Prevalence and expression of staphylococcal enterotoxin genes in *Staphylococcus aureus* isolated from food poisoning outbreaks

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Complete List of Authors:	Bastos, Caroline Peixoto; Universidade Federal de Pelotas Bassani, Milena Tomasi; Universidade Federal de Pelotas Mata, Marcia; Federal University of Pelotas, Science and Agroindustrial Technology Department LOPES, GRACIELA VOLZ; Universidade Federal de Pelotas, Food Science and Technology Silva, Wladimir Padilha; Universidade Federal de Pelotas
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1 **Prevalence and expression of staphylococcal enterotoxin genes in *Staphylococcus aureus***
2 **isolated from food poisoning outbreaks**

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4 Caroline Peixoto Bastos^a, Milena Tomasi Bassani^a, Marcia Magalhães Mata^a, Graciela Volz
5 Lopes^a, Wladimir Padilha da Silva^{a*}

6
7 ^a Federal University of Pelotas (UFPel), Faculty of Agronomy Eliseu Maciel (FAEM),
8 Department of Science and Food Technology, Pelotas, RS, Brazil

9
10 Running title: *Gene expression of S. aureus enterotoxins*

11
12 *Author for correspondence:

13 Wladimir Padilha da Silva

14 e-mail: silvawp@ufpel.edu.br

15 Tel: +55 53 32757281

16 Postal Address: Campus Capão do Leão – Universidade Federal de Pelotas, s/n – CEP:

17 96010-900 – Caixa Postal 354 – Pelotas - RS

18

19 **ABSTRACT**

20 *Staphylococcus aureus* is an important pathogen of foodborne origin. The pathogen produces
21 a variety of toxins that includes the staphylococcal enterotoxin (SE). The present study aimed
22 to evaluate the prevalence and expression of five SE genes (*sea*, *seb*, *sec*, *sed*, and *see*) in *S.*
23 *aureus* isolated from outbreaks occurred in the Rio Grande do Sul state, Brazil. All isolates,
24 with the exception of two, presented the same or higher transcriptional expression than the
25 reference strains for at least one of these genes. The presence of SE genes allied to the high
26 levels of transcriptional expression suggests that one or more SEs were involved with the
27 staphylococcal food poisoning outbreak analyzed in the present study.

28 Keywords: Staphylococcal enterotoxins; food poisoning; *Staphylococcus aureus*; gene
29 expression; qPCR

30

31 **Introduction**

32 *Staphylococcus aureus* is the most common etiological agent of bacterial diseases throughout
 33 the world due to its ability to produce a wide range of exotoxins and other virulence factors
 34 (Fusco et al. 2011). Staphylococcal food poisoning (SFP) is caused by the ingestion of food
 35 contaminated with preformed staphylococcal enterotoxins (SE) from approximately 10^6 *S.*
 36 *aureus* organisms per gram of food matrix and 100 ng of preformed toxin (Balaban and
 37 Rasooly 2000). To date, 23 SEs and SE-like toxins (SEls) have been identified around the
 38 world (Hennekinne et al. 2012). Classical SEs are classified using serological method into
 39 five emetic types: SEA, SEB, SEC, SED, and SEE (Dinges et al. 2000). They are responsible
 40 for approximately 95% of SFP outbreaks (Letertre et al. 2003, Al-Tarazi et al. 2009, Johler
 41 and Stephan 2010).

50 PCR assays have been used to evaluate the presence of the SE genes in *S. aureus*
 51 isolates (Umeda et al. 2017). Furthermore, the relative expression of the SE genes among
 52 food isolates have been described in previous studies (Lee et al. 2007; Derzelle et al. 2009;
 53 Suzuki et al. 2015). The authors suggest that the poisoning risk of *S. aureus* can be effectively
 54 evaluated based on the gene expression at the mRNA level. The reverse transcription real-
 55 time PCR (RT-qPCR) has become a very powerful tool for gene expression studies, due to
 56 their high sensitivity and specificity, reliable reproducibility and wide quantification range.

