Isolation and characterization of *Salmonella* spp. from food and food contact surfaces in a chicken processing factory

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ABSTRACT The presence of *Salmonella* serotypes is a major safety concern of the food industry and poultry farmers. This study aimed to isolate and identify *Salmonella* spp. from a chicken processing facility by PCR and pulsed-field gel electrophoresis (**PFGE**). In addition, the biofilm-forming abilities of the isolated bacteria on stainless steel, silicone rubber, plastic, and chicken skin were also investigated. PCR was used for the confirmation of *Salmonella* serotypes, and then gene similarity within the same serotype was analyzed by PFGE. As a result, 26 *S.* Enteritidis isolates were detected at a high rate from both food contact surfaces and chicken products during processing. All of them were 100% genetically identical to the same bacteria. The results indicated that the virulence factors and effective biofilm-forming ability of *S.* Enteritidis isolates could affect human health and economic revenue. It was also suggested that the visual observation of food and food contact surfaces could be a great concern in the future. The continuous monitoring of *S.* Enteritidis molecular and biofilm characteristics is needed to increase food safety.

Key words: Salmonella Enteritidis, PCR, PFGE, biofilm, chicken skin

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INTRODUCTION

Fresh poultry and meat are greatly putrefiable because of their enriched nutrient component, water activity (0.98 to 0.99), and near neutral pH (5.5 to 6.5), which is the optimal environmental condition for *Salmonella* (Acuff, 2005). Furthermore, poultry and meat could be contaminated by bacteria present in dirt and fecal material associated with slaughter and evisceration areas. In the presence of a contaminated carrier during processing, the quality of the final product could be threatened by *Salmonella*. According to the Centers for Disease Control and Prevention (CDC), nontyphoidal salmonellosis causes about 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths in the USA every year (CDC, 2019). In Korea, a large outbreak of *S.* Thompson infections caused by contaminated eggs led to 3516 patients hospitalized in 2019 (MFDS, 2019). According to a survey conducted by the Korean Ministry of Agriculture, Food and Rural Affairs, the domestic consumption of chicken per capita drastically increased by 58% in 2018 compared with 2008 (MAFRA, 2019).

The increasing demand for chicken products may have the unintended consequence of the increased risk of chicken-associated food illnesses, so hygiene management should be greatly considered during processing. *Salmonella* easily forms a biofilm on food contact surfaces (Reij et al., 2004; Rodrigues et al., 2011; Møretrø et al., 2012). Once the biofilm is formed, it protects the embedded bacteria from external physical and chemical treatment (Milanov et al., 2009; Ashrafudoulla et al., 2021). Consequently, cross-contamination between food and food contact surfaces can occur. *Salmonella* biofilm capacity has been estimated at a laboratory-scale on diverse surface materials, such as stainless steel (**SS**), rubber, glass, and synthetic plastics (**P**) (Brooks and Flint, 2008; Rodrigues et al., 2011).

Conventional methods based on selective media for the detection and identification of *Salmonella* are time-consuming, labor-intensive, and require numerous reagents. Advanced molecular biology methods, such as

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a multiplex PCR assay, could be utilized to complement these limitations. This method is cost-effective, fast, accurate, and can easily be conducted in conjunction with other bacteria typing methods, such as pulsed-field gel electrophoresis (**PFGE**) (Kim et al., 2006). By producing DNA fingerprints of bacteria, PFGE represents an important tool in identifying the origin of outbreaks (Wattiau et al., 2011). In addition, PFGE analysis is also reliable in determining genetic relationships between bacterial species (Whittam and Bergholz, 2006). The current study aimed to analyze *Salmonella* contamination in a chicken processing facility and the biofilm-forming ability on food and food contact surfaces. In addition, advanced microscopy was also considered for visual confirmation of biofilm formation ability of the tested isolates as well as morphology observation.

MATERIALS AND METHODS

Isolation and Identification of Salmonella *spp. from Chicken Processing Facility*

