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**Topic of the paper.** Tracing the source of a staphylococcal food poisoning

**Full title of the paper.** Staphylococcal poisoning foodborne outbreak: epidemiological investigation and strain genotyping

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**Keyword:** *S. aureus*, foodborne outbreak epidemiological investigation, genotyping

**Running title:** Tracing the source of staphylococcal food poisoning

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Abstract

In June 2011, an outbreak of *Staphylococcus aureus* enterotoxin food poisoning gastroenteritis occurred in Turin, Italy, following a catered dinner party given at a private home. Within a few hours, 26 of the 47 guests experienced gastrointestinal illness and 9 were hospitalized. A retrospective cohort study using a standardized questionnaire was carried out and the risk ratios (RR) for each food item were calculated. The analysis indicated consumption of seafood salad as the most probable cause of the outbreak (RR = 11.72, 95% confidence interval [CI], 1.75-78.54). Biological samples were collected from 4 of the hospitalized guests (stool and vomit), nasal mucosa swabs from 3 food handlers employed with the caterer and available food residuals. All stool and vomit samples tested positive for enterotoxigenic *S. aureus*. As residues of the seafood salad were no longer available for sampling, suspected contamination could not be verified. However no other food was found contaminated by *S. aureus* or its enterotoxins. All isolates from the biological samples were characterized at genomic level by means of two multiplex PCR protocols to determine the presence of genes encoding staphylococcal enterotoxins (SEs), pulsed-field gel electrophoresis (PFGE) and staphylococcal protein A gene (*spa*) typing to describe their genetic profiles. All the isolates presented genes encoding SEA and SEI; the PFGE genetic profiles revealed the same pulsotype in the microorganism isolated from the hospitalized guests as in one of the isolate from the nasal mucosa of one of the food handlers and their *spa* typing analysis reported two closely related *spa* types (t701 and t267), implicating the food handler as the most likely outbreak source.
Staphylococcal food poisoning (SFP) is one of the most common foodborne diseases worldwide (23). According to the European Food Safety Authority (EFSA), outbreaks of foodborne illnesses in the European Union in 2010 totalled 5262, of which 461 were caused by bacterial toxins produced by *Staphylococcus aureus*, *Bacillus*, or *Clostridium*, the fourth most common causative agent in foodborne outbreaks in Europe. In particular, 274 of these foodborne outbreaks were caused by staphylococcal enterotoxins (SEs) (6).

SFP is a foodborne intoxication that develops after the ingestion of food contaminated with enterotoxigenic *S. aureus* strains, generally improperly prepared or stored (16). *S. aureus* is a coagulase-positive staphylococcus (CPS), a facultative Gram-positive anaerobe which can grow over wide range of pH (4-10, optimally at a pH of 6-7), temperatures (7-48°C), and water activity (minimum, 8.83). Some strains are able to produce specific thermoresistant enterotoxins with a somewhat narrower production range (11). *S. aureus* colonizes both humans and domestic animals. As common opportunistic pathogen, it colonizes the skin and mucosa of humans and animals, with nasal carriage rates between 30 and 50% in the adult population (9). Skin infections, nasopharyngeal or oropharyngeal staphylococcal or streptococcal secretions also have been frequently linked to worker-associated outbreaks (18;19).

But *S. aureus* is not only a commensal colonizer. It can cause serious infections, toxinoses and life-threatening diseases, including skin and soft tissue infections, toxic shock syndrome and septicemia (20). The growth of *S. aureus* in foods makes it a potential public health hazard, and many strains are able to produce heat-stable SEs that cause food poisoning if ingested (2;3;4). Symptoms of foodborne illness include copious vomiting, diarrhea, and abdominal pain or nausea (13;15). The severity of the illness depends on the amount of toxin present in the ingested food and the general health of the victim (17). While foodborne illness symptoms subside spontaneously after 24 h in most cases, fatality rates range from 0.03% in the general population to 4.4 % in children and the elderly (5).

In the present study we describe a local SE foodborne outbreak in which we used statistical and microbiological methods to identify the likely cause. To do this, we investigated for the source of the outbreak by comparing the isolates obtained from the residual food and those from the biological samples of symptomatic patients and food handlers.

**Material and methods**

On the evening of 19 June 2011, 9 persons presented at the emergency room of two hospitals in the province of Turin because of abdominal pain and gastrointestinal symptoms (vomit and diarrhea) . All reported having attended a catered dinner party, together with 38 other guests, at a private home. An epidemiological investigation was carried out to determine the full extent of the outbreak and its probable source. Biological and food residual samples were collected and microbiological analyses performed.

