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Short communication

egc characterization of enterotoxigenic *Staphylococcus aureus* isolates obtained from raw milk and cheese

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ABSTRACT

Genes encoding staphylococcal enterotoxins (SEs) are carried by mobile genetic elements, and enterotoxin gene clusters (*egc*) are pathogenicity island-borne structures comprising several SE genes, which are frequently found among clinical *Staphylococcus aureus* isolates. In the present study, we investigated the distribution and the genetic variability of *egc* loci in *S. aureus* strains isolated from raw milk and soft cheese in Minas Gerais, Brazil. Ninety-two isolates were submitted to PCR detection of individual *egc*-borne SE genes (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*), and *egc* loci were typed using PCR-RFLP. PCR products of *egc* positive isolates were sequenced. Ninety-one isolates harbored at least one SE gene, which generated 14 different genotypes. The *sei* gene was the most widely distributed (97.8%), and was found in combination with *seg* in 49 isolates (53.3%). Altogether, a complete set of individual *egc* genes was detected in 37 isolates (40%). However, *egc* loci were detected by PCR-RFLP in only 4 isolates, and classified as *egc*1 (n = 2), *egc*3 (n = 1), and *egc*4 (n = 1). This investigation demonstrated the low occurrence of the *egc* in *S. aureus* isolated from dairy products. However, the frequency of complete sets of individual *egc*-borne genes reflects either the presence of these SE genes outside *egc* or the existence of new *egc* types in these strains.

1. Introduction

Staphylococcal enterotoxins (SEs) are a major cause of food poisoning outbreaks worldwide and milk and dairy products are foodstuffs among the most frequently associated with staphylococcal food poisoning (SFP) (Carmo et al., 2002; Cretenet et al., 2011). These cases are often caused by strains carrying one or more of the five classical SEs (SEA-SEE), although there are a few outbreaks reported to be caused by newly described SEs (Ikeda et al., 2005).

Genes encoding SEs are carried by mobile genetic elements, such as phages, plasmids and pathogenicity islands (Le Loir et al., 2003). In 2001, two research groups found SE genes present in an operon structure, later referred to as the enterotoxin gene cluster (*egc*). Polymorphisms were described in this sequence, resulting from a 15-nucleotide insertion (5′-CTCTAAAATTGATGG-3′) and a stop codon removal in the original sequence of pseudogene *Ψent1*, leading to the emergence of *selu* (Letertre et al., 2003). Thomas et al. (2006) further reported the presence of *selu*2 and *selv*, which emerged respectively from a single adenine deletion in the pseudogene sequences and from recombination events between *selm* and *sei*. According to observed nucleotide variations, the composition and organization of the *egc* loci can differ, such as for: i) *egc1* (*selo*, *selm*, *seli*, *Ψent1*, *Ψent2*, *seln*, *seg*); ii) *egc2* (*selo*, *selm*, *seli*, *selu*, *seln*, *seg*); iii) *egc3* ($selo_v$, $selm_v$, $seli_v$, $selu_v$, $seln_v$, seg_v) and iv) *egc4* (*selo*, *selv*, $selu_2$, *seln*, *seg*) (Collery et al., 2009; Schelin et al., 2011; Zhang et al., 2013). The *egc* locus may also be present in an incomplete form, lacking one or more genes, referred to as evolutionary intermediate sequences (Thomas et al., 2006). There are only a few reports focusing on the occurrence of polymorphism within *egc* clusters (Letertre et al., 2003; Collery and Smyth, 2007; Collery et al., 2009) and there is no standardized technique for the detection, identification and/or differentiation of *egc* loci. Little is known about the prevalence of *egc* in *Staphylococcus aureus* of food origin (Zhang et al., 2013). Prevalence of *egc* is remarkably high among clinical isolates (Mempel et al., 2003; Smyth et al., 2005), and *egc* is supposed to confer a selective advantage in the infection process (Jarraud et al., 2001). It is hypothesized that *egc* is a nursery of SE genes, including SE genes that are not primarily related to *egc* and that are found as monocistronic loci (e.g. *sea*, *seb*, *see*) elsewhere in the genome (Larkin et al., 2009).

Most studies assign the presence of an *egc* mainly to the PCR amplification of its constituent genes and only a few combined PCR detection

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with polymorphism identification. The aim of this study was to investigate the distribution and the genetic variability within *egc* loci in *S. aureus* strains isolated from raw milk and soft fresh cheese.

2. Material and methods

2.1. Bacterial strains

S. aureus reference strains FRI 472 (*Ψent1*, *Ψent2* [*egc*1 group], *sed* genes) and ATCC 19095 (*seu* [*egc*2], *sec*, *seg*, *seh*, *sei*, *sel*, *sem*, *sen*, *seo* genes) were used as positive controls in PCR reactions (Letertre et al., 2003), and 92 staphylococci isolates previously obtained from raw milk and fresh cheese samples were used in this study (Viçosa et al., 2010). All isolates were identified as *S. aureus* based on Gram staining, hemolytic activity on sheep blood agar, catalase production, mannitol fermentation; maltose utilization under aerobic and anaerobic conditions, growth in Baird–Parker agar supplemented with acriflavine (7 $mg \cdot L^{-1}$), coagulase tube test and thermonuclease production. *S. aureus* identification was confirmed by species-specific *femA* amplification as described previously (Mehrotra et al., 2000). Bacterial strains were grown overnight in Brain Heart Infusion at 37 °C prior to DNA extraction. All culture media were from Oxoid Ltd. (Basingstoke, England).

