Research Note: Identification and characterization of *Salmonella* spp. in mechanically deboned chickens using pulsed-field gel electrophoresis

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ABSTRACT *Salmonella* is one of the common foodborne bacteria, causing 80.3 million illnesses every year worldwide. This study was conducted to isolate and identify *Salmonella enterica* serovars from poultry samples responsible for causing foodborne poisoning in the Mississippi area, United States. A total of 55 *S. enterica* serovars—Enteritidis (6), Oranienburg (1), Schwarzengrund (8), Heidelberg (4), Kentucky (22), 4, [5], 12:i:- (1), Montevideo (2), Infantis (9), and multi serotypes (2)—were isolated from approximately 110 poultry samples. Through pulsed-field gel electrophoresis (**PFGE**) analysis, 8 to 13 bands were obtained. The profiles showed >90% similarity in strains within the same type. Consequently, PFGE could be a useful tool to determine chromosomal similarity (clonality of strains) that can be used to trace down epidemiologic sources and geographical origins of *Salmonella*.

Key words: PFGE, *Salmonella enterica* serovar, mechanically deboned chicken, bionumeric, dendrogram

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INTRODUCTION

Salmonellosis is the most commonly reported foodborne illness in the US population, affecting approximately 1 in 6 Americans per yr. As per the Centers for Disease Control and Prevention (CDC, 2019), 1,134 cases of salmonellosis associated with contaminated poultry were reported in the United States in 2019. Between 2010 and 2017, *Salmonella* was linked to 8,831 illnesses, with 1,156 hospitalizations and nearly 14 deaths (CDC, 2019). Contamination of foods with *Salmonella* can occur anywhere from farm to table, regardless of animal species and food product. About 10 to 22% of human salmonellosis cases in developed countries are attributed to exposure to contaminated poultry products (Paul et al., 2017).

The United States produces 8 billion broilers per yr, with 10% produced in Mississippi (USDA-NASS, 2011). With an increase in the consumption of poultry and poultry products, the number of *Salmonella* infections is also growing and leading to a national health issue (Paul et al., 2017). However, poultry is a highly desired meat product worldwide because of its affordable price. In particular, the mechanically deboned chicken (**MDC**) has a high consumption rate. The deboned meat is used for hamburgers, meatballs, and sausages (Mielnik et al., 2002). It is referred to as mechanically separated meat in international standards and includes distinctions of beef, pork, or chicken. Various pathogens can contaminate MDC, including *Campylobacter jejuni* and different serovars of *Salmonella enterica*. In particular, *Salmonella* Typhimurium contamination can occur in the raw carcass, with storage time and processing environment potentially adding to unsanitary conditions.

In detection of *Salmonella* contamination, there are several methods such as nonselective and selective enrichment media, plating on selective and differential agars, and some biochemical and serotype identification methods (Park et al., 2012). However, those are time consuming and require different kinds of reagent. Some common molecular methods can be used such as PCR and pulsed-field gel electrophoresis (**PFGE**). Pulsed-field gel electrophoresis is a rapid laboratory technique that can used to produce a DNA fingerprint and isolate a same type of bacteria for the purpose of epidemiologic surveillance and foodborne illness investigation

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(Wattiau et al., 2011). It separates large DNA molecules based on their migration through a gel matrix by applying an electric field that systematically changes in 2 directions for molecular subtyping foodborne microorganisms. Generally, PFGE data are considered reliable and sensitive in determining genetic relatedness between bacterial species. Owing to its high discriminative characteristics, PFGE is used regularly by the Centers for Disease Control and Prevention and the state health laboratories (Zou et al., 2013). In this study, PCR and PFGE methods were used to identify and characterize *Salmonella* spp. in various MDC samples.

