Whole-genome sequencing combined with a case-control study of an outbreak of staphylococcal food-poisoning

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Running Title: Staphylococcal food poisoning outbreak in South Korea

Abstract
This study reports an outbreak of *Staphylococcus aureus* food poisoning in Chungcheongnam-do, Korea, in 2018; a case-control study was conducted among participants of a village festival after they consumed a commercially catered buffet lunch. Whole-genome sequencing (WGS) was performed for *in silico* molecular typing and to investigate the genetic relationships among the *S. aureus* isolates. The analysis of the clustering of isolates identified by using WGS allowed us to identify a source of the outbreak. *In silico* multilocus sequence typing (MLST) and *Staphylococcus aureus* protein A (*spa*) typing analyses indicated that the majority of *S. aureus* isolates, including those from patients, food, and a food handler, showed identical sequence types (STs) and *spa* types (MLST6 and *spa* t304, respectively). The phylogenetic results showed their genetic relationship, suggesting that the contamination causing the outbreak most likely originated from the food handler. In this report, we combined WGS data with information from a case-control study to investigate a foodborne disease.

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outbreak caused by \textit{S. aureus} in Korea. This approach is a major improvement in foodborne disease outbreak detection and defines pathogen sources throughout the food chain.

**Keywords:** \textit{Staphylococcus aureus}, outbreak, foodborne, whole-genome sequencing (WGS), case-control study

**Introduction**

Foodborne diseases are major public health concerns worldwide \cite{1}. Staphylococcal food poisoning (SFP) is one of the most common causes of foodborne illness in the world \cite{2-4}. Each year in the United States, more than 240,000 people develop SFP \cite{5}, and in Europe, the number of SFP outbreaks reported by the European Food Safety Authority (EFSA) was 114 SFP outbreaks in 2018, 2.2\% of which were associated with reported bacterial toxins \cite{6}. In Korea, from 2002 to 2011, 174 SFP outbreak cases were reported, accounting for 7.4\% of all foodborne infection outbreaks \cite{7}. In recent decades (2007-2016), on average, 13.1 foodborne illness outbreaks involving 344.6 patients per year have been documented \cite{8}.

\textit{Staphylococcus aureus} produces a wide variety of toxins, including staphylococcal enterotoxins (SEs), which are causative agents of SFP \cite{9}. To date, 23 SEs have been reported \cite{10}, and a wide variety of SEs causing SFP outbreaks in humans have been reported \cite{11, 12}. Food handlers who carry enterotoxigenic staphylococci in their nostrils or on their hands are regarded as the main source of food contamination \cite{13}. The onset of SFP symptoms commonly occurs between 0.5 and 6 hours, and illness typically lasts for 1 day (up to 3 days), with rapid recovery \cite{14}. However, staphylococcal infections can be life threatening when they become systemic and spread to vital body organs \cite{15, 16}.

For the effective and rapid control of outbreaks, the early detection of outbreaks is necessary, and molecular typing has played an important role in outbreak investigations. With the rapid advances in technology in recent years, new molecular techniques, such as next-generation sequencing (NGS), have been applied to investigate outbreaks \cite{17-19}. The use of NGS in outbreak analysis facilitates rapid and accurate identification of the pathogen and identifies the transmission patterns of the pathogen, helping rapidly control an outbreak \cite{20, 21}.

In Korea, \textit{S. aureus} has been reported as a major cause of food poisoning in addition to pathogenic \textit{Escherichia coli}, norovirus, and \textit{Salmonella} \cite{8}; however, there have been no published reports regarding the use of whole-
genome sequencing (WGS) to investigate *S. aureus* outbreaks. In this study, we describe a practical and effective approach to determine the source and routes of infection in a foodborne *S. aureus* outbreak that occurred at a village festival in Korea in November 2018 by combining epidemiological studies and WGS.

**Materials & Methods**

**Epidemiological study**

On November 30, 2018, the local public health center was notified of a suspected foodborne outbreak at 6:30 pm. On that day, a village festival with 200 participants occurred on Wonsan Island, which is located in Boryeong, Chungcheongnam-do, Korea. Food was served at 11:00 am by a caterer providing a buffet of pan-fried fish filet, rice cakes, spicy pork and several other dishes. A case-control study was conducted to find evidence for or against potential sources. A case was defined as any person with an acute onset of symptoms after consuming the commercially catered buffet for lunch on the day of the festival. A control was defined as anyone who attended the buffet on that day but had not presented any symptoms. The food consumption, sociodemographic and clinical data of patients were collected by a structured questionnaire [22]. Epidemic curves were created using data from 26 symptomatic subjects. The odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the association between each food and the case or control group, in which values for unknown or no responses were excluded from analysis. The data were analyzed using Microsoft Excel 2013 (Microsoft Corp., USA).

