A Thesis for the Degree of Doctor of Philosophy

Molecular characterization of *Salmonella* spp. for traceback investigation

식중독 추적조사를 위한 살모넬라속의
분자생물학적 특성 연구

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Youngho Koh

Department of Agricultural Biotechnology
College of Agriculture and Life Sciences
Seoul National University

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지도교수 강 동 현

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위 원 장 이 도 엽 (인)
부위원장 강 동 현 (인)
위 원 이 동 우 (인)
위 원 김 순 한 (인)
위 원 박 상 현 (인)

Abstract

Student Number: 2011-31032

Koh, Youngho

Department of Agricultural Biotechnology

The Graduate School

Seoul National University

In Korea, non-typhoidal *Salmonella* is reported as one of the key organisms that frequently causing food poisoning along with pathogenic *Escherichia coli* and norovirus. The major outbreaks linked to *Salmonella* spp. occurred in 2022 were two reported cases associated with naengmyeon (cold noodle) and gimbap (seaweed roll), both of the two meals used eggs as ingredients. The average number of cases of recent five years from 2018 to 2022 increased 221% compared to previous five years from 2013 to 2017 by non-typhoidal *Salmonella* outbreaks. Salmonellosis is increasing a health burden worldwide, 10% of the world's four major causes of diarrhea, especially diarrhea under the age of 5, at 220 million yearly by the World Health Organization reports.

In particular, it is a representative food poisoning bacteria that needs to be very careful because there are mild diarrhea symptoms but also life-threatening cases. More than 2,500 serotypes have been reported biologically in *Salmonella* spp., making it difficult to test or manage them. The reason is that it is very difficult to determine whether the numerous *Salmonella* bacteria isolated from the investigation samples are the same during the investigation of the cause of food poisoning, and it is necessary to carefully approach the results as they determine the administrative responsibility. An important step in determining the cause of food poisoning is the process of determining whether the bacteria present in the food poisoning patient sample match the food consumed by the patient and the bacteria present in the related environmental sample. In this study, a laboratory method that confirming the correlation between isolated *Salmonella* strains to determine the cause of foodborne outbreak was presented through characteristic analyses and molecular typing studies.

In this study, we sought to investigate the various characteristics of *Salmonella* spp. isolated from raw chicken meats available in Korean markets. The data collected, such as food source of isolation, sampling information,

serotype, virulence, and genetic profile including sequence type, were registered in the database for further comparative analysis of the strains isolated from the traceback investigation samples. To characterize serotype, virulence, and gene sequences, we examined 113 domestically distributed chicken meat samples for contamination with *Salmonella* spp.

Phylogenetic analysis was conducted on 24 strains (21.2%) of *Salmonella* isolated from 113 commercially available chicken meats and by-products, using pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Serotyping of the isolated *Salmonella* spp. revealed *S.* Enteritidis in 11 strains (45.8%), *S.* Virchow in 6 strains (25%), *S.* Montevideo in 2 strains (8.3%), *S.* Bsilla in 2 strains (8.3%), *S.* Bareilly in 1 strain (4.2%), *S.* Dessau in 1 strain (4.2%), and *S.* Albany in 1 strain (4.2%). The genetic correlation indicated that 24 isolated strains were classified into 18 clusters with a genetic similarity of 64.4-100% between them. Eleven isolated *S.* Enteritidis strains were classified into 9 genotypes with a sequence identity of 74.4%, whereas the most distantly related *S.* Virchow was divided into five genotypes with 85.9% identity. Here, the MLST analysis indicated that the major Sequence Type (ST) of the *Salmonella* spp. isolated from

domestic chicken sold in Chungcheong Province belongs to the ST 11 and 16, which differs from the genotype of *Salmonella* isolated from imported chicken. The differential sequence characteristics can be a genetic marker for identifying causative bacteria for epidemiological investigations of food poisoning.

The strains associated with foodborne *Salmonella enterica* Thompson outbreaks in Korea have not been identified. Therefore, we characterized *S.* Thompson strains isolated from chocolate cakes linked to foodborne outbreaks in Korea. A total of 56 strains were isolated from preserved cake products, products in the supply chain distribution, the manufacturer's apparatus, and egg white liquid products used for cream preparation. Subsequently, serological typing, pathogenic gene-targeted polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), and whole-genome multi-locus sequence typing (wgMLST) were performed to characterize these isolates. The antigen formula of all isolates was 7:k:1,5, namely *Salmonella enterica* subsp. *enterica* Serovar Thompson. All 56 isolates harbored *invA*, *his*, *hin*, and *stn*, and were negative for *sefA* and s*pvC* based on gene-targeted PCR analyses. Based on PFGE results, these isolates were classified into one group

based on the same SP6X01.011 pattern with 100% similarity. We selected 19 strains based on the region and sample type, which were subjected to wgMLST. Although the examined strains showed 100% similarity, they were classified into seven clusters based on allelic differences. According to our findings, the cause of these outbreaks was chocolate cake manufactured with egg white liquid contaminated with the same *Salmonella* Thompson. Additionally, comparative analysis of wgMLST on domestic isolates of *S.* Thompson from the three outbreaks showed genetic similarities of over 99.6%. Based on the results, the PFGE and wgMLST combination can provide highly resolved phylogeny and reliable evidence during *Salmonella* outbreak investigations.

Keywords: *Salmonella* spp*., Salmonella enterica* serovar Thompson, sequence type, foodborne disease outbreak, pulsed-field gel electrophoresis, multi-locus sequence typing, whole genome multi-locus sequence typing

Contents

LIST OF FIGURES

List of Tables

Chapter I. General introduction

I-1. History of *Salmonella* Thompson outbreak, 2018

On September 5, 2018 around 12 o'clock, a report of food poisoning at two schools in Busan Metropolitan City was received by phone call and inter-governmental report system from the local public health center, and the number of suspected patients was more than 100 in each school. Around 13:00, the Ministry of Food and Drug Safety analyzed the diet the two schools consumed and determined whether the food ingredient suppliers registered in the early warning system were the same. As a result of the diet analysis, the same menu provided by the two schools was seaweed soup and chocolate cake. By around 14 o'clock, five more schools had received reports of food poisoning, and chocolate cakes were also included in the other school's diet, which was identified as suspected food for food poisoning. Around 15:00, a 'warning' was issued to all schools where the same food ingredients were supplied that food poisoning occurred from the supplier's food ingredients. In addition, the chocolate cake manufacturer visited the site, collected the remaining chocolate cake products, and conducted an inspection. As a result of collecting the same chocolate cake, which was scheduled to be delivered to a high school in Gwangju Metropolitan City around 18:00 and conducting

a rapid detection on, *Salmonella* spp. was detected around 01:00 on September 6 the following day, and *Salmonella* spp. could be suspected as the causative agent. Around 18:00, the supplier quickly obtained a list of suppliers, confirmed that it was delivered to almost 190 food facilities, and shared information with related agencies such as the Ministry of Education and the Korea Centers for Disease Control and Prevention to request close cooperation. A total of 12 food poisoning reports per day on September 5 co-occurred in five cities and provinces, including Busan, Gyeongnam-Do, Gyeongbuk-Do, Gyeonggi-Do, and Jeonbuk-Do. The spread of this chocolate cake was prevented as much as possible through a provisional ban on distribution sales around 22:00 on the same day. The seized inventory was about 3.4 tons, and the finished product was coated with chocolate cream on the surface of the cake and sprinkled with thin chocolate powder (slice) in the frozen delivery form after manufacturing the chocolate cake.

<September 6> *Salmonella* spp. was tentatively identified from rapid PCR tests using specific genes of foodborne pathogens in samples obtained from patients from six schools around 08:00 a.m., raising the possibility that the chocolate cake consumed was the suspected agent. It was confirmed that all

chocolate cakes to be provided to 2,594 students were blocked by schools issued through the 'early warning system' around 09:00. Around 10 o'clock, the company that manufactured the cake was checked, and all of the cream and cream ingredients such as egg white and finished products applied to the chocolate cake were collected and inspected. Around 12 o'clock, the Ministry of Food and Drug Safety jointly advised related agencies and related ministries not to consume problematic products, such as the current status of the investigation and the ban on distribution and sales of the cake in question. At around 18:00, a total of 28 group cafeterias where food poisoning occurred, and the number of suspected patients was 1,128.

On September 7, Same *Salmonella* spp. was detected in the egg white liquid used in the cream around 07 o'clock, and the investigation focused on the egg white liquid. The manufacturer banned the distribution and sale of other products using the same raw material and requested reinforcement of safety inspections for other raw materials. Around 18:00 on the same day, 52 facilities and 2,112 people were infected with food poisoning.

On September 8 around 09:00, we checked the distribution status of intermediate distributors who received eggs from farms that produce eggs,

secured supplies, and checked the manufacturers and bakeries that were delivered, but no further reports of food poisoning patients were confirmed. On the same day, 55 facilities for food poisoning occurred, and the number of suspected patients was 2,161.

On September 10, *Salmonella* spp. serological type, which caused this food poisoning around 15:00, was identified as *Salmonella* Thompson, and *S.* Thompson with the same genotype was identified in the specimen of patients, preserved food, egg white, and chocolate cake, and finally confirmed that the cause was *S.* Thompson. In addition, food poisoning outbreaks were additionally received at two schools at the same time, tentatively counting to 2,207 people in 12 cities and provinces and 57 locations. At around 18:00, the relevant ministries confirmed in a press release that *S.* Thompson food poisoning was caused by chocolate cake using contaminated cream with *S.* Thompson, and that all the ingredients and products in question were recalled and discarded, preventing the spread of food poisoning. The following describes the process of homogeneity analysis of *S.* Thompson, which is part of the investigation process for confirmation of the outbreak causative agent.

I-2. Limitation of current outbreak investigation

The definition of food poisoning in Article 2-14 of the Food Sanitation Act means an infectious disease or a toxin-type disease that is believed to have been caused or caused by microorganisms or toxic substances harmful to humans due to food consumption. It is stated as, and in accordance with Article 86, the obligation to report to the competent district office (public health center) when two or more people involved outbreak occur. The health center investigates and reports the patient information, such as the date of food poisoning, symptoms, the number of suspected patients, and food-consuming information to the Ministry of Food and Drug Safety and the Korea Center for Disease Control and Prevention. Immediately after the report, an epidemiological and trace back investigation of food poisoning begins, and efforts will be made to determine the cause of food poisoning with samples collected by patients, such as specimens, preserved foods, cutting boards, and knives, including environmental samples. Since it takes 3 to 7 days to separate and identify food poisoning bacteria from each sample, rapid tests such as PCR tests that can quickly check the presence of specific genes held by each food poisoning bacteria can be checked within 2 hours. The

advantage of genetic testing is that providing presumptive identification before culture methods are completed, reduces false negative results, and quickly traces the cause of contamination at food poisoning sites. For example, suppose *Salmonella* spp. is commonly detected in samples on the day of food poisoning. In that case, it is possible to infer diet and raw materials that are likely to be contaminated by *Salmonella* spp., and the estimated distribution materials are more likely to be collected from distribution chains or production facilities before they are exhausted. Preserved foods require a 3-day diet and environmental samples such as knives and cutting boards at the time of food poisoning, so the number of samples to be tested is 20 to 50, which is likely to miss the "golden time" that can be identified if rapid tests are not conducted.

In the case of a large-scale outbreak in Korea, contaminated ingredients are usually supplied to several food catering facilities, and if food poisoning occurs in one of the many group facilities, the rest of the facilities supplied by the same company should not consume the same contaminated ingredients initially. Therefore, the Ministry of Food and Drug Safety operates a system that allows all companies supplied to schools and other catering facilities to

be listed and alerted to all groups supplied from the same company if food poisoning is reported in one place. This system is called an "early warning system," and the contact information of related people, such as nutritionists who manage meals, is registered together to quickly identify and manage suspected food ingredients in the early stages of food poisoning. Since the norovirus school outbreak in 2006, the Ministry of Food and Drug Safety has been trying to improve outbreak investigation systems to prevent the spread of the outbreak and the rapid detection methods that can give species information on the day of the outbreak. A representative case that has operated in improvement for these efforts since the food poisoning report was *Salmonella* Thompson food poisoning caused by chocolate cake in September 2018, and this study attempts to describe the cause of food poisoning accidents.