MATERIALS AND METHODS

Bacterial Strains and Sample Isolation

A total of 110 MDC samples from Mississippi containing *Salmonella* spp. were analyzed. Briefly, 25 g of MDC and 225 mL of 2% buffered peptone water (Oxoid, Thermo Scientific, Lenexa, KS) enrichment broth were placed in a Stomacher bag and the top sealed. The bag was then mixed in a Seward Stomacher 400 (Seward Laboratory, London, UK) at 250 rpm for 1 min. For pre-enrichment, the samples were incubated at 37°C for 24 h. Next, 100 μL of enriched buffered peptone water was transferred to a 10-mL tube containing Rappaport-Vassiliadis medium, supplied in the *Salmonella* Rapid Test kit (Oxoid, Thermo Scientific, Waltham, MA). A 100-μL aliquot of the sample from the pre-enrichment medium was pipetted into Rappaport-Vassiliadis broth and incubated at 41.5°C for 24 h. After Rappaport-Vassiliadis streaking on xylose−lysine−deoxycholate (Oxoid, Thermo Scientific, Waltham) and brilliant green agar (Oxoid, Thermo Scientific, Waltham), with incubation at 37°C for 24 h, presumptive pink colonies with or without black centers or presumptive pink colonies surrounded by bright red medium were observed, respectively.

PCR

One to three colonies were selected from each plate, solubilized with distilled water (**DW**) in 1.5-mL graduated micro tubes (United Laboratory Plastics, Fenton, MO) and suspended in 200 μL of sterile DW. The supernatant from each sample was transferred to a PCR tube (Fisherbrand, Thermo Fisher Scientific, Waltham). One oligonucleotide primer pair was used to amplify the *invA* gene. The colonies were boiled for 5 min and then centrifuged at 14,000 × *g* for 5 min. After centrifugation, 7.5 μL of RNA-free water, 1 μL of each primer (forward: 5′-GCGTTCTGAACCTTTGGTAATAA-3'; reverse: 5′-CGTTCGGGCAATTCGTTA-3'; Sigma Life Science, St. Louis, MO), 12.5 μL of GoTaq Green Master Mix (Promega, Madison, WI), and 2 μL of the DNA template were added to a PCR tube for each sample. PCR was then performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification, consisting of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 45 s and a final extension at 72°C for 10 min. After obtaining the amplified PCR product, 8 μL was loaded for electrophoresis.

A 1.6% agarose gel with 5 μL of ethidium bromide (Invitrogen, UltraPure, Waltham, MA) was prepared using 0.5 M Tris−acetate−EDTA buffer. Eight microliters of DNA ladder was pipetted into the first well of each gel, with the samples loaded in the other wells of the gel, and gel electrophoresis was performed at 135 V for 20 min. The gel was removed from the submarine electrophoresis system and transferred to the UVDI imaging system to view band migration under UV light. The 100-bp DNA ladder (Promega) was used as a marker for *Salmonella* spp. DNA extracts from wild-type *Salmonella* were used as a template.

Pulsed-Field Gel Electrophoresis

The PulseNet (the National Molecular Subtyping Network for Foodborne Disease Surveillance in the United States) standard protocol was used for PFGE analysis. The culture grown on Tryptic soy agar (TSA, BD Difco, Franklin Lakes, NJ) plates at 37°C overnight was transferred to 1 mL of cell suspension buffer solution using a sterile polyester fiber or cotton swab. The agarose plugs were made from cell suspension, proteinase K, and 2% low-melting agarose and mixed in a pre-warmed water bath (50°C). The PFGE molds were refrigerated (4°C) for 15 min to allow the plugs to solidify. After solidification, the plugs were transferred to 5 mL of proteinase K/cell lysis buffer solution in 50-mL tubes. Lysis was performed at 40 to 45°C for 3.5 h in a shaking water bath (160 rpm). The PFGE plugs were then washed twice with preheated sterile DW (45°C) and 4 times with preheated Tris−EDTA buffer solution (45°C) for 10 min each in a shaking incubator. Afterward, the plugs were cut using a scalpel to 2-mm slices using a scalpel and kept in 5 mL of fresh Tris−EDTA buffer solution at 4°C in a microcentrifuge tube. For restriction enzyme digestion with *Xba*I, the slices were maintained in diluted restriction buffer (1X) for 15 min and then in restriction enzyme master mix (DW, 10X restriction buffer, *Xba*I) for digestion at 37°C in a water bath for 2.5 h. To stop the enzyme activity, the enzyme solution was discarded, and 500 μL of 0.5X Tris−borate−EDTA solution was added. The agarose slices of 2 mm in thickness were placed on the teeth of the electrophoresis comb horizontally. After the slices were dry, the comb was placed into the gel mold tray at a 90° angle. The 1.2% low-melting agarose at 54°C was poured into the tray and kept at room temperature for 60 min to solidify. The pulsed-field chamber (CHEF-DR II; Variable Angle System, Bio-Rad, Hercules, CA) was filled with 0.5X Tris−borate−EDTA solution and cooled down to 14°C. Once the pulsed-field agarose gel solidified, it was placed in the chamber, and electrophoresis was performed at 6 V/cm (200 V) with pulse times of 2.16 to 63.8 s and 120° angle conditions for 18 h. Afterward, the gel was stained with ethidium bromide solution (10 mg/mL) and destained with 400 mL of DW