**Laboratory tests**

Rectal swab samples collected from 26 members of the case group and 30 food handlers as well as 10 samples from the leftover food, 4 environmental surface samples, and 5 water specimens were transported to the provincial public health laboratory. Briefly, the pretreated samples were inoculated in selective and enriched media to identify the presence of bacteria (*Salmonella* spp., *Shigella* spp., *S. aureus*, *Bacillus cereus*, *Vibrio* spp., *Clostridium perfringens*, *Listeria monocytogenes*, pathogenic *E. coli*, *Campylobacter jejuni/coli*, and *Yersinia enterocolitica*) and viruses (Norovirus, Astrovirus, Enteric Adenovirus, Rotavirus, and Sapovirus). Additionally, preserved rice cakes (n=3), which were prepared in a shop located approximately 100 km away from the location of the outbreak, were tested for bacteria and viruses (Supplementary Table 1). Suspected colonies were isolated on a mannitol salt agar plate and identified as *S. aureus* by the Vitek 2 system used with
the gram-positive (GP) identification card (bioMérieux, France) and API Staph identification kit (bioMérieux, France) [22]. For the correlation analysis between the strains isolated from humans and foods, all isolated *S. aureus* were subjected to pulsed-field gel electrophoresis (PFGE) [23], and the results were plotted in a dendrogram with the dice similarity index method using BioNumerics v.5.1 (Applied Maths, Belgium).

Antimicrobial susceptibility testing (AST) was also conducted by using the broth microdilution method with the Sensititre panel and interpreted according to the CLSI guidelines [24]. Susceptibility to 18 antimicrobial agents was tested: erythromycin (ERY), clindamycin (CLI), quinupristin/dalfopristin (SYN), daptomycin (DAP), vancomycin (VAN), tetracycline (TET), ampicillin (AMP), gentamicin (GEN), levofloxacin (LEVO), linezolid (LZD), ceftriaxone (AXO), streptomycin (STR), penicillin (PEN), rifampin (RIF), gatifloxacin (GAT), ciprofloxacin (CIP), trimethoprim/sulfamethoxazole (SXT), and oxacillin (OXA). The strains for PFGE and AST were cultured in trypticase soy agar for 20 hours at 37 °C.

**Whole-genome sequencing**

We analyzed *S. aureus* isolates by using WGS. For WGS, genomic DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen, USA) according to the manufacturer’s instructions, and sample libraries were prepared using a Nextera DNA Flex library preparation kit (Illumina, Inc., USA). WGS was performed on an Illumina MiSeq platform using v3 reagent kits generating 2×300 bp paired-end reads (Illumina, USA) to obtain an average genome coverage greater than 100x for all the isolates. Sequence reads were quality filtered and trimmed using BBduk (http://jgi.doe.gov/data-and-tools/bb-tools/) and de novo assembled into contigs using SPAdes v3.12.0 [25]. The assembled WGS contigs were submitted to multilocus sequence typing (MLST) 2.0 on the Center for Genomic Epidemiology (CGE) website [26] to identify the multilocus sequence types (STs). The isolates for which the ST was classified as unknown were assigned using the *S. aureus* MLST database (https://pubmlst.org/). SCCmec typing was performed using WGS and SCCmec finder 1.2 on the CGE website in addition to multiplex PCR methods [27]. The presence of *S. aureus* virulence genes was identified in the VFDB database [28] using the BLAST tool of Geneious Prime 2019.2.1 [29]. The genes encoding enterotoxins were screened according to a previous report, which included a total of 99 sequences of staphylococcal enterotoxins and their variants [30].

**Phylogenetic analysis**
Sequences were mapped to *S. aureus* NCTC 8325, accession number NC_007795.1, as a reference genome using BioNumerics version 7.6 (Applied Maths, Belgium). A set of SNPs was deduced for each genome sequence data set using the BioNumerics whole genome SNP (wgSNP) module. The refined SNP matrix was used to generate a minimum spanning tree using the maximum bootstrap maximum likelihood approach, yielding the phylogenetic tree.

