Characterization of enterotoxin A-producing Staphylococcus aureus

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This article was published in the following Dove Press journal:
Infection and Drug Resistance

Purpose: This study aims to characterize the wild-type staphylococcal enterotoxin A (SEA)-producing Staphylococcus aureus.

Materials and methods: We identified 29 wild-type sea-positive S. aureus isolates from dairy and meat samples, as well as from patients, measured the amount of SEA produced under favorable cultivation conditions using enzyme-linked immunosorbent assay and sea mRNA transcriptional level and investigated the phage type as well as genetic diversity by means of pulsed-field gel electrophoresis and multilocus sequence typing.

Results: Among 29 sea-positive isolates, 22 were from food sources (including one outbreak case) and seven from clinical patients. Five enterotoxin gene profiles, namely, sea (14 isolates), sea+sec (9 isolates), sea+seb (4 isolates), sea+seb+sec (1 isolate) and sea+seb+sed (1 isolate), were identified. Multilocus sequence typing generated sequence type (ST)1 (13 isolates), ST6 (5 isolates), ST59 (3 isolates), ST239 (3 isolates), ST5 (2 isolates), ST188 (2 isolate) and ST15 (1 isolate). The amount of SEA per 108 colony-forming unit (CFU) after 24 h of incubation was 1.1–33.5 (mean, 8.74; SD, 7.7) ng/108 CFU. The amount of SEA per hour incubation in the log growth phase was 0.1–12.0 (mean, 2.37; SD, 3.06) ng/108 CFU. Overall, 54.2% of SEA was produced in the log growth phase. Both the transcriptional level of sea mRNA and the amount of SEA in the log growth phase correlated well with the amount of SEA after 24 h of cultivation. Four isolates, namely, SA-212, SA-217, SA-340 and SA-341, were categorized to be of high SEA production (877–1,109 ng/mL, 24 h). The total amount of SEA was mainly based on the amount of SEA in 108 CFU, not the relatively fixed bacterial cell counts (21.1–43×108 CFU/mL). Seven isolates from patients all carried the ФMu3A phage, whereas 21 of the 22 isolates from the environmental sources all carried the ФSa3ms phage.

Conclusion: The present study exhibits varied SEA production capacity of the wild sea-positive S. aureus strains. An apparent boundary in phage types between strains from the clinical samples and strains from the environment was also identified.

Keywords: Staphylococcus aureus, staphylococcal enterotoxin A, multilocus sequence typing, phage, SEA production capacity

Introduction

Staphylococcal food poisoning (SFP) typically exhibits sudden onset of nausea, violent vomiting, abdominal cramps and sometimes diarrhea. It is one of the most common forms of bacterial foodborne outbreaks worldwide and has been reported to be the fourth most common type of foodborne outbreaks in Europe in 2011.1 The major causative agents of SFP are staphylococcal enterotoxins (SEs). SEs are often produced in food contaminated with enterotoxin-producing Staphylococcus aureus (S. aureus) due to improper handling and storage. Classical SEs are classified into five types, namely, staphylococcal entero-
toxin A (SEA), SEB, SEC,SED and SEE, by the serological method. Meanwhile, 23 SE types are known, but it is not yet known how relevant some of these are for SFPs. Up to now, commercial assays only measure types A–E. The five classical SEs contribute to 95% of the SFP outbreaks. In particular, SEA is viewed to be most frequently associated with SFP and is detected in >50% of relevant outbreaks. Previous studies have proved that the minimal toxic dose of SEA for meat in standard strains is available.11

A number of experimental studies have been carried out to characterize the diverse SE gene profiles in strains from various food sources and have found that over a half of the S. aureus isolates from meat or milk are enterotoxigenic.9,10 Despite ubiquitous presence of S. aureus in the environment, whether an SFP will occur is determined largely by enterotoxin levels in contaminated food. However, studies exploring the differential enterotoxin production ability in enterotoxin levels in contaminated food. However, studies exploring the differential enterotoxin production ability in wild-type S. aureus strains are scant. Only limited knowledge on impacts of temperature, lactic acid and processed pork meat in standard strains is available.11