57 In Brazil, studies (Aragon-Alegro et al. 2007, Rall et al. 2008, 2014) have reported
 58 SFP outbreaks or presence of SE genes in *S. aureus* isolates, but there are no studies
 59 evaluating the expression of these genes. This study aimed to evaluate the prevalence and
 60 expression of the five main staphylococcal enterotoxin genes (*sea*, *seb*, *sec*, *sed*, and *see*) in *S.*
 61 *aureus* isolated from 13 different SFP outbreaks occurred between 2002 and 2004 in the Rio
 62 Grande do Sul state, Brazil.

63

64 **Material and methods**

65 **Confirmation of *S. aureus* identity**

66 *S. aureus* isolates (Table 1) from 13 different SFP outbreaks were included in this study
67 (identified as C01 for isolate 1 until C13 for isolate 13). These isolates were supplied by the
68 Laboratory of Public Health of Rio Grande do Sul state (LACEN-RS), located in southern
69 Brazil. *S. aureus* strain FRI S6 (carrying the *sea* and *seb* genes), ATCC 19095 (carrying the
70 *sec* gene), FRI 361 (carrying the *sed* gene) and FRI 326 (carrying the *see* gene), supplied by
71 the Laboratory of Food Microbiology of Federal University of Pelotas (UFPe), were used as
72 reference strains. All isolates and reference strains were previously purified using Baird
73 Parker agar (CM0961; Thermo Scientific, Hampshire, United Kingdom). The isolates were
74 phenotypically characterized as *S. aureus* as described by Brito et al. (2002) and Gandra et al.
75 (2005).

76 **Pulsed-field gel electrophoresis (PFGE)**

77 The relatedness of *S. aureus* isolates (n=13) was investigated by *Sma*I-
78 macrorestriction analysis according to previously described protocol with minor
79 modifications (McDougal et al. 2003). Pulsed-field gel electrophoresis (PFGE) was
80 performed and the resulting macrorestriction patterns were analysed using the BioNumerics
81 software package (Applied Maths, Kortrijk, Belgium). The dendrogram was derived from
82 application of the unweighted pair group method with arithmetic averages (UPGMA) cluster
83 analysis using Dice's similarity coefficient. Isolates showing patterns with at least 80%
84 similarity were classified as genetically related, but those with less than 80% similarity as
85 unrelated.

86 **DNA extraction and amplification of the enterotoxin genes by PCR assay**

87 For DNA extraction, the bacterial cells were inoculated in Trypticase Soy Agar
88 (CM0131; Thermo Scientific, Hampshire, United Kingdom) and incubated at 37°C for 24

hours. After this period, the genomic DNA was extracted according to the protocol proposed by (Matthews et al. 1997). The quality and quantity of extracted DNA were estimated by electrophoresis in a 1% agarose gel, by comparison with the Lambda DNA/HindIII Marker (SM0103; Thermo ScientificTM, Vilnius, Lithuania).

A multiplex PCR (mPCR), that targets the genes *nuc* (specific for the *S. aureus* thermonuclease gene sequence) and 16S rRNA (internal amplification control – IAC for staphylococcal DNA), was used as a molecular tool to confirm previous identification of the isolates (Baron et al. 2004). All isolates were also submitted to PCR analysis to detect the enterotoxin genes: *sea*, *seb*, *sec*, *sed*, and *see* (Table 2). The PCR amplicons were subjected to electrophoresis on 1.2% agarose gel. The gel was stained with ethidium bromide (E7637; Sigma, St. Louis, USA) and the amplified products were visualized under ultraviolet light and photographed (Loccus[®], L-Pix Touch, São Paulo, Brazil). A molecular weight marker of 100 bp DNA Ladder (15628019; InvitrogenTM, Carlsbad, CA, USA) was used as a standard.

Total RNA extraction and cDNA synthesis

The *S. aureus* isolates were inoculated in TSA and incubated at 37°C. After 24 hours, one isolated colony was removed from the TSA, inoculated in Brain Heart Infusion broth (CM1032; Thermo Scientific, Hampshire, United Kingdom) and incubated at 37°C for 24 hours. Total RNA was extracted and purified with RiboPureTM-Bacteria Kit (AM1925; InvitrogenTM, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The quality of RNA was verified on a 1.5% agarose gel. cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (4368814; Applied BiosystemsTM, Foster City, CA, USA) according to the manufacturer's instruction. The resultant cDNA was stored at -20° C.