Results

Epidemiological investigation

The case-control study involved 86 participants, including 26 participants with symptoms and 60 participants without symptoms, and all 86 participants completed the questionnaires. The outbreak affected 26 out of 200 people who attended the festival (attack rate of 13%). Approximately one hour after ingesting a variety of foods, the first guests complained about abdominal pain and diarrhea, and there were no additional patients by approximately 6:00 pm. The mean incubation time was 4.7 hours (at least 1 hour, at most 6.2 hours) (Figure 1). None of the patients had serious symptoms. The symptoms included vomiting (88.5%), diarrhea (69.2%), nausea (42.3%), abdominal pain (42.3%), and chills (34.6%). The results from the case-control study implicated that the odds of having been exposed to rice cakes in those who had symptoms was 8.87 times that of people who did not consume rice cakes (OR (95% CI): 8.87 (1.96–40.11)), suggesting that rice cakes were the most likely vehicle of food poisoning served for lunch on the festival day (Table 1).

Microbiological analysis

Among the 56 rectal swabs collected from the patients and food handlers, 17 samples were positive for *S. aureus*. Among the 17 *S. aureus* isolates, one was isolated from a food handler who was involved in the packing of the rice cakes. In addition, 7 *S. aureus* isolates were isolated from leftover food; in particular, three were isolated from the rice cake shop. No microorganisms were found in the environmental samples, including those from cookware and drinking water (Supplementary Table 1). The antimicrobial susceptibility test of all 24 *S. aureus* isolates indicated that they were all susceptible to all antimicrobials tested.

Sequencing analysis

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Based on whole-genome analysis, the STs could be assigned for 21 (87.5%) of the 24 isolates, and a *S. aureus* protein A (*spa*) type was assigned for all 24 isolates. There were two *spa* types, t304 and t11652, among the 24 isolates, and *spa* t304 was the predominant type (n=21). All *spa* type t304 isolates belonged to ST6, while the ST of *spa* t11652 was unknown. The unknown ST was assigned as ST5870. Furthermore, we performed a SCCmec analysis for all 24 isolates, and none of the isolates tested positive for the *mecA* gene [31], which was compatible with the PCR results. The screening of various virulence genes based on the WGS data indicated that virulence genes, including *selX*, *hlb*, *hld*, *hlgA*, *hlgB*, *hlgC*, *hly/hla*, *lukD*, *lukE*, *aur*, *geh*, *sspA*, *sspB*, and *sspC*, were positive in the isolates belonging to ST6. On the other hand, the other three isolates were positive for *sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seu*, *sak*, *sn*, *hld*, *hlgA*, *hlgC*, *geh*, *sspB*, and *sspC*. Notably, the staphylococcal enterotoxin-like W (*selW*) gene was identified in all 24 isolates (Figure 2, Supplementary Table 2).

**Phylogenetic tree based on SNPs**

The genetic relatedness of the 24 isolates is shown in a phylogenetic tree (Figure 2). The whole-genome phylogeny of all the isolates showed that they clustered into two distinct groups, and their grouping was completely dependent on their *spa* type and ST. The first group contained the isolates that belonged to ST6 and *spa* type t304. In this group, genotypically identical strains were isolated from 13 patients, the food handler and 7 leftover foods (rice cakes, spicy pork and pan-fried fish filet). The second group belonged to *spa* type t11652 and ST5870. Based on the topology of the tree and the lengths of the branches, we confirmed that the two groups were not genetically related. The SNP-based cluster analysis of the isolates belonging to ST6 and *spa* type t304 revealed that the maximum SNP distances were no greater than 15 SNPs (Figure 3), suggesting that these isolates were within the range of epidemiological linkage.

**Discussion**

Outbreak investigations typically rely on spatiotemporal data and PFGE, but WGS is also being increasingly used [32, 33]. WGS can provide higher discriminatory power than any other typing method. Therefore, applying WGS in outbreak investigations can enable the detection of origins of contamination quickly and accurately, resulting in a substantial reduction in the size of an outbreak [20, 34].

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The case-control study revealed that the outbreak was linked to the consumption of rice cakes. The identification of *S. aureus* in a rice cake product from the rice cake shop and on an employee who worked in the rice cake shop strongly suggested that the employee was an *S. aureus* carrier. There was no evidence of contamination in the drinking water, and the risk of illness from eating the other food items served was minimal and not statistically significant.