I-3. Objectives of this study

Identifying bacteria from patients and tracing causative foods to investigate food poisoning is a procedure that requires considerable time and effort. According to many cases of food poisoning, such if food poisoning bacteria were not detected in the specimen of patients, if food poisoning bacteria were not detected in the ingested food, and if the food poisoning bacteria detected in the specimen and ingested food were different in species or genotypes even if in same species. Epidemiological investigations eventually affect the results according to the experience and capability of investigation. This is because there are many variables in each case that this series of investigations are conducted in accordance with the guidelines but may or may not result in a theoretical agreement. In the case of catering facilities, they are obligated to hold preserved food according to Food Sanitation Act Article 88, but in many cases, there are no leftovers, such as in restaurants, so most unknown causes of food poisoning are small-scale food poisoning accidents at these places.

The cases of chocolate cake foodborne outbreaks described in the text were where a large number of patients occurred, most of them at catering facilities such as schools, and an early warning system could provide traceable supply

records of chocolate cake delivery. Follow-up measures were taken in five to six days, including the rapid operation of the outbreak reporting system, securing samples for multiple patients, and rapid supply chain investigation followed by the recall and discard of the suspected products. In other words, more than half of the time required for testing to identify and confirm food poisoning bacteria in the past was reduced compared to cases that generally took more than 7 to 14 days. PCR-based rapid detection method has a great advantage in preventing the spread of food poisoning, and if not identified, it can lead to a continuous repetition of unknown causes.

In particular, *Salmonella* food poisoning, which has many serotypes, was actively used with the most accurate culture medium and rapid detection using PCR, so it was possible to predict food poisoning by *Salmonella* spp. within 13 hours from 12:00 on the day of September 5 to 01:00 on the next day. This case was recorded as a large-scale foodborne outbreak with 2,975 patients in 60 locations when the epidemiological investigation was finalized.

The specific objectives of this study were,

(i) to investigate the prevalence of *Salmonella* spp. in chicken meat samples in Korean retail store and collect them as many as possible

(ii) to characterize the isolates what virulence factors, genotypes, and correlations between the strains to evaluate the molecular methods aiming for the application on outbreak investigation

(iii) to applicate the characterization methods on the real case of outbreak investigation to determine causative agent and compare the discrimination power of molecular methods

(iv) to establish the strategy of systematic approach for the correlation analysis between the outbreaks how to investigate the origin of contamination in laboratory research level

Chapter II. Prevalence and characteristics of *Salmonella* spp. isolated from raw chicken meat in Korea

II-1. Introduction

Many foodborne illness outbreaks of harmful bacteria in foods are reported each year, and these human health threatening incidences have had various patterns [1]. Across the globe, outbreaks of food poisoning tend to occur in groups and become larger while the key pathogenic organisms causing food poisoning include *Salmonella* spp., pathogenic *Escherichia coli*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Vibrio parahaemolyticus* [2, 3]. Among them, food poisoning from *Salmonella* has become more frequent worldwide, making up a higher share of the number of food poisoning cases reported in the Republic of Korea [2-4]. The average number of individuals in a *Salmonella* outbreak has varied significantly. For instance, from 2009 to 2020, an average of 857 cases were reported each year of individuals becoming sick with *Salmonella*, which is the third-highest, behind 1,777 people infected with pathogenic *E. coli* and 1,161 with norovirus, according to the statistics report of the Ministry of Food and Drug Administration (MFDS), Republic of Korea [4]. In particular, as seen in the case of the nationwide foodborne outbreak of 2018, which was linked to chocolate cake distributed to 190 schools and facilities, food poisoning

attributed to egg white contaminated with *Salmonella* is likely to spread easily, leading to soaring numbers of patients. In addition, according to data from the Korea Meteorological Administration, the year 2018 was one of the hottest ever, with a total of 27.8 heat wave days that had a daily maximum apparent temperature of 33℃ or higher [5]. In terms of temperature-induced proliferation of *Salmonella* in eggs, it is reported that the maximum specific growth rate (log CFU/h) of *Salmonella* contamination caused by high temperatures (35℃) during heat waves increases from 0.39 to 0.86 compared to normal temperatures (25℃) [6], which means that the growth rate of each germ per hour under 35℃ is up to three times faster than under 25℃, indicating that extra care must be taken not only to prevent *Salmonella* contamination during the manufacturing process but to maintain cold chains to prevent proliferation in the distribution and consumption stages. *Salmonella* species are a group of bacteria that can live in the intestinal tract of animals. These bacteria are widely distributed in nature, particularly in livestock such as chickens, pigs, and cattle, as well as soil and water [3, 7]. They consist of two species: *Salmonella enterica* and *S. bongori*, and so far, more than 2,500 serotypes have been reported. *Salmonella* Enteritidis and

Salmonella Typhimurium are two major serotypes that cause food poisoning [3, 8, 9, 10]. In addition, these strains cause food poisoning in both humans and animals as they have no specificity in terms of who can be infected with them and develop illness; in most cases, contaminated food, improper handling, and distribution of meat and inadequate cooking cause illness in humans [3, 11, 12]. Known causes of *Salmonella* food poisoning include diverse foods such as poultry, eggs, meat, fish, and dairy products. In particular, *Salmonella* is a pathogenic bacterium that causes diseases in humans, making it a representative, hazardous factor that poses a threat to the safety of agricultural foods [13, 14]. According to the data from the Centers for Disease Control and Prevention (CDC), it was chicken that caused the most foodborne illness in the United States between 2009 and 2015; about 3,000 people were found to have suffered from food poisoning from eating contaminated chickens, and 64 out of 149 mass food poisoning cases were attributed to *Salmonella enterica* [15]. *Salmonella* species are present in the intestinal tract of such animals as ducks, chickens, cattle, and pigs and are transmitted to humans through contaminated food, causing symptoms in the gastrointestinal tract and food poisoning, even with a small amount of 100 to

1,000 CFU [15]. *Salmonella* species are disease-causing bacteria that proliferate in livestock products including chicken, and it is crucial that we properly manage food hygiene and public health to prevent outbreaks of foodborne illness [3]. Based on this background, we aimed to isolate *Salmonella* spp. from raw chicken meat distributed domestically, and the isolated bacteria were characterized by serotyping, virulence gene-targeted PCR, and use of PFGE and MLST. Furthermore, advanced methods were seek to overcome the limitation of PFGE that reveals its poor ability to differentiate for some *Salmonella* spp.

II-2. Materials and methods

II-2-1. Sampling

From February to April 2018, 113 samples of chicken and by-products sold in department stores, large discount stores and traditional markets in Chungcheong Province were collected. After purchase, the samples were kept in a cooling box for refrigeration while being transported to the laboratory for testing.

II-2-2. Isolation and identification of *Salmonella* spp.

In accordance with the testing method of the Korean Food Code laid out by the MFDS, a 25 g sample and 225 ml of Buffered Peptone Water (Oxoid, UK) were mixed thoroughly and enriched in the incubator at 37℃ for around 24 h. The enriched culture solution was added to two enrichment media, 1 ml to 10 ml of Tetrathionate medium (Biomerieux, Spain) and 0.1 ml to 10 ml of Rappaport-Vassiliadis medium (Oxoid, UK), which underwent secondary enrichment at 37℃ in Tetrathionate medium and 42℃ in Rappaport-Vassiliadis medium for 20-24 h. The secondary enrichment culture solution was smeared on the selective media of XLD agar (Oxoid, UK) and Brilliant green sulfa agar (Remel, UK), and cultured at 37℃ for 18-24 h, and then a typical colony was selected and subcultured in the nutrient medium, which was identified by Vitek MS (Biomeriux Inc., France).

II-2-3. Pathogenic gene analysis using PCR

Genes subject to genetic characterization of *Salmonella* were *invA, his, stn*, *sefA, spvC,* and *hin* for pathogenic and serotype identification. To extract

purely isolated strain DNA, a single colony was taken and DNA was extracted using automated equipment (EZ1 Advance XL, Qiagen, UK) according to the manufacturer's methods, and this was then used as the DNA template. For *his*, *invA*, and *stn* gene detection from *Salmonella* spp., 5 μl of the template DNA was put into the mixture using a detection kit according to the method proposed by the Korean manufacturer (Kogenbiotech Co., Ltd., Korea). This brought the total to 20 μl, with which real-time PCR (7500 Fast Real-Time PCR, Applied Biosystems, USA) was performed.

For *spvC*, *sefA*, and *hin* gene identification, real-time PCR and conventional PCR were used, referencing the methods of Bugarel *et al*. [16], Seo *et al*. [17] and Kim *et al*. [18], and the primer/probe PCR conditions used are shown in Table 1.

Table 1. Primers/probe and PCR conditions used in the present study.

Target gene	Sequence (5'-3')	Size (bp)	PCR cycling conditions
spvC	F: AATGAACTACGAAGTGGGCG R: TCAAACGATAAAACGGTTCCTC P: FAM-ATGGTGGCGAAATGCAGAGACAGGC-BHQ1	112	50℃, 2m→95℃, 10m→95℃, 15s→60℃, 1m : 40cycles
sefA	F: GGCTTCGGTATCTGGTGGTGTA R: GGTCATTAATATTGGCCCTGAATA P:Cy5-CCACTGTCCCGTTCGTTGATGGACA-BHQ2	98	50℃, 2m→95℃, 10m→95℃, 15s→60℃, 1m : 40cycles
hin	F: TCCATGAGAAAAGCGACTAAAAT R: AGCCGACTAATCTGTTCCTGTTC	572	95℃, 3m→95℃, 30s→57℃, 30s→72℃, 1m : 30cycles→72℃, 2m

For *spvC*, and *sefA* genes, 5 μl of the extracted DNA, 1 μl and 1.5 μl of forward and reverse primer (10 pmole/μl), respectively, 0.5 μl of probe (10 pmole/μl) and PCR mastermix (Kogenbiotech Co., Ltd., Korea) were used, bringing the total to 20 μl, after which real-time PCR (7500 Fast Real-Time

PCR, Applied Biosystems) was performed.

For *hin* gene, 5 μl of the extracted DNA, 1 μl of forward and reverse primer (10 pmole/μl) each, and PCR mastermix (Bioneer, Korea) were used to make a total of 20 μl, and then real-time PCR (C1000 Touch Thermal Cycler, Bio-Rad, CA, USA) was performed, and with the resulting product, a specific band was verified through electrophoresis with a 2% agarose gel.

II-2-4. Serology testing

Tests were conducted in accordance with the method provided by the MFDS [4] to verify serotypes of isolated strains. Antisera (BD, Difco) by somatic (O) antigen (A, B, C, D, E, Vi) and by flagellar (H) antigens (a, b, c, d, e, h, i, k, r, y, z) were used to perform slide and tube agglutination tests for identification of serotypes.

II-2-5. Pulsed-field gel electrophoresis (PFGE)

PFGE analysis of *Salmonella* spp. was performed in accordance with PFGE Standard Testing published by the MFDS. Pure-isolated strains were put into cell suspension TE buffer (100 mM Tris, 100 mM EDTA, pH 8.0) and

adjusted the concentration to optical density 0.8-1.0 measured at 610nm using a spectrophotometer. Then, 200 μl of 1.2% Seakem Gold agarose was added to the strain suspension, mixed gently, and immediately solidified in the plug mold. The solidified plug was transferred to 1.5 ml cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0; 1% sodium-lauroyl sarcosine) to which 50 μl of Proteinase K was added, and after reaction in a 55-L shaking water bath for 1.5-2 h, the plug was washed five times with plug wash TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) for 20 min.

A 1 mm-thick slice was cut from the washed plug and reacted at 37℃ for 2 h using 40 U/μl XbaI (Roche, Switzerland).

Electrophoresis was performed with the plug gel treated with the restriction enzyme using the electrophoresis equipment at 14oC for 18 h under an initial time of 2.16 s, final time of 63.8 s, a voltage gradient of 6 V/cm, and an included angle of 120°.

S. enterica serovar Braenderup BAA-664 standards were used as the size marker, and the testing was carried out in the same way as for isolated strains. Once electrophoresis was completed, the gel was put into the SYBR gold stain (Invitrogen, Country) and dyed for 30 min, and after decoloring, UV was used

for identification. Identified pictures were analyzed using the program BioNumerics (Applied Maths, Belgium).