Real-time PCR (qPCR)

The qPCR was performed using 7500 Real Time PCR System (Applied BiosystemsTM, Foster City, CA, USA). The qPCR consisted of 12.5 µL SYBR Green PCR Master Mix (Applied BiosystemsTM, Foster City, CA, USA), 2 µL cDNA (diluted 5-fold), 100 pmol.µL⁻¹ forward and reverse primers, and nuclease-free water to a total volume of 25 µL. The samples were placed in triplicate on 96-well plates and covered with optical adhesive. Primers used for qPCR are presented in Table 3.

The temperature parameters for the qPCR were: 50°C/2min; initial denaturation at 95°C/10min, 40 cycles of three steps: denaturation at 95°C/15sec; annealing at 60°C/1min and; 72°C/1min extension, which was followed by standard dissociation curve to verify the specificity of the primers used. The efficiency of each primer pair was calculated using a standard curve of serial dilution, as described in the protocol provided by Applied BiosystemsTM. The level of gene expression was calculated based on the threshold cycle (Ct), where the 16S rRNA gene was used for normalization (internal standard) and the reference genes of each SE as calibrators. It was calculate the $\Delta\Delta CT$, which was used in the equation of the relative quantification (RQ) to express the data (Yuan et al. 2006), where $RQ = 2^{-\Delta\Delta CT}$. All the experiments were performed on three occasions, and mean and standard deviation of the relative quantification (RQ) was calculated.

Results and Discussion

Identification of *S. aureus*, molecular typing and enterotoxin genes detection

The amplification of the expected fragment of the *nuc* gene (458 bp) as well as the 16S rRNA (252 bp) was obtained in all 13 isolates from SFP outbreaks. The genetic relationship among *S. aureus* isolates based on *Sma*I-restricted DNA fragments is shown in Figure 1. In the present study, 11 *S. aureus* isolates proved to be typeable by *Sma*I, but two isolates were non-typeable. The macrorestriction analysis produced eleven (I to XI) different *Sma*I-PFGE

patterns with only two isolates (C03 and C06) showing more than 80% similarity. These isolates differed from each other in only a few DNA fragments (88.2% similarity) and maybe displayed a clonal relationship, although they came from different cities, food samples, and different isolation dates (Table 1). The persistence of *S. aureus* clones causing SFP outbreaks has already been reported. Shin et al. (2016) reported five *S. aureus* isolates associated with foodborne disease in Korea showing 100% identical PFGE pattern with ST72-SCCmec type IV although their isolation region and dates were not the same.

The *S. aureus* isolates were screened by PCR for the presence of the SE genes and all carried at least one of these genes. It was observed that the most prevalent gene was *sec* (9 isolates), followed by *sea* (8 isolates), *seb*, *sed* (4 isolates each), and *see* (2 isolates) (data not show). The prevalence of the *sec* gene in our study (20.5%) could be considered interesting, considering that the most frequently SE involved in sporadic cases and SFP outbreaks are the enterotoxins SEA and SED (Johler and Stephan 2010, Pinchuk et al. 2010). Among 46 *Staphylococcus* coagulase-positive isolates from milk and dairy products, the *sea* gene was found in 13 isolates, followed by *sed* (8), *seb* (3), *sec* (2), and none of them was carrier of *see* gene (Bendahou et al. 2009). Similarly, the *sea* was the most frequent enterotoxin gene from raw and pasteurized milk; it was found in 41% of the 57 *S. aureus* isolates (Rall et al. 2008). The *see* gene was less prevalent in our study, which corroborates with the results reported by Ostyn et al. (2010), where the SFP outbreaks involving SEE was rare. The presence of an enterotoxin gene does not necessarily imply in its expression and, consequently, in the toxin production (Pelisser et al. 2009). Thus, it is important to evaluate the expression and conditions under SE genes are expressed, so that more robust responses could be provided about their involvement in SFP outbreaks.

