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Staphylococcal food poisoning caused by *Staphylococcus argenteus* harboring staphylococcal enterotoxin genes

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ABSTRACT

Staphylococcal food poisoning (SFP) is caused by staphylococcal enterotoxins (SEs) preformed in food materials. SE genes are encoded on mobile genetic elements and are widely found across *Staphylococcus* species including *S. argenteus*, although most SFP cases are caused by *S. aureus*. *S. argenteus*, recently discriminated from *S. aureus* as a novel species, are non-pigmented staphylococci phenotypically related to *S. aureus*. In 2014 and 2015, two independent food poisoning cases occurred in Osaka, Japan, in which non-pigmented staphylococci were predominantly isolated. Several enterotoxin genes (*seb*, *seg*, *sei*, *sem*, *sen*, *seo*, and *selu2*) were found in their genome and the production of SEB was confirmed by reverse passive agglutination tests. The non-pigmented isolates from patients, food handlers, food, and cooking utensils all produced the same pulsed-field gel electrophoresis pattern. These non-pigmented isolates were coagulase-positive and biochemically identical to *S. aureus*. We performed further genetic analysis using *nucA* sequencing and multi-locus sequence typing, and identified these isolates as *S. argenteus*. We also found that *seb* was encoded on the *Staphylococcus aureus* pathogenicity island, while *seg*, *sei*, *sem*, *sen*, *seo*, and *selu2* were encoded on the enterotoxin gene cluster. From these results, we concluded that the two food poisoning outbreaks were SFP cases caused by *S. argenteus* harboring SE genes.

1. Introduction

Staphylococcal food poisoning (SFP) is caused by oral ingestion of food materials contaminated with staphylococcal enterotoxins (SEs) (Hennekinne et al., 2012). More than 20 types of SE and SE-like genes including five classical enterotoxins (*sea* to *see*) have been previously reported (Hennekinne et al., 2012; Loir et al., 2003; Ono et al., 2015). Classical SEs exhibit relatively strong emetic activity and are the cause of most SFP cases. In Japan, 83% of SFP cases that occurred from 1990 to 2010 were caused by classical SEs (Sato'o et al., 2014). Recent reports have provided evidence of SFP cases caused by newly described SEs like SEG, SEH, SEI, SEM, SEN, and SEO (Ikeda et al., 2005; Jørgensen et al., 2005; Umeda et al., 2017).

Many SE genes are encoded on mobile genetic elements (MGEs), such as prophages, plasmids, *Staphylococcus aureus* pathogenicity islands (SaPIs), and genomic islands carrying the enterotoxin gene cluster (*egc*) (Alibayov et al., 2014). MGEs can be spread by horizontal gene transfer among *Staphylococcus* species, resulting in diffusion of SEs among not only *S. aureus* but also other staphylococci (Madhusoodanan et al., 2011). Dissemination of SE genes in non-aureus staphylococci comprises a potential SFP threat, although most SFP cases to date have been evoked by SE-producing *S. aureus* strains.

S. argenteus was discriminated from *S. aureus* as a novel species in 2015 (Tong et al., 2015), which has been isolated from humans with healthcare-associated or skin and soft tissue infections (Ruimy et al., 2009; Thaipadungpanit et al., 2015). *S. argenteus* lacks staphyloxanthin, a type of carotenoid pigment and shows white-colony morphology, while *S. aureus* forms either yellow or white colonies depending on the amount of pigment production (Holt et al., 2011). *S. argenteus*, as well as *S. schweitzeri*, which forms a *S. aureus*-related complex, is biochemically similar to *S. aureus*, which makes their discrimination difficult in clinical examinations; however, these organisms have some genetic differences (Tong et al., 2015). Multi-locus sequence typing (MLST) analysis is widely used to discriminate *S. argenteus* from other *S. aureus*-related complex species (Ruimy et al., 2009; Thaipadungpanit et al., 2015).