II-2-6. Multi-locus sequence typing (MLST)

With MLST, the sequence type was identified by analyzing the sequences of seven house-keeping genes (*thrA, purE, sucA, hisD, aroC, hemD* and *dnaN*) (Table 2). As for the PCR reaction conditions, pre-denaturation was first performed at 94℃ for 10 min, followed by 35 cycles of denaturation at 94℃ for 1 min at, annealing at 55℃ for 1 min, elongation at 72℃ for 1 min, and a final extension at 72℃ for 5 min. Sequences were assembled and analyzed using Lasergene 7.2.1 software (DNAStar). Sequence type (ST) numbers were assigned by submitting the sequences and strain information to the *Salmonella* MLST website (http://www.pubmlst.org/organisms/salmonella-spp). The phylogenetic analysis was performed using MEGA 6 (version 6.05) for confirmation of their homology [19].

Table 2. PCR and sequencing primer for MLST used in this study.

Gene	Sequence (5'-3')	Product size (bp)
thrA	F: GTCACGGTGATCGATCCGGT R: CACGATATTGATATTAGCCCG	852
purE	F: GACACCTCAAAAGCAGCGT' R: AGACGGCGATACCCAGCGG	635
sucA	F: CGCGCTCAAACAGACCTAC R: GACGTGGAAAATCGGCGCC	793
hisD	F: GAAACGTTCCATTCCGCGC R: GCGGATTCCGGCGACCAG	788
aroC	F: CCTGGCACCTCGCGCTATAC R: CCACACACGGATCGTGGCG	826
hemD	F: GAAGCGTTAGTGAGCCGTCTGCG R: ATCAGCGACCTTAATATCTTGCCA	666
dnaN	F: ATGAAATTTACCGTTGAACGTGA R: AATTTCTCATTCGAGAGGATTGC	833

II-3. Results

II-3-1. Prevalence of *Salmonella* spp. from raw chicken meat

Among the 113 samples of chicken purchased in retail stores in Chungcheong

Province, 24 chicken samples (21.2%) were determined to be positive for *Salmonella* spp. (data not shown).

II-3-2. Distribution of *Salmonella* serotypes

The identified *Salmonella* serotypes are provided in Table 3. As a result of serotyping on 24 isolates of *Salmonella* bacteria, the group of O-antigens, in most cases, consisted of bacterial strains belonging to C - E. Various types were isolated, including *S*. Enteritidis in 11 strains (45.8%), *S*. Virchow in 6 strains (25%), *S*. Montevideo in 2 strains (8.3%), *S*. Bsilla in 2 strains (8.3%), *S*. Bareilly in 1 strain (4.2%), *S*. Dessau in 1 strain (4.2%), and *S*. Albany in 1 strain (4.2%).

Table 3. Serotypes of *Salmonella* spp. isolated from raw chicken meat

Sample No.	Source of isolates	Somatic Antigens			Flagellar Antigens		Serovar
		Group I	Group II	O-antigen	H phase 1	H phase 2	
1	Meat	C	Group O:8 (C2-C3)	6, 8	r	1, 2	*S.* Bsilla
2	Meat	D	Group O:9 (D1)	9, 12	g, m	-	*S.* Enteritidis
3	Meat	D	Group O:9 (D1)	9, 12	g, m	-	*S.* Enteritidis
4	Gizzard	D	Group O:9 (D1)	9, 12	g, m	-	*S.* Enteritidis
5	Meat	C	Group O:7 (C1)	$6_{1,2}$, 7	g, m, s	[1, 2, 7]	*S.* Montevideo
6	Meat	D	Group O:9 (D1)	9, 12	g, m	-	*S.* Enteritidis
7	Gizzard	D	Group O:9 (D1)	9, 12	g, m	-	*S.* Enteritidis
8	Meat	C	Group O:7 (C1)	$6_{1,2}$, 7	r	1, 2	*S.* Virchow
9	Meat	C	Group O:8 (C2-C3)	6, 8	r	1, 2	*S.* Bsilla
10	Meat	C	Group O:7 (C1)	$6_{1,2}$, 7	r	1, 2	*S.* Virchow
11	Meat	C	Group O:7 (C1)	6_{1}, 7	r	1, 2	*S.* Virchow
12	Meat	C	Group O:7 (C1)	$6_{1,2}$, 7	r	1, 2	*S.* Virchow
13	Meat	C	Group O:7 (C1)	$6_{1,2}$, 7, 14	y	1, 5	*S.* Bareilly
14	Heart	D	Group O:9 (D1)	1, 9, 12	g, m	-	*S.* Enteritidis
15	Meat	D	Group O:9 (D1)	9, 12	g, m	-	*S.* Enteritidis
16	Feet	C	Group O:7 (C1)	$6_{1,2}$, 7	r	1, 2	*S.* Virchow
17	Gizzard	C	Group O:7 (C1)	$6_{1,2}$, 7	r	1, 2	*S.* Virchow
18	Meat	E	Group O:1,3,19 (E4)	1, 3, 19	g, s ,t	-	*S.* Dessau
19	Meat	D	Group O:9 (D1)	9, 12	g, m	-	*S.* Enteritidis

20	Meat	C	Group O:8 (C2-C3)	8, 20	z_4, z_{24}	-	*S*. Albany
21	Meat	D	Group O:9 (D1)	9, 12	g, m	-	*S*. Enteritidis
22	Meat	C	Group O:7 (C1)	$6_{1,2}$, 7, 14	g, m, s	[1, 2, 7]	*S*. Montevideo
23	Meat	D	Group O:9 (D1)	9, 12	g, m	-	*S*. Enteritidis
24	Gizzard	D	Group O:9 (D1)	9, 12	g, m	-	*S*. Enteritidis

II-3-3. PCR targeted to pathogenic genes

The results from gene detection of the 24 isolates of *Salmonella* spp. are summarized in Table 4. The results reveal that all isolated *Salmonella* spp. from raw chicken meat have *invA*, *his*, and *stn* genes. On the other hand, the detection rate of *sefA*, *spvC*, and *hin* genes was 45.8% (11/24), 41.7% (10/24), and 37.5% (9/24), respectively.

Table 4. Pathogenic Gene-targeted PCR results of *Salmonella* serovars

Sample No.	**Serovar**	**Serological type**			**Real-time PCR**					**PCR**
		O-antigen Group	H Phase 1	H Phase 2	*his*	*invA*	*stn*	*sefA*	*spvC*	*hin*
1	*S*. Bsilla	C	r	1, 2	+	+	+	-	-	+
2	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
3	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
4	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
5	*S*. Montevideo	C	g, m, s	[1, 2, 7]	+	+	+	-	-	-
6	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
7	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
8	*S*. Virchow	C	r	1, 2	+	+	+	-	-	+
9	*S*. Bsilla	C	r	1, 2	+	+	+	-	-	+
10	*S*. Virchow	C	r	1, 2	+	+	+	-	-	+
11	*S*. Virchow	C	r	1, 2	+	+	+	-	-	+
12	*S*. Virchow	C	r	1, 2	+	+	+	-	-	+
13	*S*. Bareilly	C	y	1, 5	+	+	+	-	-	+
14	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
15	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
16	*S*. Virchow	C	r	1, 2	+	+	+	-	-	+

17	*S*. Virchow	C	r	1, 2	+	+	+	-	-	+
18	*S*. Dessau	E	g, s ,t	-	+	+	+	-	-	-
19	*S*. Enteritidis	D	g, m	-	+	+	+	+	- / -	-
20	*S*. Albany	C	z_4, z_{24}	-	+	+	+	-	-	-
21	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
22	*S*. Montevideo	C	g, m, s	[1, 2, 7]	+	+	+	-	-	-
23	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-
24	*S*. Enteritidis	D	g, m	-	+	+	+	+	+	-

II-3-4. Comparison of isolates of *Salmonella* spp. using PFGE

The PFGE results on the 24 *Salmonella* isolates, which were classified into 18 clusters, indicating a genetic similarity of 64.4-100%, is shown in Fig. 1. *S*. Enteritidis in 11 strains, isolated the most, was classified into 9 genotypes with a homology of 74.4%, followed by *S*. Virchow which was classified into 5 genotypes with an 85.9% homology.

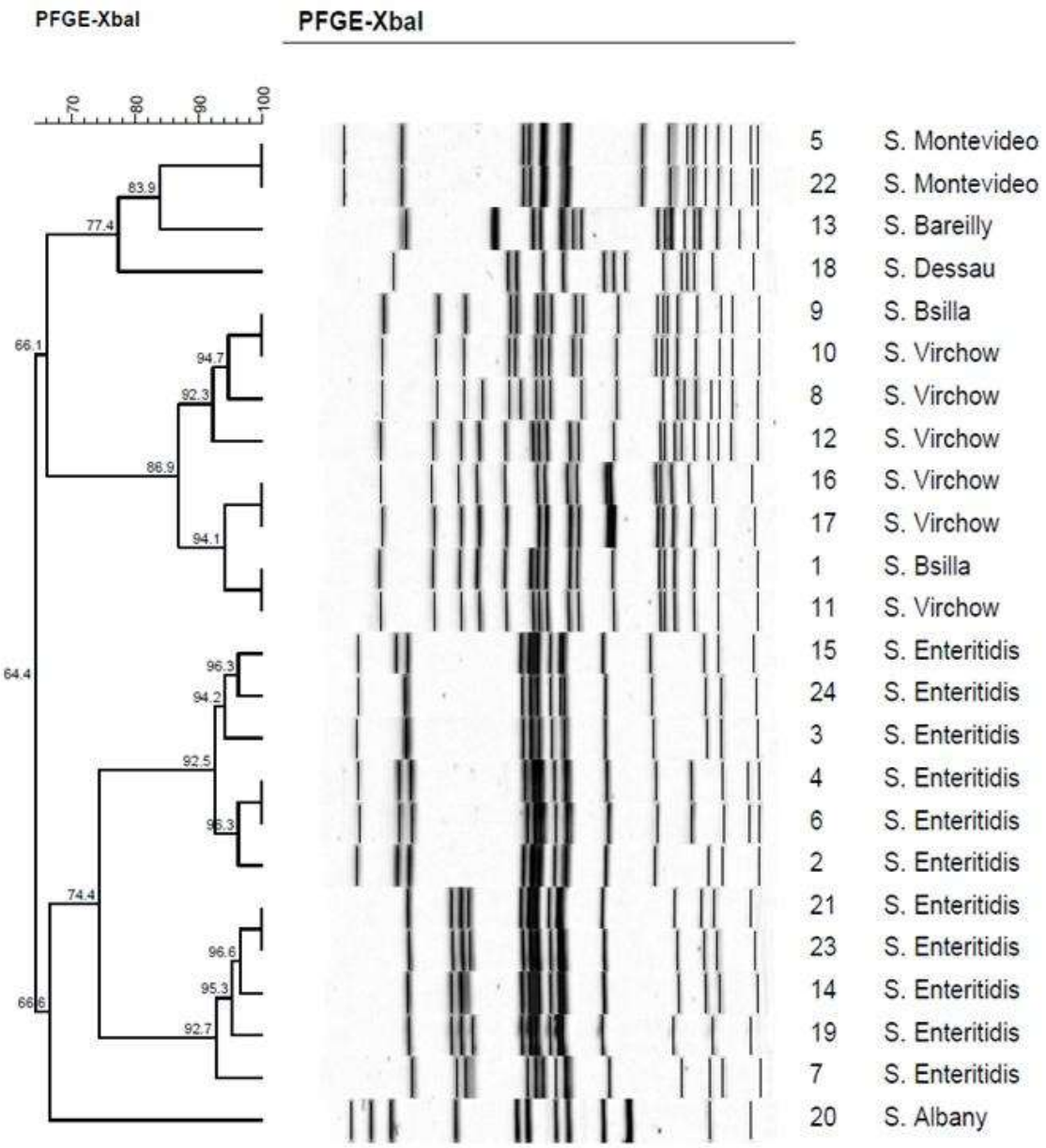


Figure 1. Relatedness of *Salmonella* spp. isolated from raw chicken meat by PFGE analysis with *Xba*I.

II-3-5. Multi-locus sequence typing analysis

Six Sequence Types (STs) from *Salmonella* spp. based on allele type for seven *loci* sequences are defined in Table 5. After being classified through the PubMLST program to verify the diversity of clones, 6 STs belonged to ST11 and 16. Of the 6 STs, ST11 (11 strains) and ST16 (8 strains) were the most common types. The rest of the strains were ST4 (2 strains), and ST203, 14, 292 (1 strain). In addition, in most cases, ST11, ST16, ST4, ST203, ST14, and ST292 appeared in isolates of bacterial species in 2001 but did not appear in those thereafter.