In an attempt to understand the routes of transmission, we obtained genomic data from the *S. aureus* isolates. *In silico* typing and the phylogenetic tree based on the SNPs of all of the isolates indicated that there were two distinct clonal strains involved in this outbreak. The three isolates that belonged to ST5870-t11652 were genetically distant from isolates that were isolated from the food handler and leftover food, suggesting that they were unrelated to the consumption of contaminated food and thus could be excluded from this outbreak case. Note that the *spa* type t11652 was previously only reported from the isolates in Ireland from 2012-2013 according to the Ridom SpaServer (https://spa.ridom.de/spa-t11652.shtml). The other 21 *S. aureus* ST6-t304 isolates formed the predominant lineage, and they were closely related based on the phylogenetic tree. These results suggested that the rice cakes were contaminated by the food handler and that the other food was cross-contaminated by the rice cakes. Therefore, the WGS analysis supports the findings of the previous epidemiological investigation. According to previous reports, the *S. aureus* ST6 strains were the most common strains associated with foodborne outbreaks in China [35, 36]. In particular, ST6-t304 *S. aureus* was the most frequent clone and was related to four outbreaks in Hangzhou, China [37]. On the other hand, in Korea, the MLST of *S. aureus* related to food poisoning has varied, including ST6, ST1, ST59 and ST30, which were the major clones [38]. ST6-t304 *S. aureus* strains were isolated from the general German population as well [39]. Additionally, ST6-t304 methicillin-resistant *S. aureus* was also isolated from hospitals in the United Arab Emirates [40] and from retail food in Singapore [41].

This study has some limitations. A retrospective cohort study that included all participants in the village festival was not performed. The outbreak occurred on an island where the only means of transportation was by ship, and many participants were elderly people. Therefore, it was difficult to interview all of the participants. In addition, a rapid investigation of the environmental surfaces on which food was prepared was not conducted, resulting in no detection of *S. aureus* from environmental samples.
Here, we showed that WGS could be applied for SFP outbreak investigation and detection. These data are in agreement with other recent reports [32, 42] that WGS can be a tool to support outbreaks and epidemiological investigations.

Sequence data access
The genomic data of this study have been deposited with links to BioProject accession number PRJNA632636 in the DDBJ BioProject database.

Acknowledgements
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Conflicts of interest
The authors have declared no conflicts of interest.

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surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin
2017; 22(50).


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Figure 1. Cases of food poisoning gastroenteritis by onset of illness during the outbreak on November 30, 2018.
Figure 2. The phylogenetic tree based on SNP analysis using the BioNumerics pipeline. The data sets on virulence genes were integrated using iTOL [43]. Eight enterotoxin genes (sea, seg, sei, sem, sen, seo, seu, and selW) were detected in the isolates belonging to ST5870-t11652, and two enterotoxin genes (selX and selW) were detected in the isolates belonging to ST6-t304. The selW gene was identified in all 24 isolates.
Figure 3. The MSTs based on SNPs of ST6-t304 S. aureus isolates. The isolates that were isolated from patients (P), served foods (SFs), and rice cakes (RCs) in the cake shop are colored white, violet, and blue, respectively. The isolate from the food handler is highlighted in pink. Detailed information about the rice cakes and served foods is as follows: RC1, rice cake 1 in the cake shop; RC2, rice cake 2 in the cake shop; RC3, rice cake 3 in the cake shop; SF1, served rice cake; SF2, served rice cake with mugwort; SF3, served pan-fried fish filet; and SF4, served spicy pork.
## Table 1. Results from analytical case-control study of food exposure in the staphylococcal food-poisoning outbreak in Korea, November 2018

<table>
<thead>
<tr>
<th>Foods</th>
<th>Patients</th>
<th>Controls</th>
<th>p-value</th>
<th>Odds ratio</th>
<th>IC 95%&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Exposed</td>
<td>Unexposed</td>
<td>Total</td>
<td>Exposed</td>
<td>Unexposed</td>
</tr>
<tr>
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<td>17</td>
<td>26</td>
<td>14</td>
<td>65</td>
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<tr>
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<td>14</td>
<td>26</td>
<td>30</td>
<td>49</td>
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<tr>
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<td>26</td>
<td>47</td>
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<tr>
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<td>12</td>
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<tr>
<td>Panfried fish filet</td>
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<td>14</td>
<td>26</td>
<td>20</td>
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<tr>
<td>Japchae&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16</td>
<td>10</td>
<td>26</td>
<td>38</td>
<td>41</td>
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<tr>
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<td>12</td>
<td>26</td>
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<tr>
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<th>1.3357</th>
<th>0.5407</th>
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</tr>
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</table>

*IC 95%-Confidence Interval (95%)*

b Japchae: mixed dish of boiled bean threads, stir-fried vegetables, and shredded meat.