We encountered two SFP outbreaks in 2014 and 2015 in Osaka, Japan. Coagulase-positive, non-pigmented staphylococci were

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independently and predominantly isolated in each outbreak. These non-pigmented isolates were presumed to be the cause of the outbreaks from epidemiological correlations based on toxigenic profiling and electrophoresis patterns of pulsed-field gel electrophoresis (PFGE).

To determine whether the non-pigmented isolates, which were biochemically similar to *S. aureus*, were *S. argenteus*, we conducted genetic analysis, including *nucA* gene sequencing and MLST. In addition, to demonstrate the evolutionary mechanisms of the enterotoxigenic *S. argenteus*, we examined the relationship between enterotoxin genes and MGEs in *S. argenteus*.

2. Material and methods

2.1. Bacterial isolation and general identification methods

Salt egg yolk (SEY) agar plates (Nissui Pharmaceutical, Tokyo, Japan) were used for bacterial isolation from fecal specimens, food samples, and swabs of cooking utensils. For enumeration of the colony forming unit (CFU), swab samples were prepared by 10-fold serial dilutions with saline and cultivated on SEY at 37 °C for 48 h. As primary screening for *Staphylococcus* species, the slide agglutination test was performed using PS latex (Eiken Chemical, Tokyo, Japan) for clumping factor and staphylococcal protein A. Coagulase production and coagulase type were determined by the tube coagulation method using rabbit plasma and a coagulase typing anti-serum kit (Denka Seiken, Tokyo, Japan). Biochemical properties were determined using ID 32 staph (BioMérieux, Paris, France) by following the manufacturer's instructions. Antimicrobial susceptibility test was conducted by the disc diffusion method (Sensi-disc, Becton Dickinson, Sparks, Maryland) according to the guideline described by Clinical & Laboratory Standards Institute. The following antimicrobial agents were tested: cefoxitin, erythromycin, penicillin, trimethoprim-sulfamethoxazole, linezolid, doxycycline, minocycline, tetracycline, vancomycin, teicoplanin, rifampin, chloramphenicol, ciprofloxacin, levofloxacin, gentamicin, amikacin, kanamycin, and norfloxacin.

2.2. DNA extraction, detection of se/sel genes and SEs

Genomic DNA was extracted using the alkaline lysis method (Yonogi et al., 2016). The primers used in this study are listed in Supplementary Table 1. SE genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selj*, *sek*, *sell*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, *ses*, and *set*) were detected by multiplex polymerase chain reaction (PCR) described previously (Omoe et al., 2005; Umeda et al., 2017). *selu* was detected using PSE2 and seu-R primers (Letertre et al., 2003). The production of classical SEs (SEA to SEE) was determined by reverse passive latex agglutination (RPLA) using a commercially available kit (enterotox-F, Denka Seiken, Tokyo, Japan) by following the manufacturer's instructions.

2.3. nucA sequencing and MLST analysis

Amplification of the *nucA* was performed using Ex Taq Hot Start Version (Takara Bio, Shiga, Japan) with nucA-F and nucA-R primers (Table S1). The PCR mix (final volume 20 μl) contained 0.5 U of Ex Taq, 250 nM of each primer, and 200 μM of deoxynucleoside triphosphates. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 30 s), extension (72 °C for 1 min), and a final extension step at 72 °C for 10 min. Primers and PCR conditions for MLST analysis were described elsewhere (Enright et al., 2000). For amplification of the *aroE*, primers designed by Ruimy were used (Ruimy et al., 2009). After treatment with Exonuclease I (Takara Bio, Shiga, Japan) and Shrimp Alkaline Phosphatase (Takara Bio, Shiga, Japan), PCR amplicons were sequenced using the ABI3130 genetic analyzer (Applied Biosystems, Foster City, California) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). MLST data were compared to the MLST database (http://saureus.mlst.net/). Phylogenetic analysis was carried out in MEGA7 software using the maximum likelihood method (Kumar et al., 2016). Reference sequences of *S. aureus* and *S. schweitzeri* were obtained from the National Center for Biotechnology Information (NCBI) database for the construction of phylogenetic trees.