**Epidemiological investigation**

All 47 dinner guests were contacted and administered a structured questionnaire by the Local Health Authority to collect information on food exposure and illness symptoms. The guests self-reported what food exposures they had and whether or not they became ill following the exposure. Information about sex, age, onset and nature of symptoms, and duration of illness were collected. From these data, the main symptoms and the time of onset were analyzed to determine the possible causes of the outbreak and to draw the epidemic curve. A retrospective cohort study was then conducted: the questionnaire responses were entered into a database, analyzed with Epi
Info™ 3.5, and the specific attack rate was calculated for each type of food served at the dinner party.

To measure the association between the food served and the illness symptoms, adjusted Mantel-Haenszel estimates of the risk ratio (RR) with a 95% confidence interval (CI) for each food item were calculated. A P value less than 0.05 was considered statistically significant.

**Laboratory investigation**

Stool and emesis samples were collected from 4 of the 9 hospitalized dinner guests and analyzed for pathogenic bacteria and toxins potentially responsible for the reported symptoms. Analyses for C. perfringens enterotoxin, S. aureus, and B. cereus were performed. Stool samples were also tested for Salmonella spp., Shigella spp., Campylobacter spp., Verotoxigenic E. coli (VTEC), Vibrio spp., Yersinia enterocolitica and Listeria monocytogenes. In addition, nasal swabs were collected from the 3 food handlers employed with the caterer and involved in the preparation of the food served at the dinner. S. aureus strains isolated from emesis, stool and nasal swab samples were sent to the Italian National Reference Laboratory for CPS (NRL for CPS - Turin) for the detection of genes encoding SEs by multiplex PCR (Method of the EU-RL CPS, Version 1, October 2009). The strains were sent to the European Reference Laboratory (EURL for CPS - ANSES France) for molecular characterization by PFGE. The pulsortypes were compared using BioNumerics® Software (Applied Maths).

Finally these isolates were analyzed with spa typing method (10). S. aureus protein A polymorphic region was amplified using forward and reverse primers, spa-1113f (5'-AAAGACGATCCTTCGGTGAGC-3') and spa-1514r (5'-CAGCAGTAGTGCCGTTTGCTT-3'). Sequences analysis was performed using BioNumerics® Software (Applied Maths), whereas numeric spa repeats and spa type codes were assigned through the Ridom StaphType server (Ridom GmbH, Germany).

Food samples were collected and sent to the Istituto Zooprofilattico of Piemonte, Liguria and Valle d'Aosta (IZS) for enumeration of CPS (ISO 6888-2), B. cereus (ISO 7932), and C. perfringens (ISO 7937). The samples were also analyzed for Salmonella spp. (ISO 6579), SEs (EU-RL Method, Ver. 5, 2010), and B. cereus diarrheal toxin (Internal Method).

**Results**

**Epidemiological and clinical characteristics of cases**

All 47 dinner guests completed the questionnaire (response rate, 100%) and 26 met the case clinical criteria definition, yielding an overall attack rate of 55%. The 26 cases reported the following clinical symptoms: vomiting (85%), diarrhea (77%), abdominal pain (69%), nausea (62%), epigastralgia (31%), chills (27%), headache (8%), sweating (7%) and bitterness in the throat (7%). Nine of these 26 (34%) guests presented at the hospital emergency room because of the severity of symptoms.

First case clinical symptoms started 1 hour and 30 minutes after the dinner and the last case occurred 10 hours and 30 minutes after the meal (Fig. 1). The median incubation period was 4.25 hours; the duration of illness ranged from 30 minutes to 36 hours (median, 8 hours and 10 minutes).

**Food risk assessment**

The data obtained through the questionnaires on food items consumed at the dinner all pointed to the seafood salad as the most likely source of the outbreak: the attack rate for persons who ate the meal was 78% and the RR was 11.72 (95% CI, 1.75-78.54; Mantel-Haenszel: 20.65 p ≤0.0005).

**Laboratory investigation**
Stool and emesis samples from 4 of the 9 hospitalized dinner guests were analysed. All the biological samples tested negative for *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *C. perfringens*, *B. cereus*, *VTEC*, *Vibrio* spp., *Yersinia enterocolitica* and *Listeria monocytogenes*. Faeces and emesis samples tested positive for enterotoxigenic *S. aureus*. A total of 14 *S. aureus* were isolated from stool and emesis samples of the 4 patients (Table 1). All 14 isolates tested with the two multiplex PCR protocols showed the same enterotoxin gene profile, presenting genes encoding for SE types A and I (Fig. 2 a and b).