Expression of staphylococcal enterotoxin genes by real-time PCR (qPCR)

All genes that were detected by PCR assay had their relative expression confirmed by qPCR. Relative quantification (RQ) of SE genes expression of each isolate from SFP outbreaks was compared to the expression of SE genes of reference strains, considering that the normalized relative expression ratio for each SE gene is equals 1. It can be seen in Figure 2 that all isolates, except C08 and C09, presented the same or bigger transcriptional expression than the reference strains for at least one of the SE genes. It should be emphasized that evaluation by PCR showed that C04 and C05 isolates did not carry *seb* gene, and the isolate C06 did not carry the *seb* and *sec* genes; however, Ct values for these genes were obtained, demonstrating the greater sensitivity of the qPCR technique (Valasek and Repa 2005).

Ct values for the *sec* genes were detected in 10 (77%) of the 13 isolates tested in this research, followed by *sea* (62%), *seb* (54%), *sed* (31%), and *see* (15%). This qPCR results corroborated the data obtained for PCR analysis. It is interesting to note that although the *sec* gene was expressed in 77% of the isolates from food poisoning outbreaks, the highest values of RQ found for *sec* gene were 3.7 (isolate C04) and 4.3 (isolate C11) times higher than the reference strain. Ct values for the *sea* gene were detected in nine isolates (62%), showing the highest overall levels of expression in C03 and C10 isolates when compared to the reference strain (Figure 2). The high level of *sea* gene expression, added to its resistance to proteolytic enzymes may suggest their participation in the food poisoning processes involved in this research. Seven isolates carried the *seb* gene (54%) and the C11 isolate showed the highest expression of this gene. The expression levels were 5.8 times higher than the reference strain (Figure 2). The *sed* gene was the fourth most prevalent (31%) in this study, with *see* being the less prevalent. All four isolates with Ct values for the *sed* gene showed a value of the RQ between 0.3 and 0.4 times higher than the reference strain RQ value. Derzelle et al. (2009)

describes that the expression levels of SE genes are distinct from each other, with *seb* and *sec* being expressed 10 times more than *sea*, *sed*, and *see*.

It was not possible to know which SE was really the causative agent of the evaluated outbreaks, considering that the factors inherent to food or the storage environment can interfere in the gene expression. However, taking into consideration the results obtained for the presence of SE genes by PCR and by the SE genes expression obtained by qPCR, it appears that one or more SE may have caused the outbreaks evaluated. Four isolates (C03, C07, C08, and C09) expressed only one of the SE genes (*sea*, *sec*, *sec*, and *sea* respectively). This result coupled with the high prevalence of SE in SFP outbreaks, suggest that those SE were responsible for the outbreaks from which those isolates were obtained. Other authors, such as, Ostyn et al., (2010), Giezendanner et al., (2009) and Asao et al. (2003), also reported SFP outbreaks involving only one SE. Different results described by Kitamoto et al. (2009), Schmid et al. (2009) and Nema et al. (2007), reported outbreaks were caused by the presence of more than one SE. The present research agreed with that, once this happened with nine outbreaks cases evaluated. The isolates C01, C02, C04, C05, C06, C10, C11, C12 and C13 expressed two or more SE genes, as demonstrated by PCR and qPCR.

Conclusion

In conclusion, the prevalence of staphylococcal enterotoxin genes in the isolates evaluated, associated with high levels of transcriptional expression, suggest that one or more enterotoxins could have been the causative agent of the food poisoning outbreaks from which these isolates were obtained.

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- Figure Legends
- Figure 1. Dendrogram analysis of SmaI-digested whole genomic DNAs of *Staphylococcus aureus* isolates from staphylococcal food poisoning outbreaks. The isolate ID, PFGE type, and SE gene profile are shown.
- Figure 2. Transcriptional expression of the classical staphylococcal enterotoxin genes in reference strains of *S. aureus* and 13 *S. aureus* isolates from staphylococcal food poisoning outbreaks (C01 to C13), occurred in the Rio Grande do Sul state, Brazil.