2.4. SaPI scanning and detection of egc locus

SaPI scanning was performed as described previously (Sato'o et al., 2013; Suzuki et al., 2014). Insertion of SaPI was estimated by the amplification of PCR products of more than 10 kb. Restriction fragment length polymorphism (RFLP) analysis was performed by digesting PCR amplicons with *Hin*dIII (Takara Bio, Shiga, Japan) at 37 °C for 1 h and electrophoresis in 0.8% agarose gel (Agarose I, Dojindo, Kumamoto, Japan). For the identification of SaPIs, RFLP patterns were compared with predicted patterns obtained by *in silico* analysis using ApE (A Plasmid Editor, http://biologylabs.utah.edu/jorgensen/wayned/ape/) and NCBI databases (http://blast.ncbi.nlm.nih.gov/). The presence of the *seb* was confirmed by PCR amplification and DNA sequencing using LA PCR products as PCR templates with SEB-1, SEB-4, GSEBR-1, and GSEBR-2 primers (Mehrotra et al., 2000; Omoe et al., 2005).

The primers and PCR conditions for the detection of the *egc* locus were described previously (Sato'o et al., 2014). For amplification and sequencing of *egc*-encoded enterotoxin genes in *S. argenteus*, we designed primers using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) based on the sequences of the *S. argenteus* MSHR 1132 type strain (accession number: FR821777): MSHR1132-16710 for *seg*, MSHR1132-16720 for *sen*, MSHR1132-16730 for *selu2*, MSHR1132-16740 for *sei*, MSHR1132-16750 for *sem*, and MSHR1132-16760 for *seo* (Table 3). The nucleotide sequences of *seg*, *sei*, *sem*, *sen*, *seo*, and *selu2* were compared to the archetypal sequences of *egc* (A900322; accession number: AF285760) and *selu2* (A900624; accession number: EF030428) by BLAST analysis.

2.5. Pulsed-field gel electrophoresis (PFGE)

Twenty-seven and 24 isolates from fecal specimens, food and cooking utensil samples from case 1 (2014) and case 2 (2015), respectively, were subjected to PFGE. Bacteria cultivated in Brain Heart Infusion broth were embedded in 0.5% SeaKem Gold Agarose (Lonza, Rockland, Maine) and treated with lysis buffer and proteinase K solution, as described previously (Hielm et al., 1998). After washing with Pefabloc SC (Roche Life Science, Penzberg, Germany), the plugs were digested with 20 U of *Sma*I (New England Biolabs, Ipswich, Massachusetts). Electrophoresis was performed using CHEF DRIII (Bio-Rad Laboratories, Hercules, California) with the following conditions: initial time 5.3 s, final time 34.9 s, for 20 h at 6 V/cm in 0.5 × Tris/Borate/EDTA (TBE) buffer. DNA was stained with ethidium bromide and visualized under long wavelength ultraviolet light.

2.6. Accession numbers

Nucleotide sequences reported in this study have been deposited in the DDBJ/EMBL/GenBank under the following accession numbers: LC275970 (*nucA*, SA14-23), LC275971 (*nucA,* SA15-03), LC275972 (*seb*, SA14-23), LC275973 (*seb*, SA15-03), LC275974 (*seg*, SA15-03), LC275975 (*seg*, SA14-23), LC275976 (*sei*, SA15-03), LC275977 (*sei*, SA14-23), LC275978 (*sem*, SA15-03), LC275979 (*sem*, SA14-23), LC275980 (*sen*, SA15-03), LC275981 (*sen*, SA14-23) LC275982 (*seo*, SA15-03), LC275983 (*seo*, SA14-23), LC275984 (*selu2*, SA15-03), LC275985 (*selu2*, SA14-23).

2.7. Ethical statements

The SFP investigations were conducted as a part of regional public