be positive for conventional PCR with an amplicon of 270-bp length (Fig. 2a) and also for SEC toxin. These *sec* positive isolates showed positive result for RT-PCR also (*entC* primers) along with subtype-specific differentiation (Table 3). The RT-PCR results obtained were compared with conventional PCR results derived from validated subtype-specific primers. Among the 91 isolates tested, 12 isolates yielded distinct melt peaks at 76 °C corresponding to *entC1*, indicating that they were SEC1-positive strains. These isolates showed amplification for ENT1 primers also. On the other hand, 27 among the 91 isolates yielded melt peaks at 82 °C corresponding to *entC2/entC3*. But when PCR was performed using validated subtype-specific primers for these isolates, 7 strains were found positive for *entC2* and 20 were found positive for *entC3* genes. The results obtained indicated the efficiency of developed assay to differentiate SEC1 from *entC2/entC3* and also its inadequacy to differentiate between C2 and C3 subtypes. The remaining SEC-positive strains (17 strains) among 56 isolates could belong to other SEC subtypes such as SEC_{bovine}, SEC_{canine}, and SEC_{ovine}.

Evaluation

Among 89 different naturally contaminated samples tested, 11 samples were found to be positive for SEC as analyzed by conventional PCR and Tecra™ kit. Of these, the developed RT-PCR was able to detect three and seven samples as *entC1* and *entC2/entC3* positive respectively based on melt curve analysis. Thus, results obtained highlighted the application of developed assay directly on naturally contaminated samples (Table 5).

Table 4 Position of SNPs on SEC subtype nucleotide sequences

SEC subtypes	SNP position among subtypes (with respect to <i>entC1</i> and <i>entC2</i> database sequences)																		
	87	101	111	121	123	127	129	132	139	140	146	147	157	158	197	255	296	402	478
<i>entC1</i> ^a	<u>C</u>	C	G	G	G	A	A	C	T	T	A	A	G	T	<u>A</u>	^b g	g	c	a
<i>entC2</i>	<u>T</u>	C	G	T	A	G	G	T	A	C	G	T	T	A	<u>T</u>	a	a	t	a
<i>entC3</i>	<u>T</u>	T	T	T	A	G	G	T	A	C	G	T	T	A	<u>A</u>	g	a	t	g

Underlined bases represent SNPs in the forward and reverse primer sequences

^a Bases inside the box (capitalized letters) represent closely located SNPs included in selected 125-bp gene stretch

^b Bases outside the box (small letters) indicate the distant SNPs which are not included for RT-PCR amplification

Discussion

During the past decade, RT-PCR has been established as a sensitive detection method in the field of molecular diagnostics (Beuret 2004; Richards et al. 2004). SYBR Green chemistry combined with melt curve analysis has revolutionized the application of RT-PCR by reducing the cost since expensive probes are not needed and yet retain the sensitivity (Varga and James 2005; Hosmillo et al. 2010). The method is simple, rapid, reproducible, and easy to perform as it requires no post-PCR steps such as gel electrophoresis which often entail carcinogenic reagents. Contamination is also precluded in the method since protocol and result interpretation can be done in a closed reaction tube.

Staphylococcal enterotoxin C is known to have highly conserved homologous and immunologically cross-reactive

subtype variants. Of these, SEC1, SEC2, and SEC3 toxins are known to possess a high degree of similarity and there is a pressing need for a highly specific detection tool that would sensitively identify and differentiate these subtypes. The high degree of homology in terms of nucleotide as well as amino acid sequences stands as a major drawback in this case. Though some of the immunological assay-based methods are available and are potent of differentiating subtypes based on specific epitopes, the presence of the toxin at detectable concentration in the analyte is a pre-requisite. Thus, the possibility of false-negative results in these cases (absence of toxin but presence of enterotoxigenic strain) may not be neglected. As an alternative strategy, we attempted to aptly utilize the technique of SYBR Green-based RT-PCR followed by melt curve analysis to specifically and differentially detect the formerly mentioned subtypes.