Isolation of Salmonella Spp A total of 182 samples were collected in triplicate from a chicken processing facility to isolate and identify of *Salmonella spp*. Every sample was collected from 7 different spots in the chicken skin facility (Yougin, Gyeonggi-do, Korea). Environmental surface, in-line food, and final food sampling were conducted by following the standard sampling guidelines (FDA, 2018). To collect microbial samples from environmental surfaces, such as the cutting board and the conveyor belt, as well those surfaces indirectly contacted, such as the box containing the food piled up in vinyl, the swab test was performed by slowly wiping the identified area (approximately 10 × 10 cm, i.e., 100 cm^2) horizontally and vertically with sterilized cotton (Pipette Swab Plus, 3M Korea Ltd., Seoul, Korea). For food sampling, the food products (chicken nugget and chicken *karaage* [Japanese-style fried chicken nuggets]) from 2 processing lines were transferred by sterile SS tongs to sterile sample bags (Nasco, Fort Atkinson, WI). The swab and food samples were transferred to the laboratory and immediately examined by the *Salmonella* qualitative test based on the modified method of the Korean Ministry of Food and Drug Safety (MFDS, 2019). For pre-enriching the food samples, 25 g of the sample and 225 mL of 2% buffered peptone water (Oxoid, Thermo Scientific, Lenexa, KS) were placed in a Whirl-Pak filter bag (Nasco) and homogenized for 2 min in stomacher at the maximum speed (Bag Mixer; Interscience, Saint Nom, France). The homogenized food samples and swab samples were then incubated at 37°C for 24 h. Next, 100 μL of each enriched sample was pipetted into 10 mL of Rappaport-Vassiliadis broth (Oxoid, Thermo Scientific, Waltham, MA) and incubated at 42°C for 24 h. Aliquots of the incubated Rappaport-Vassiliadis broth were streaked on xylose−lysine−deoxycholate agar (XLD, Oxoid, Thermo Scientific, Waltham, MA) using a loop, and each plate was incubated at 37°C for 24 h. Presumptive pink colonies with or without black centers on XLD were detected as *Salmonella* and streaked separately on fresh XLD agar plates for biochemical examination. The isolated strains were cultured in triple sugar iron agar (Difco Laboratories, Sparks, MD) and Christensen urea agar base (Oxoid, Thermo Scientific, Waltham, MA), respectively. The *Salmonella*-positive bacteria were identified, cultured and then analyzed by PCR to distinguish serotypes and PFGE to identify gene similarity.

Bacterial Strain Growth and Stock Preparation The selected *Salmonella* spp. colony from the agar plate was inoculated into 10 mL of tryptic soy broth (TSB, Difco Laboratories) and incubated at 37°C for 24 h in a shaking incubator (VS-8480, Vision Scientific, Gyeonggi-do, South Korea) maintained at 220 rpm. The cultures in TSB were centrifuged at 11,000 × *g*, 4°C for 10 min. The pellets were washed and resuspended in Dulbecco's PBS (Oxoid, Basingstoke, UK) solution (pH 7.3 ± 0.2 at 25°C) with 30% glycerol (w/v) to obtain a stock solution that was stored at −80°C until required.

Identification of Salmonella *Serotype by PCR*

PCR was used to differentiate *Salmonella* serotypes using multiplex PCR primers (**Table 1**) based on a previous study (Kim et al., 2006). A total of 12 primers were divided into 3 groups, and 1 pM of each multiplex primer was manufactured. The multiplex primers were named A−L according to the band pattern of the *Salmonella* serotypes. For instance, multiplex 3 (**Table 1**) was needed for *S.* Dublin and *S.* Enteritidis because the band patterns generated with multiplex 1 and 2 were the same. The serotypes of the *Salmonella* isolates were identified by classification of the band patterns (**Table 2**), respectively. Each isolate was incubated in 4 mL of buffered peptone water at 37°C for 24 h, then 1 mL of the *Salmonella* isolate was dispensed into a 1.5-mL Eppendorf tube for PCR. DNA was purified from the isolates using the Real Bioscience Co. (RBC) Genomic DNA Extraction Kits (Life Biomedical, Cambridge, England). Each PCR reaction (20 μL) contained the multiplex PCR primer (1 μL), the DNA template (2 μL), 2X Taq PCR Smart Mix 2 (10 μL; SolGent Co., Daejeon, Korea), and milli-Q water. The reactions were performed under the following conditions: 1 cycle of 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, then a final extension at 72°C for 5 min.

Electrophoresis

A 2.5% agarose gel was prepared from 1X Tris−borate−EDTA (**TBE**) buffer consisting of 90 mM Tris, 90 mM borate, and 20 mM EDTA (pH 8.0). A 1.0-kb (0.1 to 10 kb) DNA ladder (Smobio Technology, Inc., Hsinchu, Taiwan) was used as a molecular weight marker, and a representative strain of *S.* Enteritidis

Table 1. Primers used for deciding *Salmonella* serotypes.