To investigate the SEA production capacity of S. aureus isolates harboring the sea gene, we identified 29 wild-type S. aureus isolates that were isolated from dairy and meat samples, as well as from patients, measured the amount of SEA produced in the log growth phase and after 24 h of incubation under favorable cultivation conditions using enzyme-linked immunosorbent assay (ELISA) and relevant transcriptional level of sea mRNA and investigated the phage types, as well as genetic diversity by means of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Materials and methods

**Staphylococcus aureus isolates**

The S. aureus isolates used in this study were recovered from SFP outbreaks, dairy and meat samples as well as from clinical samples as part of the routine hospital laboratory procedure. The isolates were identified using standard laboratory techniques including Gram stain, plasma coagulase test, biochemical reactions by the Vitek 2 Gram-positive identification card (bioMérieux, Marcy l’Etoile, France) and polymerase chain reaction (PCR) detection of the *nuc* gene.12

**DNA extraction and PCR amplification**

Genomic DNA of S. aureus isolates was extracted using bacterial genomic DNA extraction kits (TaKaRa MiniBEST, Ver.3.0; TaKaRa, Kawasaki, Japan). The *sea, seb, sec, sed* and *see* genes were amplified according to the methodologies reported in a previous study. Staphylococcus aureus ATCC25923 and a sea-positive S. aureus proved by sequencing were used as a negative and a positive control for the *sea* gene, respectively. Isolates with the *sea* gene were used to determine the SEA production capacity.

**MLST and PFGE**

MLST was conducted on sea-positive S. aureus isolates by means of amplifying seven house-keeping genes (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*) listed on the S. aureus MLST website (http://saureus.mlst.net/). The allelic profile of a S. aureus isolate appearing as an array of seven allele numbers was derived by sequencing internal fragments of these seven house-keeping genes (Sangon Biotech Company, Shanghai, China). The sequence types (STs) were assigned on the basis of the array of seven allele numbers using the same website.

PFGE was conducted to investigate genetic relatedness of the sea-positive S. aureus isolates according to the PulseNet standardized protocol.13 All image patterns were normalized against the pattern of the *Salmonella* Braenderup H9812 reference standard. Image data were transferred into software BioNumerics 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) and processed to identify clusters of isolates. Dendrograms of the PFGE patterns were constructed using the unweighted pair-group method.

**Culture conditions for the determination of SEA**

Favorable cultivation was done in brain heart infusion (BHI) broth or on BHI agar at 37°C as described previously with some modifications.11 S. aureus isolates with the *sea* gene were transferred from a stock to BHI agar for overnight incubation, and one colony was inoculated into 10 mL of broth for overnight culture prior to the experiment. Portions of 10 mL culture were inoculated into 50 mL of broth at a final cell concentration of 10⁶ colony-forming unit (CFU)/mL and cultivated with shaking at 150 rpm. Culture was taken at 5 h of growth time from the 50 mL inoculated broth and used as a seed culture for the following assay. Then, the seed culture was inoculated into 50 mL of broth at a final cell concentration of 10⁶ colony-forming unit (CFU)/mL and cultivated with shaking at 150 rpm. Culture was taken at 5 h of growth time from the 50 mL inoculated broth and used as a seed culture for the following assay. 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ELISA for the determination of SEA

The concentration of extracellular SEA was determined for the sea-positive isolates using Ridascreen SET A, B, C, D and E (R-Biopharm, Darmstadt, Germany) following the manufacturer’s instructions. In short, S. aureus liquid cultures were centrifuged at 3,500 × g at 10°C for 10 min. The supernatant was diluted to 1:100, 1:200 and 1:500 (v/v) with 0.85% NaCl solution to ensure that the resulting values were within the detection limit of the ELISA kit. SEA standard (0.1 ng/mL; Toxin Technology Inc., Sarasota, FL, USA) and culture supernatants were added onto the plate (100 μL/well) at appropriate dilutions. The optical density (OD405) of each well was measured using a microplate reader (Multiskan Ascent, Thermo Electron Corporation, Waltham, MA, USA). OD405 values were plotted against SEA concentrations. SEA concentrations for each isolate were calculated from linear regression. Three replicate measurements were obtained to ensure the reliability of results.