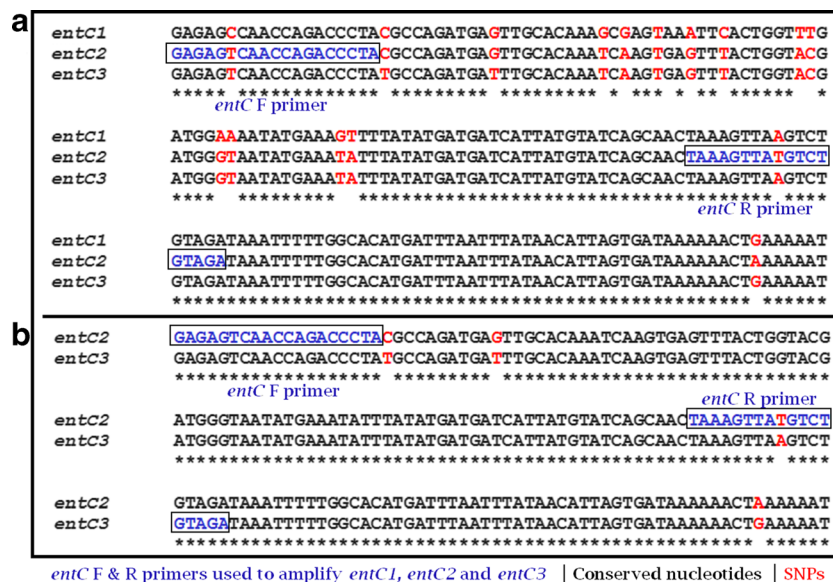


Fig. 1 The Clustal Omega multiple sequence alignment data showing conserved and polymorphic regions between SEC subtype nucleotide sequences. The sequence alignment was color coded for easy identification (blue = *entC* primer sequence, black = identical, red = SNPs). Alignment

was considered from 82nd position with respect to *entC1* and *entC2* database sequences. **a** Alignment result between *entC1*, *entC2*, and *entC3*. **b** Alignment result between *entC2* and *entC3*

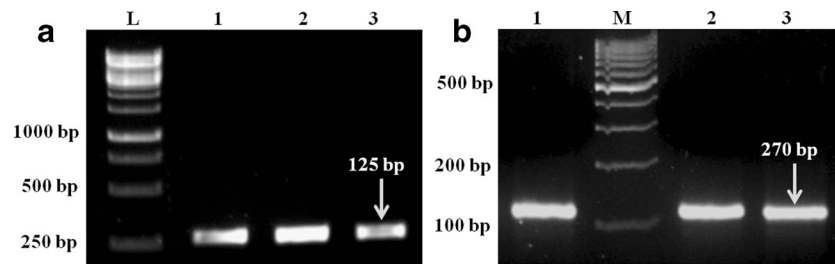


Fig. 2 An example of PCR amplification using primers employed in the present study. **a** Agarose gel electrophoresis showing 270-bp product amplified using *sec* specific primers. **b** Agarose gel electrophoresis showing 125-bp product amplified using *entC* primers. Lanes: 1, DNA

from *S. aureus* ATCC 19095 (SEC1); 2, DNA from *S. aureus* FRI 361 (SEC2); 3, DNA from *S. aureus* ATCC 700699 (SEC3); L, standard 1-kb ladder; M, standard 100-bp DNA ladder

The major objective of the study was to determine regions of variation/heterogeneity among *entC1*, *entC2*, and *entC3* and employ the promising variation/heterogeneity in differentiation of the three toxin subtypes using SYBR Green-based RT-PCR combined with melt curve analysis. This was challenging, due to the high degree of homology exhibited by these variants. With the aid of multiple sequence alignment, highly conserved and gene-specific SNPs among the three subtypes were identified. Based on these results, the work was designed to develop a single novel primer pair that amplifies the gene stretch of all three variants comprising of SNPs followed by melt curve analysis. Since shape and peak location of the melt curve varies with GC/AT ratio, length, and sequence of the fragment, melt peaks will be generated at different temperatures, thus allowing differentiation of closely related amplicons based on melt curve (Ririe et al. 1997; Wehrle et al. 2009). The same strategy was followed to achieve subtype differentiation.