Primer	Primer sequence (5′−3′)	Amplicon size (bp)	Result label
Multiplex 1			
STM 1	F: AACCGCTGCTTAATCCTGATGG R: TGGCCCTGAGCCAGCTTTT	187	A
STM 2	F: TCAAAATTACCGGGCGCA R: TTTTAAGACTACATACGCGCATGAA	171	B
STM 3	F: TCCAGTATGAAACAGGCAACGTGT R: GCGACGCATTGTTCGATTGAT	137	C
STM 4	F: TGGCGGCAGAAGCGATG R: CTTCATTCAGCAACTGACGCTGAG	114	D
STM 5	F: TGGTCACCGCGCGTGAT R: CGAACGCCAGGTTCATTTGT	93	E
Multiplex 2			
STY 1	F: TGGTATGGTTAAGCGGAGAATGG R: GAGAGTCATAGCCCACACCAAAG	301	F
STY 2	F: GGCTGGAGCAGCCTTACAAAA R: AAGAGTTGCCTGGCTGGTAAAA	262	G
STY 3	F: AATCCCCCCCCCTCAAAAA R: GGTACACGTTTACTGTTTGCTGGA	220	H
STM 6	F: ATATCTCATCGTCTCCTTTTCGTGT R: GAAGGTCCGGATAGGCATTCT	181	I
STY 4	F: AATTACGGAGCAGCAGATCGAGG R: TGCGGCCAGCTGTTCAAAA	124	J
Multiplex 3			
PT 4	F: GGCGATATAAGTACGACCATCATGG R: GCACGCGGCACAGTTAAAA	225	K
STM 7	F: CATAACCCGCCTCGACCTCAT R: AGATGTCGTGAGAAGCGGTGG	101	L

(ATCC 13076) was also used as a reference. After electrophoresis at 50 V for 80 min, the gel was dyed with ultrapure ethidium bromide (Life Technologies, Waltham, MA) for 20 min. Gel images were acquired on a Gel Doc XR system (Bio-Rad, Hercules, CA), and the bands were visualized under UV light.

Table 2. Classification of PCR band pattern for *Salmonella* serotypes in this study.

Salmonella serotype	PCR pattern	Reference
Typhi	AFGHJ	(Kim et al., 2006)
Thompson	BCEJ	
Stanley	ABEG	
Saintpaul	ABCDEL	
Paratyphi	ABDE	
Javiana	ABHJ	
Infantis	BG	
Heidelberg	ABDEG	
Enteritidis	BCEHK	
Derby	ABCEJ	
Weltevreden	ABDEFIJ	
Westhampton	ABCEGJ	
Oranienberg	ND	
Ohio	BEJK	
Muenchen	ABJ	
Montevideo	EGI	
Mbandaka	BCEGJ	
Hadar	BCE	
Dublin	BCEH	
Chester	ABG	
Braenderup	BEL	
Brandenburg	ABG	
Bovismorbificans	BCEG	
Berta	BCEGH	
Anatum	ABCE	
Agona	BCJ	
Newport	ABCE	
Typhimurium	ABCDEI	

PFGE

To check the gene similarity among the 35 strains of *Salmonella* spp., all isolates were analyzed by PFGE using the standard protocol of PulseNet for *Salmonella* (CDC, 2017), with some modifications. The *Salmonella* isolates and a standard strain (*S.* Braenderup BAA-664) were incubated in tryptic soy agar at 37°C for 24 h. The optical density at 610 nm (OD_{610}) was measured using a spectrophotometer (Shimadzu, Scientific Support, Inc., Hayward, CA). The concentrations of bacteria taken by swab were adjusted to 0.8 to 1.0 OD_{610}.

Next, 0.12 g of SeaKem Gold agarose (Lonza, Rockland, ME) was added to 10 mL of plug wash TE buffer (10 mM Tris−1 mM EDTA, pH 8.0) to obtain a 1.2% solution. After thorough stirring, it was microwaved to dissolve completely and kept at room temperature for 10 s. Before use, it was placed in a water bath at 55°C. An aliquot (200 μL) of the bacterial suspension was dispensed into a 1.5-mL Eppendorf tube, then 20 μL of proteinase K (20 mg/mL) was added. The solution was mixed carefully with 200 μL of the agarose solution to prevent bubbles. The PFGE molds were refrigerated (4°C) for 5 min to completely solidify. The solid plugs were transferred to 50-mL tubes containing 30 μL of proteinase K and 5 mL of cell lysis buffer (50 mM Tris−50 mM EDTA, pH 8.0 + 1% sodium lauryl sarcosine). Each tube was covered with a screen cap and agitated at a constant speed (175 rpm) in a shaking water bath (Gyratory Water Bath Shaker G76, New Brunswick Scientific, Edison, NJ) at 55°C for 2 h. After eliminating the reaction solution, 15 mL of preheated plug wash TE buffer was added, the screen cap was attached, and the tube was placed in the shaking water bath (55°C for 10

min) with the test solution fully submerged. This washing process was repeated another 4 times. The plugs were placed on a glass slide and cut with a razor blade into 2-mm slices. The remaining plugs were stored in a tube with 1.5 mL of fresh plug wash TE buffer at 4°C.