Transcriptional level of sea mRNA in the log growth phase

The total RNA of culture in the log growth phase was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the product manual. Pelleted RNA was resuspended in RNA storage solution (TaKaRa). RNA purity and concentration were verified by electrophoresis and OD260/OD280 absorption ratio >1.95 using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was synthesized from the suspended RNA extraction (1 μg) in two separate reverse transcription assays using specific reverse primers for the sea gene and the 16S rRNA gene

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<th>Gene Primers (5’→3’)</th>
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<td>16sRNA-2: GTTTACCGGCGGAATCA</td>
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Table 1 Primers used in this study

Abbreviation: SEA, staphylococcal enterotoxin A.
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Chen et al (accession no. NC_009782) and the end of 1,391 bp-long gene of ФSa3ms (accession no. NC_002953). For each reaction of 50 μL, 25 μL of PCR Master Mix (Promega Corporation, WI, USA), 2 μL of each primer (c=10 μM), 2 μL of DNA template and 21 μL of water were added. PCR cycling conditions included the following: 5 min hot start at 95°C, followed by 30 amplification cycles (denaturation at 95°C for 45 s, annealing at each set of primer-specific annealing temperature for 45 s and elongation at 72°C for 45 s), a final elongation step at 72°C for 10 min and a cooling step. Target-specific amplicons were confirmed by electrophoresis using a 1% agarose gel.

**Results**

A total of 29 wild-type *S. aureus* isolates with the *sea* gene were identified from 168 *S. aureus* isolates. These *sea*-positive isolates comprised 22 from food sources (including one outbreak case) and seven from patient samples (Table 2). The following five SE profiles were identified in 29 isolates: *sea* (14 isolates), *sea*+*sec* (9 isolates), *sea*+*seb* (4 isolates), *sea*+*seb*+*sec* (1 isolate) and *sea*+*seb*+*sed* (1 isolate), revealing 55% of isolates having more than one of five classical SE genes.

Seven STs were identified in 29 isolates, including ST1 (13 isolates), ST6 (5 isolates), ST59 (3 isolates), ST239 (2 isolates), ST188 (2 isolates), ST15 (1 isolate) and ST212 (1 isolate). Table 2 summarizes the SEA production capability of 29 *sea*-positive *Staphylococcus aureus* in terms of amount of SEA per hour in the log growth phase and after 24 h of cultivation.

![Table 2](image)

**Abbreviations:** CFU, colony-forming unit; SE, staphylococcal enterotoxin; SEA, staphylococcal enterotoxin A; SFP, staphylococcal food poisoning; ST, sequence type.
Enterotoxin A-producing Staphylococcus aureus (3 isolates), ST5 (2 isolates), ST188 (2 isolates) and ST15 (1 isolate). The genetic typing results demonstrated a predominant sea-positive ST1 lineage. PFGE also proved a similar cluster around the ST1 lineage. Especially, the only one outbreak-related isolate also belonged to ST1 (Figure 1 and Table 2).

The values of bacterial cell counts after 24 h of incubation showed limited variation, most focusing on 29–39×10^8 CFU/mL. After 6 h of incubation, on the average, bacterial cell counts accounted for 53% (18.26/34.37) of the maximal cell counts of 24 h of incubation (Table 2).

The amount of SEA after 24 h of incubation ranged from 1.1 to 33.5 ng/10^8 CFU, with an average of 8.74 and most values at lower part of the range (Table 2 and Figure 2A). The amount of SEA per hour in the log growth phase ranged from 0.1 to 12.0 ng/10^8 CFU, with an average of 2.37 and most values also at lower part of the range (Table 2 and Figure 2A). Upon comparing the amount of SEA after 24 h of incubation with the amount of SEA of the 2 h log growth phase (amount of SEA per hour [2.37]×2), it was found that 54.2% (4.74/8.74) of SEA was produced in the log growth phase. This value was very close to the proportion (53%) of bacterial cell counts in the log growth phase. Two linear regressions illustrated that both the transcriptional level of sea mRNA and the amount of SEA in the log growth phase correlated well with the 24-h amount of SEA. When arbitrarily setting the 24-h amount of SEA that was over the median value of the amount of SEA range as a cutoff value for high SEA production, four isolates, namely, SA-212, SA-217, SA-340 and SA-341, were categorized to be of high SEA production (Table 2).