Taking into consideration that short amplicons yield the most consistent results in RT-PCR technique, a 125-bp region with considerable variation/heterogeneity was

selected for primer designing. Thus, designed primer pair was found to encompass one or two SNPs near to 5' end in both forward and reverse primer sequences. Though studies have reported the detrimental effects of primer mismatches in terms of reduced sensitivity and efficiency of PCR, the effect is more when the mismatch is towards 3' end (Guy et al. 2004; Bru et al. 2008). However, in our study, mismatch was away from 3' end (6th position in forward primer and 9th position in reverse primer). Hence, when amplification was carried out, the oligos were able to hybridize and amplify all three subtypes regardless of SNPs as confirmed further by sequencing analysis. But, a difference of two logs was observed in C_T value (loss of sensitivity) even when equal concentration of template DNA was used for amplification (Fig. S2). Optimization of RT-PCR assay was successful using the designed primer pair. Post-RT-PCR melt curve analysis revealed that, based on the melt peaks, *entC1* was successfully differentiable from *entC2/entC3*. However, with dispersed and limited number of SNPs within the selected 125-bp gene stretch in the case of *entC2* and *entC3*, both genes resulted in identical melt

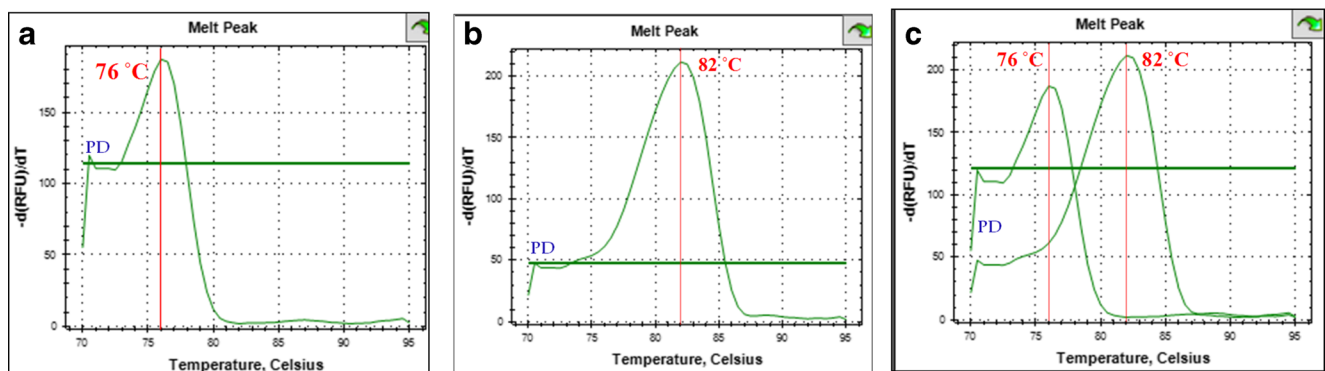


Fig. 3 Fluorescence melt curves of SEC1 and SEC2 subtypes with distinct melt peaks after SYBR Green RT-PCR amplification using *entC* primers. PD—a non-specific peak at relatively low temperature (around 70 °C) represents primer dimer. **a** Characteristic melt peak at 76 °C in presence of DNA from SEC1-positive *S. aureus* ATCC 19095 strain. **b**

Characteristic melt peak at 82 °C in presence of DNA from SEC2-positive *S. aureus* FRI 361. Similar peak was obtained for SEC3-positive *S. aureus* ATCC 700699 strain. **c** Comparison of C1 and C2 peaks by overlapping results obtained