For the restriction enzyme treatment, the slices were transferred to 100 μL of diluted 10X restriction buffer (1X; Thermo Fisher Scientific, Waltham, MA) for 10 min before the buffer was removed. Restriction enzyme master mix (100 μL consisting of 87 μl of reagent water, 10 μL of 10X restriction buffer, 0.5 μL of bovine serum albumin, and 2.5 μL of *Xba*I) was added and reacted in a water bath at 37°C for 2 h. After the solution was discarded, 200 μL of TE buffer was added and left for 5 min. The plug slices were placed on the end of the comb lane and dried for 5 min. The comb was set up at an angle of 90° and the gel caster was slowly filled with 1% SeaKem Gold agarose (0.5X TBE, thiourea, and agarose). When about 1 mL of agarose solution remained, it was kept in the water bath at 37°C. Once the cast agarose had solidified at room temperature, the comb was removed, and the remaining void was filled with the preheated agarose solution. The pulsed-field chamber (CHEF-Mapper XA, Bio-Rad) was filled with 2 L of 0.5X TBE, followed by the addition of 1 mL of 1.5 M thiourea. The gel was placed on the frame of the PFGE chamber at an angle of 120°, and electrophoresis was conducted under conditions of 2.16 s initial switch time and 63.8 s final switch time at a gradient of 6 V/cm (200 V) for 18 h (CDC, 2017)

After electrophoresis, the gel was dipped in a mixture of 50 μL of SYBR Gold solution (10 mg/mL) and 500 mL of sterile distilled water and dyed for 20 min, then decolored in distilled water for 90 min, and again in fresh, sterilized distilled water for a further 30 min. Finally, the band pattern was visualized under UV light using a UVDI imaging system (Major Science, Saratoga, CA) and photographed using BioNumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). The band patterns were compared using Dice's coefficient with a 1.0% band position tolerance and the unweighted pair group method using arithmetic averages (Zhao et al., 2007).

Preparation of Food Contact Surfaces, Biofilm Formation, and Detachment Population of Food Contact Surfaces

SS (2 × 2 × 0.1 cm; Type-304; Chung-Ang Scientific, Inc., Seoul, Korea), silicone rubber (**SR**) (2 × 2 × 0.2 cm; Komax Industrial Co., Ltd., Seoul, Korea), and P (2 × 2 × 0.5 cm; ultra-high-molecular-weight polyethylene, JINIL Tec-Pla Co., Ltd., Seoul, Korea) coupons were used as representative food contact surfaces based on the frequency of use. The food contact surfaces were prepared for biofilm formation by following previous methods (Bae and Lee, 2012; Shen et al., 2012) with modifications. The SS coupons were washed with detergent solution, ultrasonicated for 5 min, immersed in 15% phosphoric acid (Daejung, Siheung, Korea) with agitation for at least 30 min rinsed with sterile distilled water. The SR and P coupons were immersed in 70% ethanol for 1 h and washed with sterile distilled water. Afterward, all the coupons were autoclaved at 121°C in a sealed, sterilized beaker and placed in a dry oven for a minimum of 24 h.

A single *S.* Enteritidis strain was selected for biofilm formation considering that *S.* Enteritidis is the highest-detected serotype and has strong gene similarity in PFGE. For biofilm formation, 100 μL of cell suspension was resuspended into 0.1% peptone water (**PW**; Oxoid, Basingstoke, UK) to obtain a final cell concentration of 5 log CFU/mL. Prepared SS, SR, and P coupons were fully dipped in sterile 50-mL conical tubes (SPL Life Science Co., Ltd., Gyunggi-Do, Korea) containing TSB inoculated with the cell suspension. The tubes were incubated at 37°C for 24 h to allow the formation of stable biofilms. The biofilms on coupons were detached according to a previously reported method (Giaouris et al., 2005) with modifications. To eliminate planktonic cells, every coupon was washed twice with PBS and transferred to a 50-mL conical tube containing 10 mL of 0.1% PW and 10 sterile glass beads (diameter, 3 mm). After mixing by vortex for 2 min, the detached suspension cells were diluted with 0.1% PW and spread on XLD agar. The biofilm populations (expressed as CFU/cm^2) were compared among the surface materials.

Chicken Skin Preparation and Bacterial Inoculation

Fresh chicken skins were obtained from a local market in Anseong, Korea, and stored at 4°C before use. Each sample was cut into pieces (10 ± 0.2 g) using sterile SS scissors and placed in sterile Petri dishes. Immediately, each side of the samples was irradiated with UV light at 1000 μW/cm^2 for 5 min to eliminate background microorganisms and then washed twice with sterile distilled water for 2 min. Natural contamination of bacteria was investigated using tryptic soy agar and was below the detection limit (<1.0 log CFU/g). Before inoculation with the cell suspension, the samples were dried on a clean bench for 10 min. Chicken skins were spot-inoculated with 100 μL of *S.* Enteritidis suspension (Sagong et al., 2011), followed by refrigeration at 4°C for 1 h to allow attachment. The cooled samples were immediately transferred to Whirl-Pak filter bags (Nasco) containing 90 mL of 0.1% PW and homogenized using a stomacher (Bag Mixer 400) at speed 4 for 2 min to detach bacteria from the chicken skin. For enumeration of *S.* Enteritidis, the sample was diluted in 0.1% PW serially, spread on XLD agar, and incubated at 37°C for 24 h.