The nasal swab samples collected from the 3 food handlers involved in the dinner preparation tested positive for *S. aureus* (Fig. 3 a and b) carrying the enterotoxin gene profile shown in Table 2. The PCR results confirmed that the *S. aureus* isolated from the nasal mucosa of one food handler (no. 3) carried the same encoding sequences (*sea* and *sei*) as those deriving from the 14 biological samples of the patients.

In order to characterize at the genomic level of the 15 isolates and to determine their genetic correlation, we carried out PFGE and *spa* typing analysis. With PFGE method the 15 isolates were found to belong to the same clade, with a 100% degree of homology between their profiles (Fig. 4). The presence of the same genetic band pattern, as analyzed with BioNumerics® software, confirmed the genetic correlation between the *S. aureus* isolates. According to *spa*-typing, the 15 isolates were grouped into the same comparisons database and two *spa* types was assigned: t701 for 14 isolates (93.33% degree of homology) including 13 patients biological samples and *S. aureus* isolated from the nasal mucosa of one food handler (no. 3). The other isolate deriving from biological sample resulted in t267 *spa* type.

Microbiological analyses of the food residues other than the seafood salad (no more of which was available) showed a CPS concentration below the limit of quantification (LOQ) of the method; no significant positivity for other microbiological contaminations was found in the food samples.

**Discussion**

The results of the present investigation shed light on the epidemiological and genetic aspects of this foodborne illness outbreak. One of the criteria for confirmation of a staphylococcal food poisoning outbreak is isolation of an organism belonging to the same strain from stool or vomit from two or more ill people (21). Microbiological analysis permitted to isolate enterotoxigenic *S. aureus* from the stool and emesis samples of the hospitalized cases and from the nasal swabs of the food handlers employed with the caterer. The *S. aureus* isolates were evaluated using PCR methods to identify the SE genes. Multiplex PCR allows to identify numerous SE targets when other more traditional methods, such as ELISA, are unavailable for identifying all toxins. PCR demonstrated that the 15 isolates, 14 from the patients’ biological samples and one from the nasal mucosa of 1 food handler, carried the same SE encoding genes (*sea* and *sei*). The presence of the *sea* gene in *S. aureus* deriving from food matrices, and its encoded toxin, is in line with previous data (7;8).

This study confirms that the combined use of molecular methods would be a practical tool to match clinical isolates with food isolates and/or swabs. Furthermore, such an approach would help to identify potential outbreaks that would otherwise not be possible using traditional microbiological methods. *S.aureus* isolates (n=15) resulted to belong at t701 and t267 *spa*-types: they own similar repeats (10 units), differing in number of one repeating unit (figure 5). Patients biological samples isolates (n=13) and *S. aureus* isolated from the nasal mucosa of one food handler (no. 3) belong to the same *spa* type (t701), already reported in a case of outbreak.
(22), whereas the other belong to t267 spa type. PFGE characterization, currently considered the gold-standard technique for molecular typing of isolates deriving from a suspected common source (1), allowed us to determine the genetic relatedness of the 15 S. aureus isolates (100% homology degree), providing convincing evidence about the source of food contamination. Because of the spa type percentage of similarity is based on the analysis of repeated units of shared or analogous sequences, 88% of similarity degree permits to consider genetically related two or more strains. Matching molecular data permits to confirm epidemiological correlation between patients strains and food handler. Due to the different discriminatory power of the two techniques, as demonstrated in a previous study (22), there is the possibility to have the same PFGE patterns but different spa types.

In contrast, microbiological analysis of the residual food samples tested negative for S. aureus. However, the statistic epidemiological analysis of food risk assessment demonstrated a significant attack rate for the seafood salad that was no longer available for sampling for microbiological analysis.

The molecular characterization provided epidemiological evidence and the microbiological testing of the biological samples allowed us to identify as the possible source of contamination an enterotoxigenic S. aureus strain deriving from one of the food handlers employed with the caterer. Although the study lacks complete microbiological data for all food samples, the results offer clues that the seafood salad could have been contaminated with S. aureus strains able to synthesize SEA and SEI toxins in that food matrix. The growth of enterotoxigenic S.aureus strains to more than 10^6 cells per g of food is generally considered necessary to produce a sufficient amount of enterotoxin to cause foodborne intoxication (14). As demonstrated in a previous study, (12) food temperature is one of the most important factors that affect SEs production: the study demonstrated that the amount of SE is positively correlated to the growth of S. aureus at various storage temperatures, except for 17°C.