Table 1. *Staphylococcus aureus* isolates used in this study.

Isolate ID	Year	Food Vehicle	City	Involved people (n)	Sick people (n)	SE genes
C01	2003	Rice	Uruguaiana	69	29	<i>sea, seb, sec</i>
C02	2002	Ground beef	Taquara	5	5	<i>sea, sec, sed</i>
C03	2003	Beef	Veranópolis	700	150	<i>sea</i>
C04	2002	Homemade cake	Santa Maria	50	6	<i>sea, sec</i>
C05	2003	Chicken salad with mayonnaise	Canoas	-	-	<i>sec, sed</i>
C06	2002	Mayonnaise	Nova Prata	112	106	<i>sea, sed</i>
C07	2003	Potato salad with mayonnaise	Veranópolis	700	150	<i>sec</i>
C08	2003	White sauce	Porto Alegre	3	3	<i>sec</i>
C09	2003	Beef parmigiana	Porto Alegre	3	3	<i>sea</i>
C10	2003	Mayonnaise	Eugênio Castro	70	47	<i>sea, sec</i>
C11	2003	Chocolate Cake	Uruguaiana	69	29	<i>sea, seb, sec</i>
C12	2003	Fruit salad with sour cream	Porto Alegre	2	2	<i>seb, see</i>
C13	2004	Pie	Três de Maio	-	-	<i>seb, sec, sed, see</i>

- : Information is not available.

Table 2. Primers used for detection of the classical staphylococcal enterotoxin genes.

Primers	Sequence 5' - 3'	Amplicon size (pb)	Target	Reference
SEA1	ACGATCAATTTTACAGC	544	<i>sea</i>	Rosec e Gigaud (2002)
SEA2	TGCATGTTTTCAGAGTTAATC			
SEB1	ATTCTATTAAGGACACTAAGTTAGGGGA	404	<i>seb</i>	Jarraud et al. (2002)
SEB2	ATCCCGTTCATAAGGCGAGT			
SEC1	GACATAAAAGCTAGGAATTT	257	<i>sec</i>	Rosec e Gigaud (2002)
SEC2	AAATCGGATTAAACATTATCCA			
SED1	CAAATATATTGATATAATGA	330	<i>sed</i>	Zocche et al. (2009)
SED2	AGTAAAAAAGAGTAATGCAA			
SEE1	CAAAGAAATGCTTTAAGCAATCTTAGGC	482	<i>see</i>	Jarraud et al. (2002)
SEE2	CACCTTACCGCCAAAGCTG			

Table 3. Primer sequences used for qPCR analysis.

Primers	Sequence 5' - 3'	Amplicon size (pb)	Target	Reference
SEAR1	AAAATACAGTACCTTTGGAAACGGTT	92	<i>sea</i>	Klotz, et al. 2003
SEAR2	TTTCCTGTAAATAACGTCTTGCTTGA			
SEBR1	ACACCCAACGTTTTAGCAGAGAG	81	<i>seb</i>	Klotz, et al. 2003
SEBR2	CCATCAAACCAGTGAATTTACTCG			
SECR1	AATAAAACGGTTGATTCTAAAAGTGTGAA	80	<i>sec</i>	Klotz, et al. 2003
SECR2	ATCAAAATCGGATTAACATTATCCATTC			
SEDR1	TGATTCTTCTGATGGGTCTAAAGTCTC	115	<i>sed</i>	Klotz, et al. 2003
SEDR2	GAAGGTGCTCTGTGGATAATGTTTT			
SEER1	TCAATGTGCTGGAGGCACACCAA	51	<i>see</i>	This study
SEER2	ACACCCCGTACATACATGCTGTT			
16SR1	AAGTCCCGCAACGAGCGCAA	86	<i>16S rRNA</i>	This study
16SR2	CCTCCGTTTGTACACCGCA			

SmaI Restriction

217x122mm (300 x 300 DPI)

195x122mm (300 x 300 DPI)