Transmission Electron Microscopy (TEM) Sample Preparation and Imaging

The purpose of the TEM analysis was to evaluate morphological structure of *S.* Enteritidis to confirm the

visual characteristics of chicken processing factory isolates with other recognized pathogenic *S.* Enteritidis. A TEM sample was prepared by minor modifications of previously published reports (Golding et al., 2016; Ashrafudoulla et al., 2019). The samples were centrifuged at 8,000 × *g*, 4°C, for 12 min and washed with PBS at least twice. A 10-μL aliquot of bacterial suspension was dispensed on filter paper and overlaid with a carbon-coated 400-mesh copper grid for 1 min. The grid was washed 3 times in distilled water, 2% methylamine tungstate (Nano-W, Nanoprobes, Yaphank, NY) was used to stain the sample negatively and imaged under FEI Tecnai 20 TEM (FEI Company, Hillsboro, OR).

Field-Emission Scanning Electron Microscopy (FE-SEM)

The biofilm-forming ability of the representative single *S.* Enteritidis isolate was examined by FE-SEM. The surfaces were prepared as described in our previous report (Ashrafudoulla et al., 2020) with some modifications. The samples containing bacterial biofilm were fixed with 2.5% glutaraldehyde approximately 3 to 4 h after washing with PBS at normal temperature. The samples were then treated with solutions of varying ethanol concentrations (50 to 100%) for 15 min each, followed by successive dehydration with ethanolic solutions of hexamethyldisilazane at concentrations of 33 to 100% for 15 min each. Then it was sputter-coated with platinum for evaluation under an FE-SEM (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

In this study, biofilm formation was performed 3 times, and viable cells were expressed in log scale. The Microsoft Excel (Tenth version of Microsoft Office 2016, a productivity suite), GraphPad Prism 5.03 version for Windows (Version 5.03; GraphPad Software, Inc., La Jolla, CA), and SAS software (version 9.2; SAS Institute, Inc., Cary, NC) were performed for statistical analysis. Duncan's new multiple range test was used for statistical analysis. Significant value was also determined by Duncan's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Isolation and Identification of Salmonella Spp. from Chicken Processing Facility

Identification of Salmonella Serotypes by PCR The serotypes of *Salmonella* isolated from a chicken skin facility were determined by PCR analysis. **Table 3** presents the specified genes of each serotype. A total of 35 isolates were detected from 7 spots on environmental surfaces and food from 2 processing lines (chicken nuggets and chicken *karaage*). The PCR results demonstrated that *S.* Enteritidis, *S. infantis*, and *S.* Montevideo were detected at 4 of the 7 spots on food contact surfaces and 3 spots on the food samples during processing. In this study, the sampling names cp1, cp3, and cp16 denote raw meat at the preprocessing room, the food at the processing line after molding, and the food at the processing line after mixing the batter, respectively. The terms cs30, cs73, cs74, and cs75 denote the surfaces of the conveyor belt after molding, the feed hopper of the cutter used for raw meat, the workbench edge of the cutter, and the conveyor belt of the cutter, respectively. Every *Salmonella* was detected at some spots between food contact surfaces and food. It suggests that contamination between surface materials and food leads to increased contamination during processing and, consequently, of the final product. In addition, *S.* Enteritidis was highly isolated from the chicken processing facility in this study. *Salmonella* Enteritidis is one of the prevalent serotypes and the main pathogen in poultry and its products worldwide (Hugas and Beloeil, 2014). In the USA, *S.* Enteritis was continuously ranked first in association with outbreaks from 2010 to 2016 (Cheng et al., 2019). In Korea, the *Salmonella* serotypes are becoming increasingly diverse each year (2008 to 2015), but, like in the USA, *S.* Enteritidis is still the most frequent (Kim and Lee, 2017).

Table 3. *Salmonella* serotypes isolated from a chicken processing facility.