![Figure 1 PFGE, MLST and SE profiles of 29 sea-positive Staphylococcus aureus isolates.](image)

**Abbreviations:** MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; SE, sequence type.
As a measure indicating the production speed of SEA, the amount of SEA per hour in the log growth phase correlated well with the concentration of SEA at 24 h (Figure 2A). Similar correlation existed with the SEA expression level in the log growth phase and the amount of SEA at 24 h (Figure 2B). Upon integrating the bacterial cell counts, the amount of SEA in 10⁸ CFU at 24 h and total amount of SEA at 24 h, we could see that the total amount of SEA (y axis in Figure 2C and D) was determined largely by the amount of SEA in 10⁸ CFU at 24 h (x axis in Figure 2C) and almost no apparent correlation with the bacterial cell counts (x axis in Figure 2D).

As to types of phages that carry the sea gene, seven isolates carried the ФMu3A phage and 22 isolates carried the ФSa3ms phage (Table 2). It is important to note that the isolates from patients all carried the ФMu3A phage and 21 of the 22 isolates from environmental sources carried the ФSa3ms phage. This phenomenon generated a distinct boundary of phage type between two types of isolation sources.

Discussion
The present study on the SEA production capacity of wild sea-positive S. aureus isolates yielded the following findings: although all isolates produced SEA at varied levels, isolates with high production capacity accounted for a small proportion of all isolates; production speed in the log growth phase decided the final SEA concentration, which further decided the total amount of SEA; transcriptional level of sea mRNA in the log growth phase could also well reflect the SEA production capacity; and there was an apparent boundary of phage types between isolates from clinical samples and isolates from the environment.

As shown by PFGE and MLST distribution, wild sea-positive S. aureus isolates were found to focus on limited lineages genetically. Isolates of ST1 (n=13) and ST6 (n=5) accounted for most of the environmental isolates (82%, 18/22) (Figure 1 and Table 2). ST6 was previously reported to be the most dominant clone in China. ST5 and ST188 are also among the most found STs in SE-producing strains.
both from foodborne cases and environmental sources.\textsuperscript{19,20} However, the remarkably large proportion of ST1 isolates in this study indicates a clustering trend in such strains. Regarding the hospital isolates in this study, ST239, ST5 and ST59 were most found STs.

This study proved that after 24 h of incubation all wild \textit{sea}-positive \textit{S. aureus} isolates could produce SEA at varied levels of amount. Several previous studies were performed concerning the influential factors such as temperature, prophage induction and pH.\textsuperscript{11,17} The results of these studies varied greatly due to such different influential factors on SEA production. Under favorable cultivation conditions at 37°C for 24 h, it has been observed that 50–300 ng/mL of SEA is reached,\textsuperscript{11} and another report showed that \textasciitilde{}608–739 ng/mL has been detected on pork sausage,\textsuperscript{21} which is very close to the results of this study (average: 305 ng/mL; range: 362–1,109 ng/mL) after 24 h of incubation. In this study, the amount of SEA produced after 24 h of incubation by four highest SEA-producing isolates, namely, SA-212, SA-217, SA-340 and SA-341, was 10 times that produced by four lowest SEA-producing isolates, namely, SA-2, SA-19, SA-187 and SA-189. This gap was much smaller than a reported one that was 4 log units and with a production speed in the log growth phase. We also identified a distinctive boundary between environmental strains and hospital strains. As to the SEA production capacity, SA-212 and SA-217 with \textit{Phi}Sa3ms and SA-340 and SA-341 with \textit{Phi}Mmu3A had high SEA-producing capacity, indicating that both phages had the ability to produce large amounts of SEA, although only in a few strains (Table 2).

**Conclusion**

The present study exhibits varied SEA production capacity of the wild \textit{sea}-positive \textit{S. aureus} strains in terms of SEA concentration and total amount of SEA after 24 h of incubation, as well as production speed and transcriptional level in the log growth phase. We also identified a distinctive boundary of phage types between strains from clinical samples and the environment.

**Acknowledgments**

This study was supported by the Natural Science Foundation of Ningbo (no. 2013A610246 and 2017A610273) and Wenzhou Science and Technology Project (Y20170148). This study also made use of the Multi Locus Sequence Typing website (http://www.mlst.net) at Imperial College London developed by David Aanensen and funded by the Wellcome Trust.

**Disclosure**

The authors report no conflicts of interest in this work.
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