Table 5. ST definitions based on allele type for each of seven loci sequenced and assigned by the *Salmonella enterica* database

ST	Allele type							No. of isolates	% of total
	thrA	*purE*	*sucA*	*hisD*	*aroC*	*hemD*	*dnaN*		
11	11	6	6	7	5	3	2	11	45.8
16	14	8	10	10	6	10	7	8	33.3
4	4	34	13	13	43	16	41	2	8.3
203	17	68	12	12	81	36	69	1	4.2
14	13	7	8	8	7	8	6	1	4.2
292	48	104	9	78	104	54	100	1	4.2

II-4. Discussion

In this study, *Salmonella* spp. were isolated from 24 domestic raw chicken meat samples out of a total 113 samples for a 21.2% detection rate. According to Pilar *et al*. [20], *Salmonella* was detected in chicken collected from retail stores at a rate of 42%, and from supermarkets at 36%; a detection rate of 17.41% was found by a study conducted by Rodriguez *et al*. [21], and 8.3% from one by Anisa *et al*. [22], showing the difference in detection rate with this study. *Salmonella* bacteria infecting humans through contaminated eggs, poultry meats, and byproducts [23, 24, 25] can cause cross-contamination through diverse routes in the process of distribution. The serotypes of *Salmonella* spp. isolated from chicken meat were identified as *S*. Enteritidis (11/24, 45.8%), *S*. Virchow (6/24, 25%), *S*. Montevideo (2/24, 8.3%), *S*. Bsilla (2/24, 8.3%), *S*. Bareilly (1/24, 4.2%), *S*. Dessau (1/24, 4.2%), and *S*. Albany (1/24, 4.2%). Lee *et al*. [26] reported that, of isolates from 24 *Salmonella* bacterial strains, *S*. Enteritidis was isolated the most, at 70.8%; according to a study conducted by Kim et al. [27] on serotypes of *Salmonella* bacteria isolated from chicken meat, *S*. Enteritidis and *S*. Montevideo were found to be widely distributed. As a result of a study by Jung et al. [28] on

serotypes of *Salmonella* bacteria in chickens from 2003 to 2004, *S*. Enteritidis was reported to be present in about 52% (39/75). A study by Yang et al. [29] also revealed that of five serotypes, *S*. Enteritidis, *S*. Newport, *S*. Typhimurium, *S*. Derby, and *S*. Galinarum, *S*. Enteritidis was detected the most, at 46%. Based on earlier studies, the serotype isolated the most from chicken produced locally was *S*. Enteritidis, showing the same pattern as in the past. In terms of distribution of *Salmonella* bacteria in foreign countries from a Canadian study, *S.* Typhimurium (44.4%, 123/277) showed the highest rate of frequency, with Kentucky (32%, 120/382), Heidelberg (20%, 78/382) and Enteritidis (16%, 62/382), showing a difference from cases in South Korea in terms of the distribution pattern. The major serotypes causing *Salmonella*-derived food poisoning were reported to be *S*. Typhimurium, *S*. Heidelberg, *S*. Enteritidis, *S*. Thompson, and *S*. Montevideo [30]. In particular, *Salmonella* Enteritidis and *Salmonella* Typhimurium are bacterial strains that are most frequently related to food poisoning both worldwide and in South Korea. Various serotypes are isolated from source foods infected with *Salmonella*, including chicken and by-products, of which *S*. Enteritidis is found to be the most prevalent. With respect to genotypes of isolated

Salmonella spp. in this study, as described in the results, the *invA*, *his*, and *stn* genes were detected in all isolated *Salmonella* spp. Conversely, the detection rate of *sefA*, *spvC*, and *hin* genes was 45.8% (11/24), 41.7% (10/24), and 37.5% (9/24), respectively. Of genes related to *Salmonella* bacteria, those identified were: *inv*, related to adhesion and invasion into epithelial cells [31], *his*, involved in regulating histidine transport [32], *sef*A, which encodes fimbria and specifically detects *S.* Enteritidis [33], *spv*, that causes cytotoxicity by moving into the host cell [11], *Salmonella* enterotoxin (*stn*), which causes diarrhea by *Salmonella* invading the intestines, and *hin*, expressing flagella corresponding to the two flagellar antigens phase 1 and 2. PCR is used to specifically detect *Salmonella* by identifying *Salmonella*-related genes [34]. In this study, all the *Salmonella* spp. isolated from monitoring had genes such as *invA*, *stn,* and *his,* showing the same trend of earlier studies that *Salmonella* bacteria carried the *inv*A gene [35, 36]. The *sef*A gene that encodes thin filamentous fimbria of *S.* Enteritidis was detected in 11 *Salmonella* serogroup D isolates from this study, and there is a report that it is observed specifically in serogroup D1 [34, 37]. In the case of *spv*, a gene that expresses pathogenicity, derived from a plasmid that can specifically detect *S.*

Enteritidis, studies conducted by Araque and Chaudhary *et al*. [38, 39] reported that *spv*C was not detected in any isolates of *S.* Enteritidis. On the other hand, Soto *et al*. [40] reported that *spv*C was detected in all 60 strains of *S.* Enteritidis, indicating that plasmid-derived genes show different results depending on the bacterial strains used; it was found that all *S.* Enteritidis isolated in this study, had *spv*C. In addition, PCR with *hin* could identify a *hin*-specific product of 572 bp in 9 bacterial strains of 24 *Salmonella*. According to Kim *et al*. [18], it was found that *Salmonella* strains with monophasic flagella do not have the *hin* gene and that all monophasic *Salmonella* were expressed as phase 1. Furthermore, the study also reported that in the case of *Salmonella* bacteria with no *hin* gene, the composition of O-antigens and phase 1 of H antigens could identify serotypes of *Salmonella* bacteria without conducting a phase 2 test, similar to this study.

In this study, the serotype of *Salmonella* isolated from raw chicken and by-products was determined as *S.* Enteritidis, a representative serotype that causes food poisoning in humans. As shown here, *Salmonella* spp. isolated from domestic chicken sold in Chungcheong Province showed specific sequence types as the ST of *Salmonella* spp., which several studies reported

were isolated from Brazilian poultry. *Salmonella* Typhimurium isolated from poultry revealed ST-19 [41, 42] and most of the *Salmonella* Dublin isolates (n = 112) from human and animal presented ST-10 (n = 68), ST-3734 (n = 28), and ST-4030 (n = 9) [43]. The Sequence Type determined by the MLST can be used as an important clue for traceback investigation particularly when multiple outbreaks of foodborne illness derived from the same *Salmonella* spp. occur. For example, useful information can be obtained relatively fast when we analyze the suspected source of contamination between two or more independent outbreak cases. To be a meaningful clue, species identification, serotyping, and pathogenic gene-targeted PCR are carried out in advance according to the traceback investigation manual by the National Institute of Food and Drug Safety (NIFDS). If all the test results are decided to be identical between the strains, the sequence type determined by MLST is a useful marker for the final confirmation. Considering that the aim of an outbreak investigation is to find its source through comparing many strains isolated from a specimen, whether that may come from a sample of ingested food or from the environment, MLST can provide evidence for the coincidence of strains within the same outbreak. To that end, an accumulation

of data on *Salmonella* spp. as a food source of isolation, sampling information, serotype, virulence, and genetic data including sequence type, has been registered in the Integrated Foodborne Pathogen Data System operated by the NIFDS for further comparative analysis between strains. This study is in line with the data construction of *Salmonella* spp. with its virulence characteristics and sequence type of isolated from domestic poultry.

Along with the rapid growth of the global food trade, the consumption of food or ingredients has become highly dependent on importation, and the possibility of food poisoning sources from imported food is increasing. In the case of an outbreak suspected to be caused by an imported food source, a traceback investigation is conducted by authorities in the importing and exporting countries and the investigating country requests the gene sequence data of isolated pathogens from the suspected source, which becomes important scientific evidence for the investigation. So, it is crucial to monitor the prevalence and gene sequence profile of pathogens isolated from domestic products through sustainable national surveillance programs in response to a foodborne illness outbreak investigation as well as to protect the health of people and the agricultural industry. In this study, we attempted to investigate

the various characteristics of *Salmonella* including the prevalence of serotype, and gene sequence profile isolated from domestic raw chicken meats. As *Salmonella* food poisoning repeatedly occurs along with the consumption of poultry products worldwide, these gene sequence characteristics can be used as important clues to identifying causative bacteria for epidemiological investigations and traceback studies. Additionally, sustainable monitoring programs at the national level are necessary to establish gene sequence profiles of *Salmonella* isolates from various conditions, such as domestic and imported products, regional data, food type, and seasonal data.

Chapter Ⅲ. Comparative genomic analysis of *Salmonella enterica* subsp. *enterica* Serovar Thompson isolates associated with foodborne disease outbreaks in Korea

III-1. Introduction

In Korea, non-typhoid *Salmonella* represents one of the most frequent causes of foodborne disease outbreaks, following norovirus and pathogenic *Escherichia coli*. Food poisoning caused by *Salmonella* spp. is a global health threat, with 80.3 million cases reported annually [44]. Among the different species of *Salmonella*, *Salmonella enterica* has long been subclassified into serovars based on differential antibody reactions [45]. The use of specific antibodies that can identify distinct cell-surface antigens within lipopolysaccharides and flagella has led to the identification of over 2,500 serovars that differ in their antigenic formulas [46].

S. Enteritidis and *S*. Typhimurium that cause human salmonellosis are major serotypes found globally. Recently, mass infection cases caused by major serotypes as well as other serotypes have been reported. According to data from the National Institute of Health, the Korea Disease Control and Prevention Agency, infections related to the serotype of *Salmonella* in Korea show a diverse isolation trend every year, and in 2015, major serotypes isolated were in the following order: *Salmonella* Enteritidis, *Salmonella* I 4,[5],12:i:-, *S*. Bareilly, *S*. Typhimurium, and *S*. Montevideo [47, 48].

Due to the foodborne outbreak caused by *S.* Thompson in 2018, which is addressed in the present study, a total of 3,516 patients infected with *Salmonella* spp. were identified in 2018, including 2,975 patients from 12 cities and provinces across the country, and a prevalence of 3.6 times the annual average was recorded according to the statistical report of the Ministry of Food and Drug Safety (MFDS) [49].

In September 2018, 60 school meal services, including those in kindergartens, reported foodborne outbreaks [50], and 56 isolates were collected from the traceback investigation by regional public health centers and the MFDS. This outbreak involved the second-highest number of cases to occur in a school outbreak nationwide, following the norovirus school outbreak (over 3,000) in 2006 and the highest number of cases in a *Salmonella* outbreak in Korea.

When foodborne outbreaks occur, one of the most important laboratory analyses involves the determination of correlation among the strains. For example, if a strain isolated from a specimen of the patient matches exactly with that isolated from food samples, then the strain is confirmed as the cause of the outbreak. To investigate whether the source and isolates of these outbreaks were identical or independent and further matched with the isolates

obtained from specimens, we analyzed the phenotypic and genotypic characteristics of 56 strains in this study. Various methods are used to determine the similarities between strains, and the pulsed-field gel electrophoresis (PFGE) analysis method has long been used as the gold standard worldwide. Recently, various whole-genome sequencing analysis methods with relatively higher accuracy and precision have been researched with the development and generalization of next-generation sequencing technology. One of them is whole-genome multi-locus sequence typing (wgMLST), which is used as a standard method in PulseNet International [51]. In the present study, wgMLST was used for the analysis of correlation with outbreak strains, leading to more accurate results with high resolution, and we described the laboratory procedure for confirming the sources of outbreak via characterization analysis.