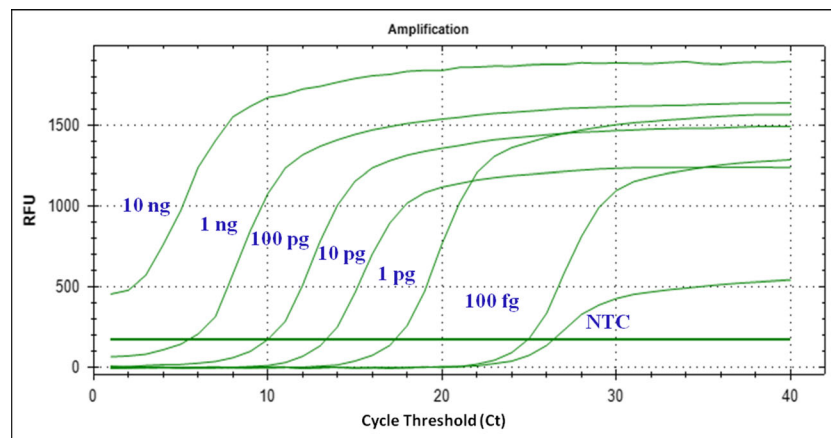


Fig. 4 Determination of sensitivity of RT-PCR in presence of different concentrations of DNA from *S. aureus* strain ATCC19095 using *entC* primers. NTC represents no template control containing all PCR components except DNA. Tenfold serial dilutions of DNA were used

ranging from 10 ng to 100 fg. The C_T values increased with decreasing DNA concentration and the limit of detection was obtained at a concentration of 100 fg of template DNA

peak. Hence, the developed RT-PCR coupled with melt curve analysis failed to distinguish between *entC2* and *entC3*. Further, since priority was given to design a single primer pair containing maximum number of SNPs, parameters such as primer dimer formation was not taken into consideration. As a result, a small non-specific peak was observed in melt curve analysis. However, since it melts at a low temperature than that of actual amplicon, it was clearly distinguished from the intended product and thus could be ignored.

According to the best of our knowledge, this is the first report of unambiguously discriminating *entC1* from *entC2/entC3* using a single primer pair. However, due to the high degree of homology, the developed assay was inadequate to discriminate between *entC2* and *entC3* genes. Perhaps, sensitive methods such as high-resolution melt (HRM) curve analysis can be exploited coupled with the developed assay to utilize the limited number of SNPs encountered between *entC2* and *entC3* and thus achieve reliable differentiation. High detection

sensitivity was achieved which enable amplification even in presence of low number of DNA copies/bacterial cell load. Thus, in addition to rapid and sensitive detection of *sec* in any sample, the assay developed facilitates molecular discrimination of its subtypes in epidemiological surveillance of type C staphylococcal enterotoxin relevant to food poisoning cases. Moreover, the differentiation ability on naturally contaminated samples emphasized its real-time application on naturally contaminated food samples. With the benefit of short amplicon size, the assay protocol and result interpretation requires minimal time. To conclude, the SYBR Green-based real-time PCR assay described in this study can be implemented as an excellent tool for rapid, sensitive detection and differentiation of *entC1* from *entC2/entC3* with a single primer pair using inexpensive RT-PCR approach. The assay needs to be evaluated for its detection potential from different sample analytes so as to understand the possibility of employing the method in the diagnosis of SEC-mediated food poisoning cases.

Table 5 Detection of SEC and its subtypes in natural samples

Type of sample	No. of samples positive for <i>sec</i> using conventional PCR	No. of samples positive for SEC using Tecra™ kit	No. of samples positive for RT-PCR	
			(T_m 76 °C) <i>entC1</i>	(T_m 82 °C) <i>entC2/entC3</i>
Milk ($n = 28$)	5	5	2	3
Cheese ($n = 22$)	3	3	–	2
Ice cream ($n = 15$)	2	2	1	1
Chicken ($n = 12$)	1	1	–	1
Mutton ($n = 12$)	–	–	–	–
Total ($n = 89$)	11	11	3	7

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement This article does not contain any studies with human participants or animals performed by any of the authors.

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