	Multiplex[2]			
Sample[1]	1	2	3	Serotype
cp3-1	E	GI	-	Montevideo
cp3-2	E	GI	-	Montevideo
cp3-3	E	GI	-	Montevideo
cp3-4	BCE	H	K	Enteritidis
cp3-5	E	GI	-	Montevideo
cs30-1	B	G	-	Infantis
cs30-2	B	G	-	Infantis
cs30-3	B	G	-	Infantis
cs30-4	B	G	-	Infantis
cs30-5	B	G	-	Infantis
cs73-1	BCE	H	K	Enteritidis
cs73-2	BCE	H	K	Enteritidis
cs73-3	BCE	H	K	Enteritidis
cs73-4	BCE	H	K	Enteritidis
cs73-5	BCE	H	K	Enteritidis
cs74-1	BCE	H	K	Enteritidis
cs74-2	BCE	H	K	Enteritidis
cs74-3	BCE	H	K	Enteritidis
cs74-4	BCE	H	K	Enteritidis
cs74-5	BCE	H	K	Enteritidis
cs75-1	BCE	H	K	Enteritidis
cs75-2	BCE	H	K	Enteritidis
cs75-3	BCE	H	K	Enteritidis
cs75-4	BCE	H	K	Enteritidis
cs75-5	BCE	H	K	Enteritidis
cs73-P1	BCE	H	K	Enteritidis
cs73-P2	BCE	H	K	Enteritidis
cs73-P3	BCE	H	K	Enteritidis
cp1-1	BCE	H	K	Enteritidis
cp1-2	BCE	H	K	Enteritidis
cp1-3	BCE	H	K	Enteritidis
cp1-4	BCE	H	K	Enteritidis
cp1-5	BCE	H	K	Enteritidis
cp16-1	BCE	H	K	Enteritidis
cp16-2	BCE	H	K	Enteritidis
Total: 35 strains				

[1]Sample name: "cs" indicates the swab test samples taken from environmental surfaces; "cp" denotes the food samples.

[2]Multiplex primer: letters (B, C, E, G, H, I, and K) indicate the PCR plasmid profiles.

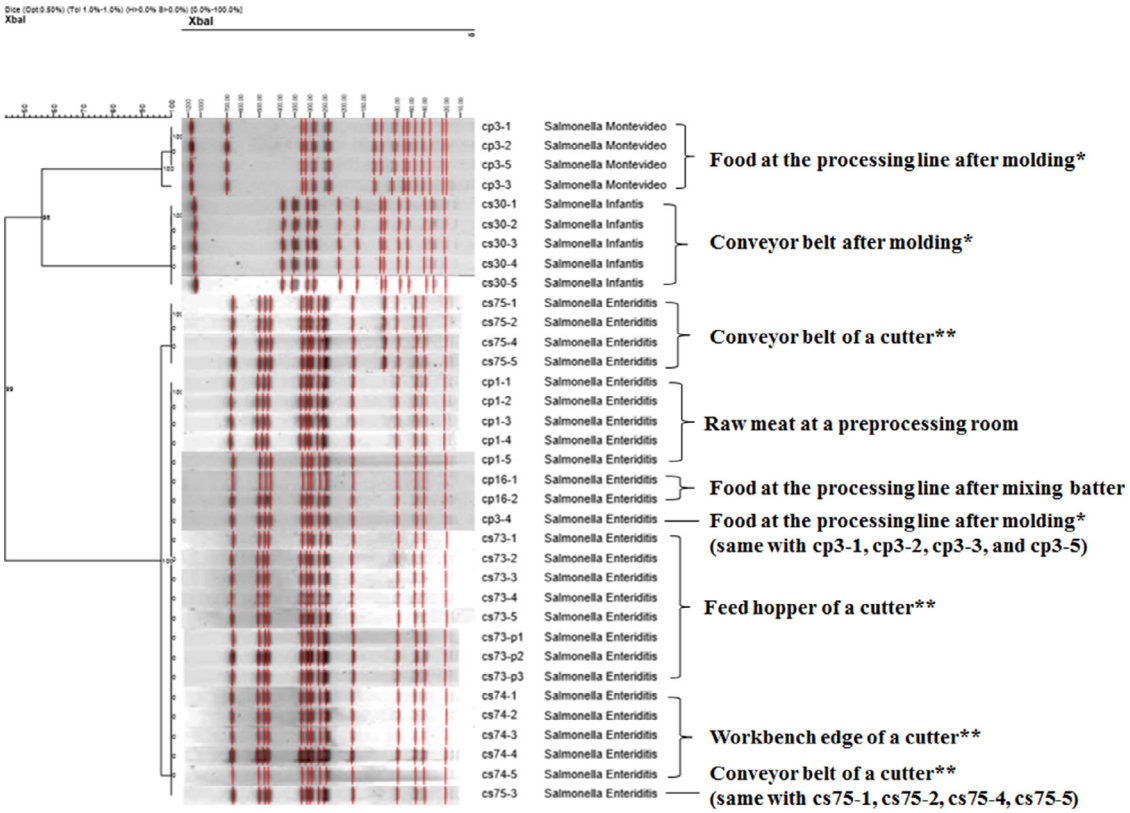

Figure 1. PFGE dendrogram analysis of 35 *Salmonella* isolates. (optimization: 0.5%, tolerance: 1.0%). *, in same processing procedure. **, a cutter is a same machine for cutting raw meat in chicken processing facility.