III-2. Materials and methods

III-2-1. *S.* Thompson isolation

S. Thompson strains were isolated according to the methods of the Korean Food Code and Guidelines for Laboratory Analysis of Foodborne Outbreaks

for food and environmental samples collected during the traceback investigation in six cities (Seoul, Busan, Daegu, Gwangju, Ulsan, and Jeju) and four provinces (Chungbuk, Gyeongbuk, Gyeongnam, and Jeonbuk) in South Korea. A total of 56 strains were isolated from preserved cake products, products in the supply chain distribution, the manufacturer's apparatus, and egg white liquid products used for cream preparation. A 25 g sample and 225 mL of buffered peptone water (Oxoid, Hampshire, United Kingdom) were mixed thoroughly and enriched in an incubator at 37°C for approximately 24 h. The enriched culture solution was added to two enrichment media, 1 mL of culture was added to 10 mL of tetrathionate medium (BioMérieux, Madrid, Spain) and 0.1 mL was added to 10 mL of Rappaport-Vassiliadis medium (Oxoid), which underwent secondary enrichment at 37°C in tetrathionate medium and 42°C in Rappaport-Vassiliadis medium for 20–24 h. The secondary enrichment culture solution was smeared on selective media including XLD agar (Oxoid) and brilliant green sulfa agar (Remel, London, United Kingdom) and cultured at 37°C for 18–24 h, and then a typical colony was selected and subcultured in the nutrient medium following identification using Vitek MS (BioMérieux Inc., Marcy-l'Étoile, France).

III-2-2. Serology testing

Serological tests were conducted in accordance with the method provided by the MFDS [47] to verify serotypes of isolated strains. Antisera (BD, Difco) with somatic (O) antigen (A, B, C, D, E, and Vi) and flagellar (H) antigens (a, b, c, d, e, h, i, k, r, y, and z) were used to perform slide and tube agglutination tests to identify the serotypes.

III-2-3. Pathogenic gene analysis using PCR

Virulence genes were screened for using PCR for 56 isolates. To extract DNA from pure strains, a single colony was taken and DNA was extracted using automated equipment (EZ1 Advance XL; Qiagen, Manchester, United Kingdom) according to the manufacturer's specifications and then used as the DNA template. The target genes were *invA, his, Salmonella* enterotoxin (*stn*)*, sefA, spvC*, and *hin* according to the following references: For *his*, *invA*, and *stn* gene detection in *Salmonella* spp., 5 μL of the template DNA was added to the mixture using a detection kit Powercheck™ *Salmonella* spp. (Kogenbiotech Co., Ltd., Seoul, Korea) according to the method proposed by the manufacturer and made up to a total volume of 20 μL, using which real-

time PCR (7500 Fast real-time PCR; Applied Biosystems, Carlsbad, CA, USA) was performed. For *spvC*, *sefA*, and *hin* gene detection, real-time PCR and conventional PCR were performed according to the methods of Bugarel *et al*. [52], Seo *et al*. [53], and Kim *et al*. [54]; the primer/probe PCR conditions used are listed in Table 6.

Table 6. Primers/probe and PCR conditions used in the present study

Target gene	Sequence(5'-3')	Size (bp)	PCR cycling conditions
spvC	F: AATGAACTACGAAGTGGGCG R: TCAAACGATAAAACGGTTCCTC P: FAM-ATGGTGGCGAAATGCAGAGACAGGC-BHQ1	112	50℃, 2m→95℃, 10m→95℃, 15s→60℃, 1m : 40cycles
sefA	F: GGCTTCGGTATCTGGTGGTGTA R: GGTCATTAATATTGGCCCTGAATA P:Cy5-CCACTGTCCCGTTCGTTGATGGACA-BHQ2	98	50℃, 2m→95℃, 10m→95℃, 15s→60℃, 1m : 40cycles
hin	F: TCCATGAGAAAAGCGACTAAAAT R: AGCCGACTAATCTGTTCCTGTTC	572	95℃, 3m→95℃, 30s→57℃, 30s→72℃, 1m : 30cycles→72℃, 2m

For *spvC* and *sefA* detection, 5 μL of the extracted DNA, 1 μL and 1.5 μL of forward and reverse primers (10 pmol/μL), respectively, 0.5 μL of probe (10 pmol/μL), and PCR master mix (Kogenbiotech) were used to make a final volume of 20 μL, and real-time PCR (7500 Fast real-time PCR, Applied Biosystems) was performed.

For *hin* gene detection, 5 μL of the extracted DNA, 1 μL of forward and reverse primers (10 pmol/μL) each, and PCR master mix (Bioneer, Daejeon, Korea) were made up to a final volume of 20 μL and real-time PCR (C1000 Touch Thermal Cycler; Bio-Rad, Hercules, CA, USA) was performed. Based on the PCR products, a specific band was verified via electrophoresis on a 2% agarose gel.

III-2-4. Pulsed-field gel electrophoresis

PFGE analysis of *Salmonella* spp. was performed in accordance with the PFGE Standard Testing published by the MFDS. Cultures of pure strains were added to cell suspension TE buffer (100 mM Tris and 100 mM EDTA, pH 8.0), and the concentration was adjusted to an optical density of 0.8–1.0 measured at 610 nm using a spectrophotometer. Subsequently, 200 μL of 1.2% Seakem Gold agarose was added to the strain suspension, mixed gently, and immediately solidified in a plug mold. The solidified plug was transferred to 1.5 mL cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0; 1% sodium lauroyl sarcosine) to which 50 μL of proteinase K was added. After incubation in a 55°C shaking water bath for 1.5 to 2 h, the plug was washed five times

with a plug wash TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 min. A 1-millimeter thick slice was cut from the washed plug and incubated at 37°C for 2 h with 40 U/μL XbaI (Roche Diagnostics, Basel, Switzerland). Electrophoresis was performed using the plug gel treated with XbaI at 14°C for 18 h under the conditions: initial time 2.16 s, final time 63.8 s, gradient 6 V/cm; and an angle of 120°. *S. enterica* serotype Braenderup BAA-664 standards were used as the size marker, and testing was performed in the same manner for the isolated strains. After electrophoresis, the gel was placed into SYBR gold stain (Invitrogen, Waltham, MA, USA) and dyed for 30 min, after which UV was used for identification. Identified pictures were analyzed using the program, BioNumerics (Applied Maths, Ghent, Belgium).

III-2-5. Whole genome multi-locus sequence typing analysis

For whole-genome sequencing, genomic DNA was extracted from *S.* Thompson using the MagListo™ 5 M Genomic DNA Extraction Kit (Bioneer). Sequencing libraries were prepared using the Nextera™ DNA Flex Kit (Illumina, San Diego, CA, USA), and paired-end reads were generated using the MiSeq Reagent Kit v2 (Illumina) using the MiSeq sequencing

platform (Illumina) [55]. After the entire genome sequencing process, allele calls with genome assembly were determined for wgMLST analysis. BioNumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) suite of software applications was used to perform wgMLST analysis [56]. Cluster analysis of categorical values of allelic numbers for wgMLST was used to construct phylogenetic networks using the unweighted pair group method with arithmetic mean algorithms. Among the 56 strains analyzed in this study, wgMLST analysis was performed for 19 strains, excluding strains with the same sample characteristics and isolation area. The 19 strains analyzed included two strains from two cakes (MFDS1011643 and MFDS1011640), two strains from egg white liquid products (MFDS1011653 and MFDS1011655), one strain from the manufacturing apparatus: whipper (MFDS1011714), and 14 strains from preserved food samples (MFDS1011687, 1011692, 1011683, 1011679, 1011694, 1011682, 1011686, 1011691, 1011693, 1011704, 1011705, 1011712, 1011730, and 1011702).

III-2-6. Correlation analysis among *S.* Thompson isolates related to foodborne outbreaks

The correlation between the strains isolated from foodborne diseases in 2018 and *S.* Thompson isolates from the previous two outbreaks in Korea was analyzed using the PFGE method. The two strains from outbreaks in 2014 and 2015, which showed highly similar PFGE patterns with those of the strains isolated from egg white liquid samples, were additionally analyzed using the wgMLST analysis method.

III-3. Results

III-3-1. Isolation and identification of *Salmonella* spp.

S. Thompson strains were isolated from the samples collected during the outbreak investigation process in six cities, including Seoul, Busan, Daegu, Gwangju, Ulsan, and Jeju, and four provinces, including Chungbuk, Gyeongbuk, Gyeongnam, and Jeonbuk. The list of 56 *S.* Thompson strains isolated from a total of 56 samples, including three cakes produced by a manufacturer, one used manufacturing apparatus, two egg white liquid products, and 50 preserved foods, is shown in Table 7.

Table 7. Food and environmental isolates analyzed in the present study.

No.	Isolates	Strain # of MFDS	Region	Sample Type
1	*S.* Thompson	1011630	Kwangju	Cake (chocolate)
2	*S.* Thompson	1011640	Seoul	Cake (strawberry)
3	*S.* Thompson	1011643	Seoul	Cake (white)
4	*S.* Thompson	1011653	Seoul	Egg White Liquid
5	*S.* Thompson	1011655	Seoul	Egg White Liquid
6	*S.* Thompson	1011678	Daegu	Cake (Preserved in elementary school)
7	*S.* Thompson	1011679	Daegu	Cake (Preserved in kindergarten)
8	*S.* Thompson	1011680	Daegu	Cake (Preserved in elementary school)
9	*S.* Thompson	1011681	Daegu	Cake (Preserved in middle school)
10	*S.* Thompson	1011682	Busan	Cake (Preserved in middle school)
11	*S.* Thompson	1011683	Busan	Cake (Preserved in middle school)
12	*S.* Thompson	1011684	Busan	Cake (Preserved in middle school)
13	*S.* Thompson	1011685	Busan	Cake (Preserved in elementary school)
14	*S.* Thompson	1011686	Busan	Cake (Preserved in high school)
15	*S.* Thompson	1011687	Busan	Cake (Preserved in high school)
16	*S.* Thompson	1011688	Busan	Cake (Preserved in high school)
17	*S.* Thompson	1011689	Busan	Cake (Preserved in middle school)
18	*S.* Thompson	1011690	Busan	Cake (Preserved in elementary school)
19	*S.* Thompson	1011691	Jeju	Cake (Preserved in elementary school)
20	*S.* Thompson	1011692	Chungbuk	Cake (Preserved in middle school)

21	*S.* Thompson	1011693	Chungbuk	Cake (Preserved in high school)
22	*S.* Thompson	1011694	Gyeongbuk	Cake (Preserved in elementary school)
23	*S.* Thompson	1011695	Gyeongbuk	Cake (Preserved in high school)
24	*S.* Thompson	1011696	Gyeongbuk	Cake (Preserved in elementary school)
25	*S.* Thompson	1011697	Gyeongbuk	Cake (Preserved in high school)
26	*S.* Thompson	1011698	Gyeongbuk	Cake (Preserved in middle school)
27	*S.* Thompson	1011699	Gyeongbuk	Cake (Preserved in middle school)
28	*S.* Thompson	1011700	Gyeongnam	Cake (Preserved in high school)
29	*S.* Thompson	1011701	Gyeongnam	Cake (Preserved in high school)
30	*S.* Thompson	1011702	Gyeongnam	Cake (Preserved in (High school)
31	*S.* Thompson	1011703	Gyeongnam	Cake (Preserved in (High school)
32	*S.* Thompson	1011704	Gyeongnam	Cake (Preserved in Middle school)
33	*S.* Thompson	1011705	Gyeongnam	Cake (Preserved in High school)
34	*S.* Thompson	1011706	Gyeongnam	Cake (Preserved in Elementary school)
35	*S.* Thompson	1011707	Gyeongnam	Cake (Preserved in Middle school)
36	*S.* Thompson	1011708	Gyeongnam	Cake (Preserved in Middle school)
37	*S.* Thompson	1011709	Gyeongnam	Cake (Preserved in Middle school)
38	*S.* Thompson	1011710	Gyeongnam	Cake (Preserved in Elementary school)
39	*S.* Thompson	1011711	Gyeongnam	Cake (Preserved in high school)
40	*S.* Thompson	1011712	Ulsan	Cake (Preserved in middle school)
41	*S.* Thompson	1011713	Ulsan	Cake (Preserved in high school)
42	*S.* Thompson	1011714	Seoul	Whipper
43	*S.* Thompson	1011718	Jeonbuk	Cake (Preserved in elementary school)

44	*S.* Thompson	1011719	Jeonbuk	Cake (Preserved in middle school)
45	*S.* Thompson	1011720	Jeonbuk	Cake (Preserved in middle school)
46	*S.* Thompson	1011721	Jeonbuk	Cake (Preserved in elementary school)
47	*S.* Thompson	1011722	Jeonbuk	Cake (Preserved in elementary school)
48	*S.* Thompson	1011723	Jeonbuk	Cake (Preserved in elementary school)
49	*S.* Thompson	1011724	Jeonbuk	Cake (Preserved in high school)
50	*S.* Thompson	1011725	Jeonbuk	Cake (Preserved in elementary school)
51	*S.* Thompson	1011726	Jeonbuk	Cake (Preserved in elementary school)
52	*S.* Thompson	1011727	Jeonbuk	Cake (Preserved in elementary school)
53	*S.* Thompson	1011728	Jeonbuk	Cake (Preserved in elementary school)
54	*S.* Thompson	1011729	Jeonbuk	Cake (Preserved in middle school)
55	*S.* Thompson	1011730	Jeonbuk	Cake (Preserved in elementary school)
56	*S.* Thompson	1011731	Jeonbuk	Cake (Preserved in elementary school)

III-3-2. Confirmation of serotypes

Serological testing of 56 *Salmonella* isolates revealed that the O-antigen group contained a bacterial strain that belonged to serotype C. *In silico* serotyping predicted an antigenic profile of 7:k:1,5. All strains showed O:7 agglutination, flagella H-antigen phase 1 on k, and phase 2 on 1 and 5. Finally, 56 strains with identical serotypes were confirmed to be *S.* Thompson according to the Kauffman-White scheme [57].