Food safety authorities require that food processors identify and control steps critical to food safety: good practice codes (Good Manufacturing Practices [GMP] and Good Hygienic Practices [GHP]) comprise the fundamental principles, procedures, and means for ensuring an environment is suitable for safe food production (24). GMP programmes cover both the safety and quality aspects of food production, including GHP and Hazard Analysis and Critical Control Points (HACCP). GHP comprises the essential principles of food hygiene applicable throughout the food chain so that food is safe and suitable for human consumption; while the HACCP system identifies, evaluates, and controls hazards relevant for food safety. A solid GHP system is a prerequisite to implementing HACCP. Our study underscores the importance of applying GHP in each step of food treatment and the presence of S. aureus as a common opportunistic pathogen in the food chain.

Acknowledgments

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Figure legends

FIGURE 1. Epidemic curve of the outbreak.

FIGURE 2 (a). Enterotoxin gene profiles of *S. aureus* strains isolated from emesis and stool of patients. Agarose gel electrophoresis of multiplex PCR products for detection of *sea* to *see* and *ser* genes: lane M, marker (50-bp ladder); lanes 1-3, strains from emesis of patient n.1; lanes 4-6, strains from emesis of patient n. 2; lanes 7-8, strains from emesis of patient n. 3; lane 9-10-11-12, positive controls; lanes 13-15, strains from stool of patient n. 3; lanes 16-18, strains from stool of patient n. 4; lane 19, negative control (free of DNA). (b). Enterotoxin gene profiles of *S. aureus* strains isolated from emesis and stool of patients. Agarose gel electrophoresis of multiplex PCR products for detection of *seg* to *sej* and *sep* genes: lane M, marker (50-bp ladder); lanes 1-3, strains from emesis of patient n. 1; lanes 4-6, strains from emesis of patient n. 2; lanes 7-10, strains from emesis of patient n. 3; lane 8-9, positive controls; lanes 11-13, strains from stool of patient n. 3; lanes 14-16, strains from stool of patient n. 4; lane 17, negative control (free of DNA).

FIGURE 3 (a). Enterotoxin gene profiles of *S. aureus* strains isolated from nasal mucosa of food handlers. Agarose gel electrophoresis of multiplex PCR products for detection of *sea* to *see* and *ser* genes: lane M, marker (50-bp ladder); lane 1, strain from food handler n. 1; lane 2, strain from food handler n. 2; lane 3, strain from food handler n. 3; lane 4-5-6-7, positive controls; lane 8, negative control (free of DNA). (b). Enterotoxin gene profiles of *S. aureus* strains isolated from nasal mucosa of food handlers. Agarose gel electrophoresis of multiplex PCR products for detection of *seg* to *sej* and *sep* genes: lane M, marker (50-bp ladder); lane 1, strain from food handler n. 1; lane 2, strain from food handler n. 2; lane 3, strain from food handler n. 3; lane 4-5, positive controls; lane 6, negative control (free of DNA).

FIGURE 4. PFGE profiles of *S. aureus* isolated strains. Lane M, reference strain (*S. aureus* NCTC 8325); lanes 1-3, strains from emesis of patient n.1; lanes 4-6, strains from emesis of patient n. 2; lanes 7 and 8, strains from emesis of patient n. 3; lanes 9-11, strains from stool of patient n.3; lanes 12-14, strains from stool of patient n. 4; lane 15, strain from nasal mucosa of food handler n. 3.
Table 1. Number of *S. aureus* strains isolated from emesis and stools of patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Emesis samples (strain number)</th>
<th>Stool samples (strain number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>No. 2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>No. 3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>No. 4</td>
<td></td>
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</tbody>
</table>

Table 2. Enterotoxin gene profile of *S. aureus* isolated from food handlers.

<table>
<thead>
<tr>
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<th>Multiplex PCR protocol no.1</th>
<th>Multiplex PCR protocol no.2</th>
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<td></td>
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<td>seg seh sei sej sep</td>
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<td>No. 1</td>
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<td>pos</td>
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<tr>
<td>No. 2</td>
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</tr>
<tr>
<td>No. 3</td>
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<td>pos</td>
</tr>
</tbody>
</table>
Figure 2 (a)
Figure 2 (b)
Figure 3 (a)
Figure 3 (b)