PFGE Analysis The PFGE analysis was needed to evaluate gene similarity between different serotypes or within the same serotype because the chicken processing facility receives the raw meat from various companies. During monitoring, 4 strains of *S.* Montevideo, 5 strains of *S. infantis*, and 26 strains of *S.* Enteritis were isolated. Restriction enzyme analysis using PFGE with *Xba*I is a widely successful approach for identifying gene differences between *Salmonella* isolates (Nesse et al., 2003) and has been evaluated as a standard for subtyping of *Salmonella* (Woo et al., 2003). It can also decide whether or not the samples share a common origin because the PFGE results show genetic similarity among samples from diverse locations (Melendez et al., 2010). *Salmonella* Braenderup strain H9812 was used as a standard, which may differ from other bacterial species (CDC, 2018). From the cluster of 35 isolated strains, 14 to 16 bands were obtained and showed genetic similarity (**Figure 1**). The cluster shows some factors that are important in predicting the heterogeneity among isolates. Clusters cs75-1, cs75-2, cs75-4, and cs75-5 were different from the other strains of *S.* Enteritidis, and cp3-4 was also different from the others (cp3-1, cp3-2, and cp3-5). However, the difference in one band statistically means approximately 100% similarity. As a result, every isolate had almost 100% gene similarity, and so the samples have the same origin in this study.

Comparison of Biofilm-forming Ability on Food and Food Contact Surfaces

Bacterial cells may attach within a matrix of extracellular polymeric substances to develop biofilm on biotic or abiotic surfaces (Ashrafudoulla et al., 2019, 2021). Biofilms have been associated with many outbreaks of pathogens and up to 80% of microbial infections (Epstein et al., 2011). Therefore, this study demonstrated the biofilm-forming ability of *S.* Enteritidis isolates on food and various food contact surface materials. Polyethylene and SS are used in food processing as food contact surfaces (Jun et al., 2010). Food processing facilities also often use SR as a surface material. **Table 4** shows the biofilm populations of *S.* Enteritidis on SS, SR, and P at 24 h. These data verify that there was no significant difference ($P > 0.05$) among the biofilm populations on SS, SR, and P coupons at 24 h adhesion time. Previously, it was demonstrated that the biofilm formation of *S.* Poona was similar

Table 4. Biofilm formation of *Salmonella* Enteritidis on stainless steel, silicone rubber, plastic, and chicken skin at 24 h.

	Stainless steel	Silicone rubber	Plastic (polyethylene)	Chicken skin
Biofilm formation (log CFU/cm^2)	6.17 ± 0.04	6.30 ± 0.08	6.28 ± 0.05	6.11 ± 0.06

Values are mean ± standard deviation; (n = 3).