III-3-3. PCR analysis of pathogenic genes

PCR analysis of the strains isolated from the samples revealed that all bacterial strains harbored *invA*, *his*, *hin*, and *stn* and were negative for *sefA* and s*pvC* (Table 8). The following genes related to *Salmonella* spp. were detected: *inv* is related to adhesion to and invasion into epithelial cells [58], *his* is involved in regulating histidine transport [59]; *sefA* encodes fimbria to specifically detect *S.* Enteritidis [60], *spv* causes cytotoxicity upon moving into the host cell, *stn* causing diarrhea when *Salmonella* spp. invade the intestines, and *hin* expresses flagella corresponding to two flagellar antigen phases 1 and 2. PCR is used to specifically detect *Salmonella* by identifying the related genes [61]. In this study, all *S.* Thompson isolates had the following genes: *invA, hin*, *stn*, and *his* PCR analysis of *hin* identified a *hin*-specific product of 572 bp in all *Salmonella* strains.

Table 8. PCR and serological typing result of *Salmonella* Thompson isolates

Strain	Serovar	Serological type			Real-time PCR					PCR
		O-antigen Group	H Phase 1	H Phase 2	*his*	*invA*	*stn*	*sefA*	*spvC*	*hin*
56 isolates	*S.* Thompson	C	k	1, 5	+	+	+	-	-	+

III-3-4. PFGE patterns of *S.* Thompson

Based on PFGE results, the 56 *S.* Thompson isolates were classified into one cluster with the same pattern as shown in Figure 1, which showed 100% identical genetic similarity.

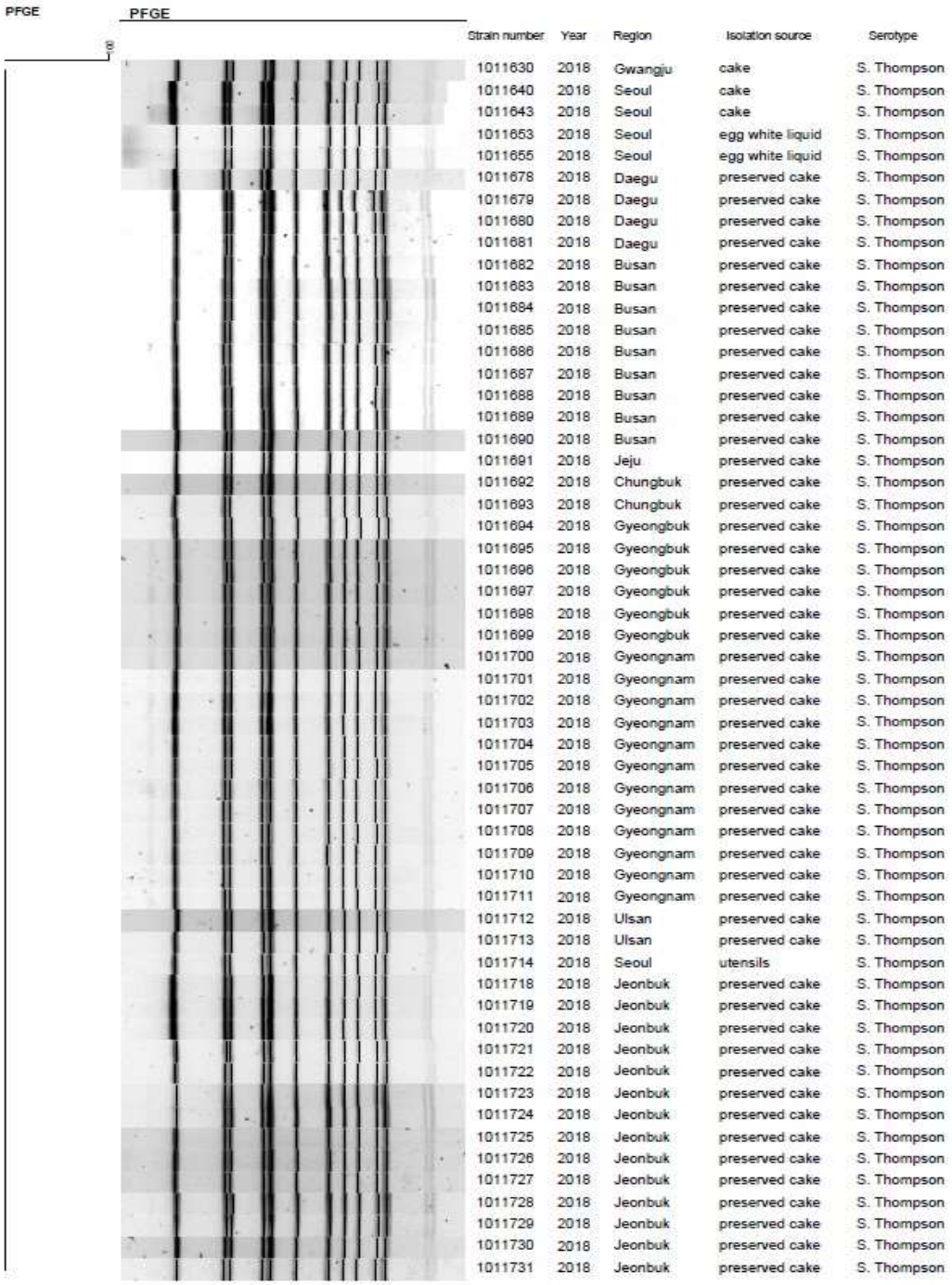
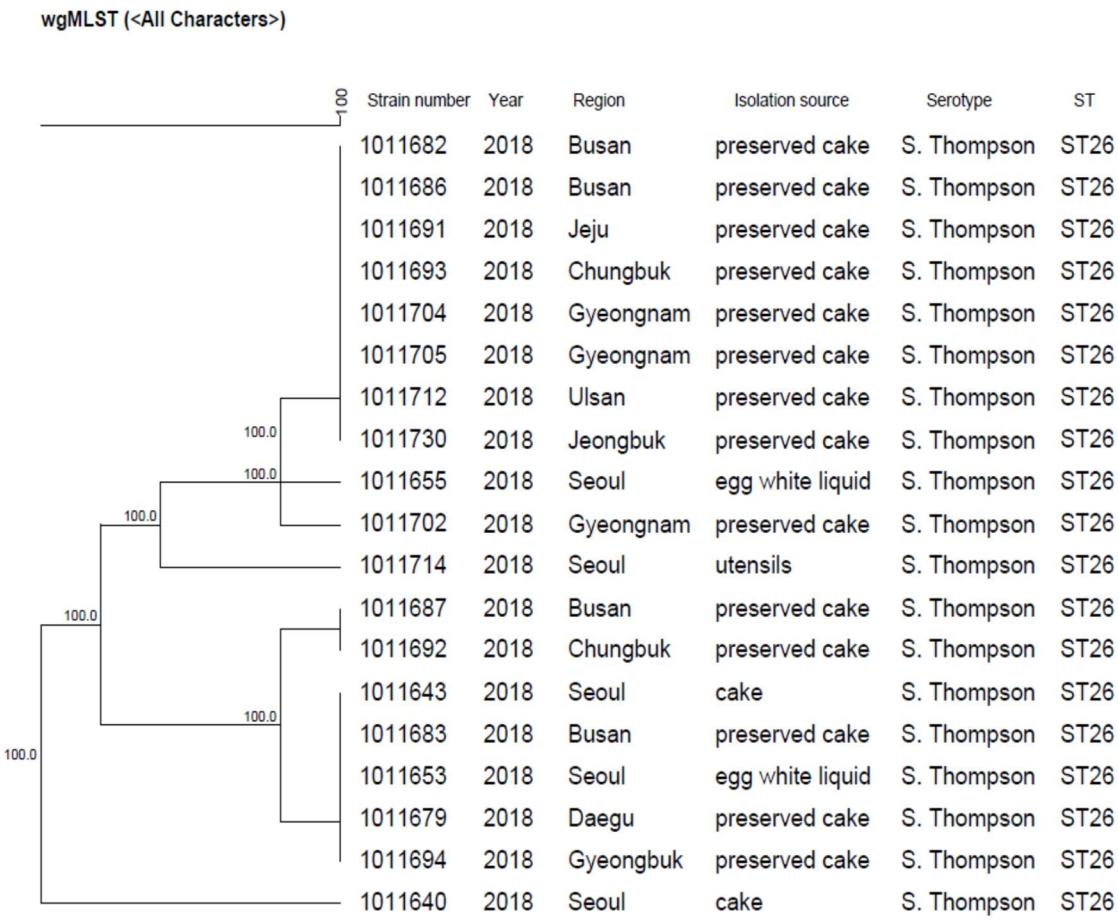


Figure 2. Similarity of 56 *Salmonella* Thompson isolates determined via pulsed-field gel electrophoresis analysis using XbaI.

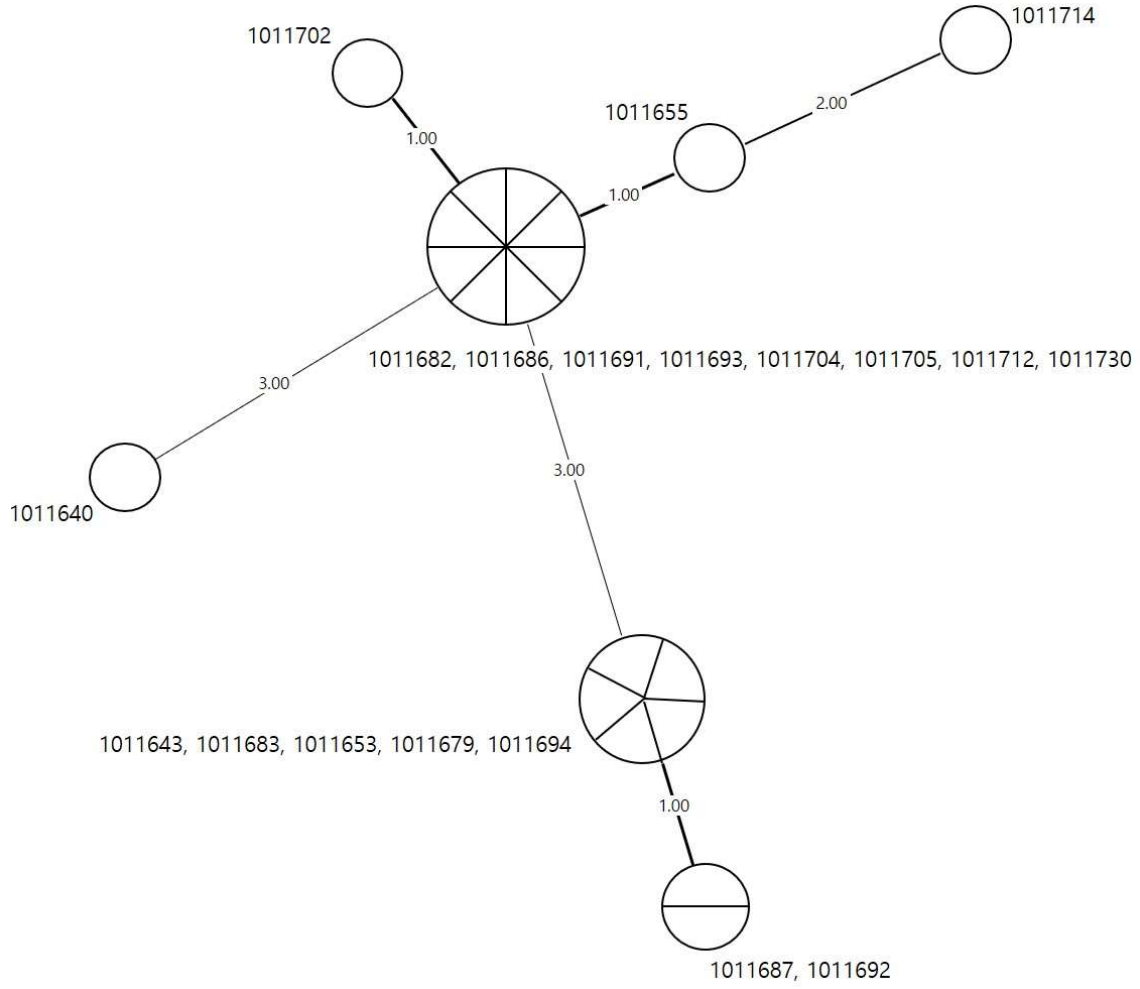
III-3-5. Whole genome multi-locus sequence typing analysis