2.2. DNA extraction

Total DNA from bacterial strains was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, USA), according to the manufacturer's instructions. DNA quantity and quality were assessed by horizontal electrophoresis using a mixture of 2 μL of obtained DNA, 2 μL of Blue/Orange Loading Dye 6× (Promega Corp.) and 1 μL of GelRed™ 20× (Biotium Inc., Hayward, CA, USA). DNA preparations were kept at −25 °C until PCR amplifications.

2.3. PCR detection of individual SE genes associated with the egc locus

Presence of SE genes was determined either by multiplex (*seg* + *sei*, *sem* + *seo*) or by simplex (*sen*, *seu*) PCR. Multiplex PCR was performed in a final volume reaction of 25 μL, containing 12.5 μL of GoTaq® Green Master Mix 2× (Promega Corp.), 8.5 μL of nuclease free water, 200 nM of each primer and 2 μL of DNA template. For *sen* and *seu* detection, 400 mM of each primer were incorporated into a 25 μL-volume reaction. Primers sequences and PCR amplification conditions used in these reactions were previously described (Bania et al., 2006; Jarraud et al., 2002; Nashev et al., 2007). PCR products were separated by horizontal electrophoresis on 2% (w/v) agarose gels in 0.5× TBE buffer alongside 100 bp DNA Ladder (Promega Corp.), revealed in a GelRed™ 3× solution and visualized under UV light.

2.4. Detection and differentiation of egc locus

To detect the distribution and verify the nature of the *egc* locus, primers designed by Letertre et al. (2003) were used in a PCR-RFLP protocol described by Collery and Smyth (2007) and Collery et al. (2009), with some modifications. Primer combinations used in PCR reactions were PSE1/PSE4, PSE2/PSE4 and PSE2/PSE6. PCR reactions contained 12.5 μL of GoTaq® Green Master Mix 2× (Promega Corp.), 8.5 μL of nuclease free water, 400 nM of each primer and 2 μL of DNA template, in a final volume of 25 μL. Five microliter of each amplified product was analyzed in 2.5% agarose gels (w/v), revealed in a GelRed™ 3× solution and visualized under UV light.

Subsequently, in order to differentiate isolates bearing pseudogenes (*Ψent1* and *Ψent2*) from those containing *selu* or $selu_v$, PCR products obtained with the PSE1/PSE4 primer set were submitted to differential cleavage using *Bcc*I (New England Biolabs Inc., Ipswich, USA), *Hph*I (New England Biolabs Inc.) and *Hind*III (Promega Corp.) restriction endonucleases. Additionally, the same PCR products were also submitted to *Bbv*I and *Tse*I cleavages, to differentiate *egc*2 and *egc*3 groups. All cleavage reactions were conducted according to Collery and Smyth (2007) and Collery et al. (2009), except for *Bcc*I endonuclease, in which 20 μL of PCR product were added to 5 μL of NEB Buffer 1 (10 mM BisTris-propane HCl, 10 mM $MgCl_2$, 1 mM DTT pH 7.0) and 1 μL of enzyme (10 U/μL), and incubated overnight at 37 °C. All resulting products were separated by horizontal electrophoresis on 2.5% (w/v) agarose gels alongside 100 bp DNA Ladder (Promega Corp.), revealed in a GelRed™ 3× solution and visualized under UV light. Isolates were classified into distinct *egc* groups according to the resulting PCR-RFLP restriction pattern.

PCR products amplified by the PSE1/PSE4 primers were purified and sequenced by Macrogen Inc. (Seoul, South Korea) in order to confirm their *egc* nature. The obtained sequences were analyzed using the software Sequencher® v.4.1.4 (Gene Codes Corporation, Ann Arbor, USA) and compared to *egc* sequences previously deposited in the GenBank database (National Institutes of Health, Maryland, USA).

3. Results and discussion

3.1. PCR detection of individual SE genes of egc locus

All isolates were confirmed as *S. aureus* based on biochemical tests and amplification of *femA* gene.

Ninety one out of 92 *S. aureus* isolates (98.9%) carried at least one of the SE genes investigated in this study. *sei* was the most commonly detected gene (97.8%), while *seu* presented the lowest frequency, being present in 35 isolates (38.0%). In our study, *seg* and *sei* were simultaneously detected in 49 isolates (Table 1). Although *seg* and *sei* coexist within the *egc* locus, as described by Jarraud et al. (2001), *sei* may also occur independently of *seg* in staphylococci isolates of food origin. McLauchlin et al. (2000) reported the occurrence of *seg* and *sei* simultaneously in 19 out of 129 isolates, while *sei* alone was detected in one isolate. These findings were in accordance with Aydin et al. (2011), who found 28 isolates harboring both *seg* and *sei*, while 3 isolates harbored only *sei*, in 1070 isolates of various food samples in Turkey. In the present study, the frequency of *sei* was shown to be much higher than that of *seg* in *S. aureus* isolates from raw milk and fresh cheese, reinforcing the hypothesis that *sei* may exist in another genetic element, outside an *seg*-carrying *egc*. PCR results for individual SE genes of *egc* locus generated 14 different SE gene combinations. Among these, the combinations *seg sei sem sen*

Table 1
Genotypes defined on the basis of PCR reactions for enterotoxin genes in *Staphylococcus aureus* strains isolated from raw milk and fresh cheese.

Genotypes	Frequencies
seg sei sem sen seo seu	21[a]
seg sei sem sen seo	16
sei sem sen seo	14
sei sem seo	8
sei	6
seg sei sem seo	5
sei sem sen seo seu	4
sei sem seo seu	4
sei sen	4
seg sei sem seo seu	3
seg sei	2
seg sei sen seo seu	2
sem sen seo	1
sei sen seu	1
None	1
Total	92

[a] Four of these 21 isolates were positive for *egc* and typeable by the PCR-RFLP method developed by Collery and Smyth (2007) and Collery et al. (2009).