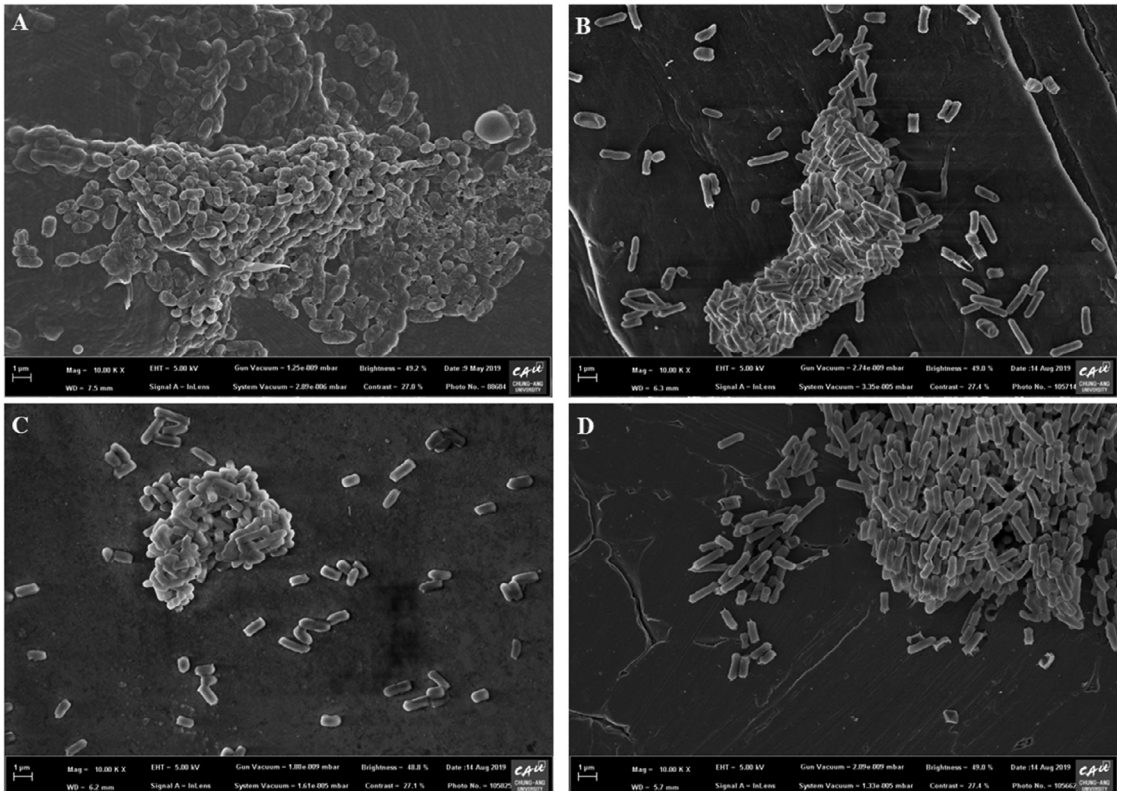

Figure 2. Representative FE-SEM images of biofilms on food and food contact surfaces. (A) Chicken skin, (B) plastic, (C) silicone rubber, and (D) stainless steel.

between surfaces of SS and P (ultra-high-molecular-weight polyethylene) (Manijeh et al., 2008). The current study showed that *S.* Enteritidis isolates have a biofilm-forming ability. In addition, the biofilms of *S.* Enteritidis could remain on food contact surfaces and increase the risk of cross-contamination between food and food contact surfaces in the processing line. Other strains of *Salmonella* spp. and the environmental isolate *S.* Enteritidis also possess biofilm-forming ability on chicken skin (Seo et al., 2019). **Table 4** represents the meaningful biofilm-forming ability of *S.* Enteritidis on chicken skin.

FE-SEM

The FE-SEM images of *S.* Enteritidis on different surfaces are shown in **Figure 2**. The environmental strain *S.* Enteritidis demonstrated biofilm-forming ability on food (chicken). The biofilms displayed a structural organization with intact cell-to-cell connections and looked smooth with an intact cell membrane (**Figure 2**). The SEM technique has been used previously to examine the biofilm-forming ability of *S.* Typhimurium on chicken skin (Seo et al., 2019) and *S.* Blockley on food contact surfaces (Dhowlaghar et al., 2018). *Salmonella* Enteritidis is known to form biofilm on different food industry surfaces at low temperatures (Webber et al., 2019).

Morphology Observation by TEM

The morphological structure of *S.* Enteritidis was evaluated through TEM (Figure 3). The bacterial cell looked rod-shaped with smooth cell membrane and clear flagella. This microscopic analysis indicated that isolated *S.* Enteritidis from chicken processing factory may exist same function as other recognized pathogenic *S.* Enteritidis. In our previous study we also checked the morphological structure of *Vibrio parahaemolyticus* isolated from seafood (Ashrafudoulla et al., 2019).

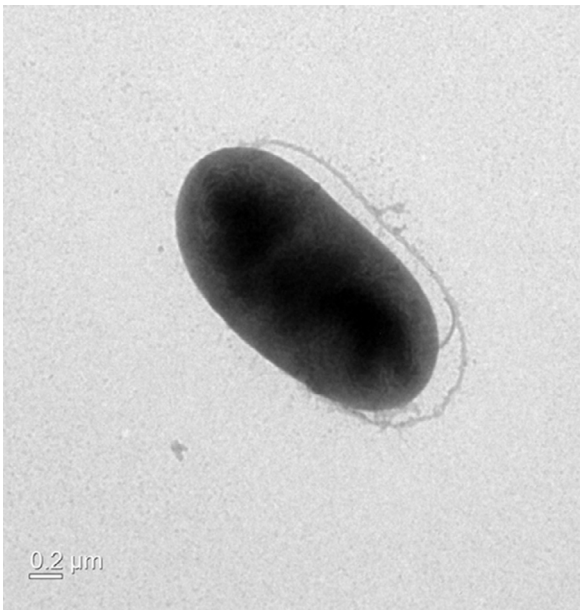

Figure 3. Representative TEM image of *S.* Enteritidis environmental isolate.

CONCLUSION

Salmonella still accounts for a high percentage of contamination in the poultry industry. To prevent cross-contamination of the carrier and equipment in the food processing line, it is important to determine the major sources and routes of contamination. This study evaluated *Salmonella* contamination in a chicken processing facility and biofilm-forming ability on various food contact surfaces. *Salmonella* isolates were mainly detected in the raw meat, a cutter that handled the raw meat, and surfaces of the equipment and chicken products in the processing lines. Above all, cross-contamination was identified between food and food contact surfaces in this study. Furthermore, all *Salmonella* serotypes had almost 100% genetic similarity and the same origin. Data obtained in this study on the prevalence and biofilm formation of *Salmonella* will help refine sanitation management guidelines in poultry processing facilities to prevent *Salmonella* outbreaks. Moreover, wild strains of *S.* Enteritidis could have the ability to form biofilm on various materials (SS, SR, and P), as well as food (chicken skin), which could be of great concern and should be investigated in further study.

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DISCLOSURES

There are no potential conflicts of interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101234.

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