Nineteen strains were analyzed which comprised 14 strains isolated from the preserved cakes selected by region and sample type, including one strain at a time, two strains from egg white liquid products, two strains from the cake products in the supply chain distribution, and one strain from the manufacturer's apparatus, a whipper. These strains showed the same genotype in PFGE and 100% similarity in wgMLST; however, they were classified into seven clusters. The clusters were classified based on the number of allelic differences, which were small based on 2 to 3 alleles. Therefore, in terms of similarity (%), the difference was trivial, leading to the conclusion that they have the same similarity level.



Strain number	Year	Region	Isolation source	Serotype	ST
1011682	2018	Busan	preserved cake	S. Thompson	ST26
1011686	2018	Busan	preserved cake	S. Thompson	ST26
1011691	2018	Jeju	preserved cake	S. Thompson	ST26
1011693	2018	Chungbuk	preserved cake	S. Thompson	ST26
1011704	2018	Gyeongnam	preserved cake	S. Thompson	ST26
1011705	2018	Gyeongnam	preserved cake	S. Thompson	ST26
1011712	2018	Ulsan	preserved cake	S. Thompson	ST26
1011730	2018	Jeongbuk	preserved cake	S. Thompson	ST26
1011655	2018	Seoul	egg white liquid	S. Thompson	ST26
1011702	2018	Gyeongnam	preserved cake	S. Thompson	ST26
1011714	2018	Seoul	utensils	S. Thompson	ST26
1011687	2018	Busan	preserved cake	S. Thompson	ST26
1011692	2018	Chungbuk	preserved cake	S. Thompson	ST26
1011643	2018	Seoul	cake	S. Thompson	ST26
1011683	2018	Busan	preserved cake	S. Thompson	ST26
1011653	2018	Seoul	egg white liquid	S. Thompson	ST26
1011679	2018	Daegu	preserved cake	S. Thompson	ST26
1011694	2018	Gyeongbuk	preserved cake	S. Thompson	ST26
1011640	2018	Seoul	cake	S. Thompson	ST26

Figure 3. a) Phylogenetic tree of *Salmonella* Thompson strains: wgMLST results show the evolutionary distance of the 19 strains of isolates which were confirmed to be 100% identical.



b) Minimum spanning tree of 19 strains shows the allelic distance between each strain that is closely correlated; the seven digits indicate strain number and the number between the circles shows the allele distance. MST was constructed in BioNumerics 7.6 using wgMLST data for isolates sequenced in this study. wgMLST, whole-genome multi-locus sequence typing.

III-3-6. Correlation among *S.* Thompson strains isolated in Korea

To determine the correlation between isolated *Salmonella* and foodborne outbreaks, three strains associated with three foodborne outbreaks were analyzed using PFGE and wgMLST. In addition to one strain isolated from the outbreak in 2018, two strains isolated from a restaurant in Jeonnam-do (2014) and a restaurant in Jeonbuk-do (2015) were analyzed. The strain isolated from an ingredient of gimbab (crab stick) in 2014 and from rolled egg omelets provided by a restaurant in 2015 belonged to the strain numbers MFDS1004024 and MFDS100681, respectively. The three strains had the same genotype, sequence type, and characteristics, as summarized in Table 9.

Table 9. Strains isolated from *S.* Thompson related foodborne outbreaks in Korea and their characteristics

Collection Date	Source	Point-of-Service	Serotype	Harboring Genes	Gene Bank No.	Genetic Info.
5-Sep - 2014	Crab-stick	restaurant	*S.* Thompson O:7, H(k:1,5)	*his*(+), *inv*A(+) *hin*(+) *stn*(+)	MFDS1004024	ST-26
29-June-2015	Egg (Shell)	restaurant	*S.* Thompson O:7, H(k:1,5)	*his*(+), *inv*A(+) *hin*(+) *stn*(+)	MFDS1006818	ST-26 SP6X01.009
9-Sep-2018	Egg White Liquid	School	*S.* Thompson O:7, H(k:1,5)	*his*(+), *inv*A(+) *hin*(+) *stn*(+)	MFDS1011653	ST-26 SP6X01.011

The results of PFGE analysis of the three strains showed that the two strains (MFDS1004024 and MFDS1006818) isolated from different outbreaks in 2014 and 2015, respectively were 100% identical as shown in Figure 4, and

showed 80.8% similarity with MFDS1011653, a strain from 2018, indicating a relatively lower level of similarity.

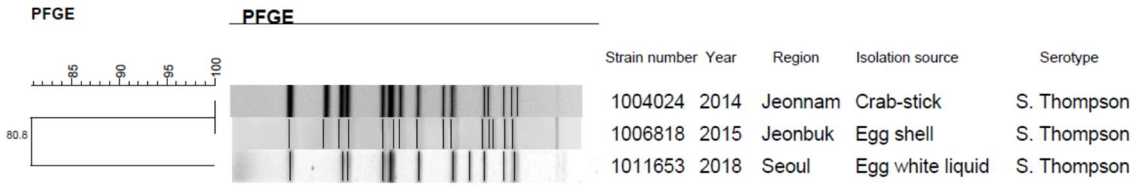
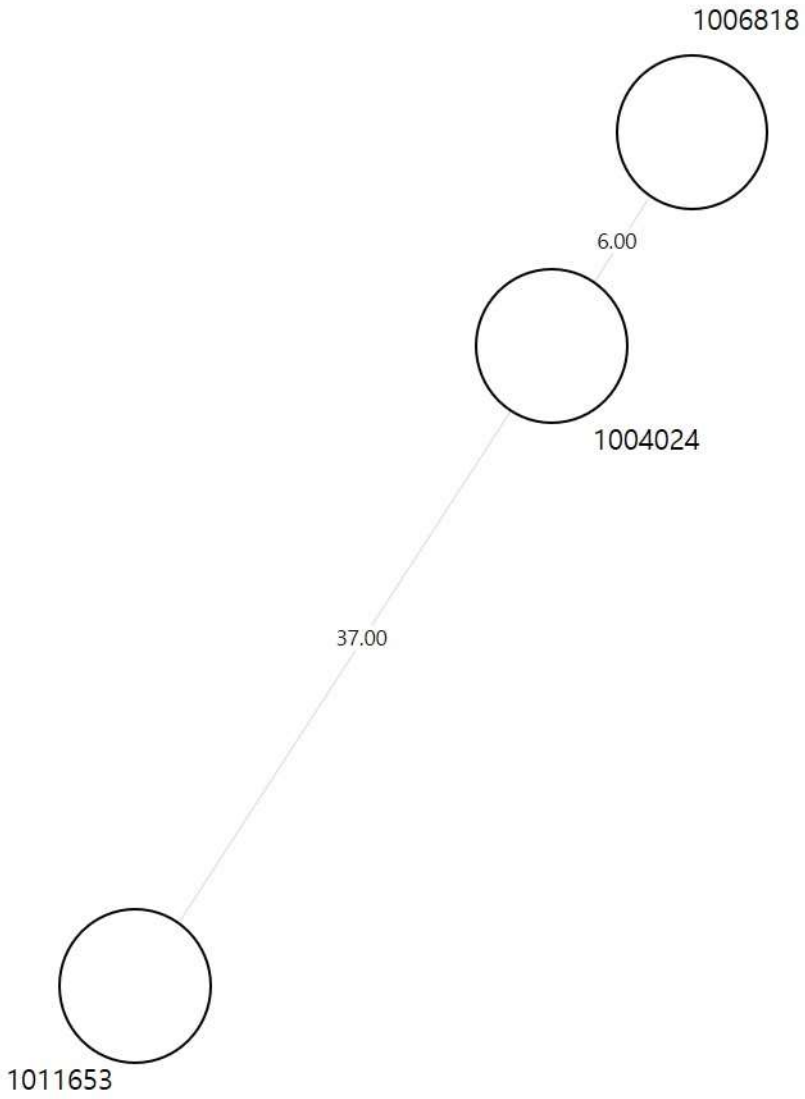


Figure 4. Pulsed-field gel electrophoresis patterns of *Salmonella* Thompson isolates obtained from three *Salmonella* outbreaks in Korea.

The results of wgMLST used to determine the correlation between the three strains are shown in Figure 5. Strain MFDS1004024 has 99.9% similarity with strain MFDS1006818, and the similarity between these two strains and strain MFDS1011653 was 99.6%, which contradicts the PFGE analysis. In conclusion, PFGE analysis showed that the two strains MFDS1004024 and MFDS1006818 had a high correlation and these two strains showed relatively low similarity (80.8%) with the strain MFDS1011653. In contrast, wgMLST

results showed that the similarity of the three strains was ≥99.6%, indicating a very high similarity.

Figure 5. a) Similarity between domestic *Salmonella* Thompson isolates determined via wgMLST analysis. The isolates of MFDS1004024 and MFDS1006818 showed 99.9% similarity. MFDS1011653 showed 99.6% similarity with MFDS1004024 and MFDS1006818.



b) Minimum spanning tree of three isolates showed 37 allelic differences between MFDS1004024 and MFDS1011653 and 6 allelic differences between MFDS1004024 and MFDS1006818. MST was generated in BioNumerics 7.6 using wgMLST data for isolates sequenced in this study. wgMLST, whole-genome multi-locus sequence typing.

III-4. Discussion

A multiple-school foodborne outbreak of *Salmonella* occurred in September 2018, which affected a total 2,975 patients, and this was recorded as the largest bacterial outbreak in Korea. The present study showed that the cause of this outbreak reported by approximately 60 school meal services was a single food source and originated from the same contaminated product of egg white liquid upon analyzing the characteristics of *S.* Thompson strains isolated from the investigation samples. We confirmed that *S.* Thompson strains with the same genotype were detected in the chocolate cake consumed by patients directly, chocolate cakes in distribution, whipper used by chocolate cake manufacturer, and egg white liquid which is one of the main ingredients of the cream applied on the chocolate cakes. Fifty-six strains harbored *stn* that causes diarrhea when *Salmonella* invade the intestines, *inv*A which facilitates adhesion to and invasion into epithelial cells, *his* which is involved in regulating histidine transport, and *hin* which expresses a flagellum corresponding to two flagellar antigen phases 1 and 2, which are associated with pathogenicity. Generally, it has been reported that the primary process of salmonellosis involves the invasion of *Salmonella* into intestinal cells

followed by proliferation. *Salmonellosis* is caused by the invasion of *Salmonella* into intestinal cells and fluid secretion, which causes inflammation of the lamina propria. Various virulence factors are involved in this process. Virulence factors are substances that are produced by pathogens and cause infections and diseases in the host. After exposure to pathogens, bacteria adhere to the skin or mucus membrane, and the adhered bacteria invade the host cell through the epithelium [62]. Genes in *Salmonella* associated with pathogenicity are involved in plasmid and chromosomal DNA replication, including *Salmonella* pathogenicity islands (SPIs). Upon invasion into the cells of an animal host, the type 3 secretion system encoded by SPIs is induced to secrete various virulence factors into the host cytoplasm to disrupt normal intracellular functions, leading to the promotion of invasion and proliferation of *Salmonella* in the cells. The *invA* gene is involved in this process and is an essential gene required for the adhesion and invasion of pathogenic strains that cause food poisoning. It has a *Salmonella*-specific sequence that is used to detect *Salmonella* in various samples [63, 64]. The present study found a trend similar to that reported in earlier studies in which *Salmonella* strains have been found to harbor *inv*A [65, 66]. The *sefA* gene,

which encodes thin filamentous fimbria of *S.* Enteritidis, was not detected in *S.* Thompson isolated in this study, and it has been reported to be observed specifically in serogroup D1 [61, 67]. In the case of *spv*, a gene that confers pathogenicity derived from a plasmid that can specifically detect *S*. Enteritidis, studies conducted by Araque and Chaudhary *et al.* [68, 69] reported that the gene has not been detected in any *S.* Thompson isolates. The *invA*, *stn*, and *hin* genes were detected in the *Salmonella* isolates analyzed in this study, indicating that they are closely related to the occurrence of food poisoning. According to Kim *et al.* [70], *Salmonella* strains with monophasic flagella do not harbor the *hin* gene and all monophasic *Salmonella* strains show phase 1. In addition, the study reported that in the case of *Salmonella* bacteria that lack *hin*, the composition of O-antigens and phase 1 of H antigens can identify *Salmonella* serotypes without conducting a phase 2 test, as in this study. The *hin* gene is involved in *fliA*, *B*, and *C* regulation that express flagella of phases 1 and 2, and one type of flagella is occasionally generated owing to the loss of the *hin* gene. The toxin gene *stn* causes inflammation and facilitates intestinal invasion of *Salmonella* [71, 72] along with the regulation by Hin recombinase expressed by the *hin* gene in *Salmonella* chromosome.

The PFGE pattern was identified to be the same for the 56 strains, demonstrating that the foodborne outbreaks that occurred in ten different areas were caused by the same strains and sources. Additional wgMLST analysis revealed that the isolated strains were 100% identical, with small differences in the number of alleles by 2 to 3 alleles. The analysis led to the conclusion that outbreaks were caused by consuming chocolate cake, a single source, and that the egg white liquid used for preparing the cream of the chocolate cake was contaminated with the same *S.* Thompson strain.

The occurrence of global outbreaks associated with *S.* Thompson have been linked to smoked salmon (Netherlands in 2012), chicken (Shawarma; Canada in 2016), cilantro (California, USA in 2001), and contamination of beef and bread [73–76]. Of the 403 food poisoning cases caused by *Salmonella* for 10 years from 1998 to 2008, six cases were caused by *S.* Thompson, accounting for 1.5% of the total cases [77]. The most common *Salmonella* serotypes detected in foodborne outbreaks in Korea over the past five years are *S.* Enteritis, *S.* Heidelberg, and *S.* Infantis, and there have been only two cases of food poisoning caused by *S.* Thompson in Korea until this outbreak occurred in 2018. In relation to the *S.* Thompson food poisoning incident

compared to that in the past, one of the two strains isolated was a strain previously isolated from a crab stick in 2014 (MFDS1004024), an ingredient of gimbap left at a restaurant in Hwasun-gun, Jeonnam-do after serving gimbap; 25 individuals who consumed gimbap at that restaurant had reported food poisoning symptoms. In addition, the strain isolated in 2015 (MFDS1006818) was detected in eggshells; eggs are used as an ingredient in rolled omelets served during breakfast and lunch at restaurants located in Jeonju-si, Jeonbuk-do. In total, 24 individuals who consumed the lunch box reported symptoms of food poisoning and were hospitalized. In cases of food poisoning at restaurants, investigations are often limited as the food does not remain at the time of the investigation, and additional examination of distribution is affected owing to missing distribution records or a narrow scope of food poisoning incidents. In particular, the outbreak that occurred in 2015 was found to be mainly caused by the fact that eggs, which are the main ingredients of rolled egg omelets, were stored at room temperature and that the rolled egg was cooked the night before and served the next day.

Based on the findings of the present study, the three strains isolated from the three outbreaks in Korea harbored the same genes and high genetic similarity.

Since strains with the same genetic characteristics were detected continuously from 2014 to 2018, it may be possible that the land or water in the area was contaminated with *S.* Thompson or that the areas had the same source of contamination. This result can be considered as the basis for tracking and preventing contamination of the source related to *S.* Thompson. The investigation of an outbreak is complex and is limited based on human resources and time taken to trace the contamination route of origin in many cases of small-scale outbreaks. Therefore, tracking methods using advanced genetic technologies may represent an important alternative to overcome these limitations. If the contaminated food source and causative organisms are identified and independent outbreaks are found to be caused by similar strains, as is the case in this study, it can be used to determine the cause of contamination in the investigation process. Gene similarity can be used as a standard for identifying common features regarding the cause of independent outbreaks, distribution processes, and production stages, making it possible to identify the cause of outbreaks with limited manpower. In addition, if the contaminated food source has been discarded or is identified for independent outbreaks, and the characteristics and similarity of strains are matched, an

analysis can be conducted to determine whether there are common contaminated foods or sources to identify the cause of food poisoning.

There are few reported cases of *S*. Thompson food poisoning worldwide, including Korea; however, this study identified the cause of *S.* Thompson foodborne outbreaks and analyzed the genetic characteristics of the strains. In addition, PFGE analysis showed that the three strains were not the same, while wgMLST analysis results indicated that the three strains had a significant correlation of similarity considering the rates of mutation of generation during the time of 4 years which was reported as Base-Pair Substitution (BPS) of 5.54~34.00 x 10^{-4} per genome per generation [78]. This result was supported by the finding that single nucleotide polymorphism (SNP) differences among the three strains were less than 60 SNPs (data not shown).

In conclusion, wgMLST analysis was able to provide highly resolved phylogeny and the combination of characterization methods of PFGE with wgMLST analyses can be effective in providing evidence during outbreak investigation.

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Abstract in Korean

우리나라에서 비장티푸스성 살모넬라균은 병원성대장균, 노로바이러스 등과 함께 다발성 식중독 요인 중 하나로 보고되고 있다. 살모넬라에 의한 식중독은 세계적으로도 보건학적 부담을 초래하고 있으며, 세계 설사 원인의 4대 주요 원인균 중 하나로서 이로 인해 세계인구의 연 10%가 장관성 설사질환을 경험하고 특히 5세이하의 설사를 경험하는 수가 2억2천여명으로 세계보건기구에서는 보고하고 있다. 특히 가벼운 설사 증상도 있지만 생명을 위협하는 경우도 있어 매우 주의가 필요한 대표적 식중독균이다. 살모넬라속은 생물학적으로도 혈청형이 2,500여 종 이상이 보고되고 있어 이에 대한 검사와 관리가 까다로운 특성이 있다. 그 이유는 식중독 원인조사 과정 중 조사 시료에서 분리되는 수많은 살모넬라균들이 동일한지 밝혀내는 과정이 매우 어렵고, 그 결과에 따라 행정적 책임 소재를 판단해야 하므로 신중하게 접근해야 하기 때문이다. 식중독의 원인을 밝히는 매우 중요한 근거가 되는 단계는 식중독 환자 시료에 존재하는 균과 환자가 섭취한 식품과 관련 환경 시료에 존재하는 균이 일치하는지 판단하는 과정이라고 할 수 있다.

본 연구에서는 살모넬라 식중독 원인 규명을 위하여 분리된 살모넬라 균주 간의 일치 여부를 확인하는 실험실적 방법을 살모넬라의 특성 분석 및 분자생물학적 연구를 통해 제시하였다. 이를 위하여 우리나라 소매상에서 유통되는 계육에서 살모넬라의 분포도를 확인하고, 분리된 살모넬라 균의 특성 연구를 통해 유사도를 판단하였다. 유사도를 판단하는 실험실적 과정은 살모넬라의 동정, 혈청형 확인,

병원성 유전자 확인, 펄스장 겔 전기영동(Pulsed-Field Gel Electrophoresis, PFGE), 다좌위 서열 구분법(Multi-locus Sequence Typing, MLST)을 수행하였다. 총 113건의 계육을 대상으로 살모넬라를 분리 동정한 결과 24건의 시료에서부터 24 종의 살모넬라를 분리하여 21.2%의 분포율을 확인하였고, 혈청형은 *S.* Enteritidis 11건(45.8%), *S.* Virchow 6건(25%), *S.* Montevideo, *S.* Bsilla 각 2건(8.3%), *S.* Bareilly, *S.* Dessau, *S.* Albany 각 1건(4.2%)으로 나타났다. 병원성 유전자는 유전자 6개 (*his, invA, stn, sefA, spvC, hin*)를 중심으로 PCR을 통해 확인한 결과 모든 분리된 살모넬라 균주는 *his, invA, stn*에 양성을 보여 식중독을 일으킬 수 있는 병원성을 보유한 것으로 판단되었다. PFGE 결과 24종의 살모넬라는 18개의 그룹으로 나뉘었으며, 64.4~100% 유사도를 보였다. 11종의 *S.* Enteritidis는 9개 그룹의 유전형으로 구분되고 74.4%의 유사도를 보인 반면, *S.* Virchow는 5개 그룹의 유전형, 85.9%의 유사도를 보였다. MLST 결과 국내 충청지역 유통 계육에서의 주요 서열유형(Sequence Type)은 ST-11, ST-16이었으며, 수입산 계육과 차이를 보임을 확인할 수 있었다. 따라서 MLST를 통한 서열 유형도 식중독 원인 식품을 규명하는 하나의 표지로 활용될 가능성을 확인하였다. 본연구에서는 2018년 9월에 발생한 전국 규모의 학교 집단식중독의 원인 조사를 사례연구로 원인조사 시료에서 분리된 살모넬라 균종 간의 특성과 유사도를 분석하였다. 발생 시설에서 보존되어 있는 초코케익 제품을 포함, 유통 중인 초코케익 완제품, 제조사의 제조기구(whipper), 케익 제조용 크림의 원료인 난백액 제품 시료에서 56개의 살모넬라 균종을 대상으로 하였다. 이들 살모넬라의 특성은 앞서 분포도 연구와 동일한 방법으로 정확한 동정을 거쳐 혈청형 확인, 병원성 유전자 확인,

PFGE를 수행하였으며, 다좌위 서열 구분법은 전장유전체 다좌위 서열 구분법(whole genome Multi-locus Sequence Typing, wgMLST)을 수행하였다. 모든 연구 대상의 살모넬라 균주는 혈청형 확인 결과 7:k:1,5 항원 반응 조합의 *S.* Thompson이었으며, 병원성 유전자는 *invA, his, hin, stn* 양성, *sefA, spvC* 음성을 나타내었다. PFGE 수행 결과 유전형은 1개 그룹의 100% 유사도를 보여 균주 간의 유전자적 차이가 없음을 알 수 있었다. Whole genome MLST는 56개 균종 중 시료 종류와 지역의 중복 균주를 제외한 19개 균종을 대상으로 수행하였다. 그 결과 100% 유사도를 보였으며, 추가적으로 allele 차이를 기준으로 7개의 유전형 그룹으로 구분됨을 확인할 수 있었다. 이러한 실험실적 특성 분석 결과는 본 연구에 포함되지 않았지만 환자 시료와 동일하게 일치함을 확인하여 이를 근거로 2018년 발생한 학교 식중독의 원인 식품은 살모넬라 톰슨이 오염된 난백액으로 제조한 크림이 도포된 초코케익이었으며, 단일 섭취식품에 의한 식중독임을 입증할 수 있었다. 국내에서 *S.* Thompson 과 관련된 식중독은 본 식중독 발생 이전 두 건이 보고되었으며, 2014년, 2015년 각 1건의 조사과정에서 분리된 균주와 2018년 균주를 대상으로 특성을 분석하였다. 그 결과 세 균주 모두 동일한 특성과 높은 유전자적 유사도(<99.6%)를 보여 본 연구에서 수행한 실험실적 유사도 분석 방법은 향후 오염원 규명에 매우 유용할 것으로 판단된다.

핵심어: 살모넬라속, 살모넬라 톰슨, 유전자서열 유형, 집단식중독, 펄스장겔전기영동, 다좌위 서열 구분법, 전장유전체 다좌위 서열 구분법