for 30 min each. The PFGE pattern was documented under UV light by EagleEye II (Stratagene, La Jolla, CA).

Statistical Analysis

The PFGE results were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The banding patterns were compared using Dice's coefficient with a 1.5% band position tolerance and the unweighted pair group method with arithmetic averages.

RESULTS AND DISCUSSION

The presence of *Salmonella* was confirmed by PCR using the *invA* gene, a highly conserved gene present in almost all *Salmonella* serotypes. A total of 61 of 110 samples (52.73%) showed positive result for *Salmonella* spp.

A total of 55 *Salmonella* serotypes were analyzed in electrophoresis chamber. As per the PFGE fingerprints, there were 9 different pulsotypes (strains of bacteria separated by PFGE)— *Salmonella* Enteritidis (6), *Salmonella* Oranienburg (1), *Salmonella* Schwarzengrund (8), *Salmonella* Heidelberg (4), *Salmonella* Kentucky (22), *Salmonella* 4, [5], 12:i:- (1), *Salmonella* Montevideo (2), *Salmonella* Infantis (9), and *Salmonella* multiserotypes (2). Lanes 4, 8, 12, and 15 are the reference strain of *Salmonella* Braenderup H9812 (Figure 1). Conventional PFGE using the restriction enzymes *Xba*I and *Bln*I has been reported widely as a successful approach for differentiating strains of *Salmonella* isolates (Nesse et al., 2003). After *Salmonella* strains were examined by PFGE and digested by the macrorestriction enzyme *Xba*I, between 8 and 13 fragments were obtained (Figure 1).

The PFGE plugs of H9812 were tested with the *Salmonella* PulseNet standard protocol. Strain H9812 is being used profitably by PulseNet for PFGE analysis of *Salmonella*, *Escherichia coli*, and *C. jejuni* (Hunter et al., 2005). It shows a stable PFGE pattern on subculture and is susceptible to antimicrobial agents typically used to treat severe *Salmonella* infections (Hunter et al., 2005). Currently, *S.* Braenderup H9812 is used as a universal standard because it comprises the entire range of band sizes detected in foodborne pathogens traced via PulseNet despite that the pattern of the H9812 standard is likely to differ based on electrophoresis conditions and the species (e.g., *Listeria monocytogenes*, *C. jejuni*, and *Vibrio* spp.).

Figure 2 represents several clusters with different degrees of similarity. It is important to note that using the PFGE molecular typing method, heterogeneity was observed within *Salmonella* belonging to the same serovar. Serologic relatedness did not show correlation with genetic relatedness as previously reported (Fois et al., 2017). If there is a similarity >90% in a baseline, it indicates that the isolates are from the same origin. The 9 clusters that have the similarity >90% are S4 and S68 (*S.* Enteritidis), S7 and S10 (*S.* Schwarzengrund), S92 and S94 (*S.* Montevideo), S79 and S81 (*S.* Kentucky), S20 and S21 (*S.* Kentucky), S39 and S40 (*S.* Kentucky), and S41 and S42 (*S.* Kentucky) (Figure 2). Based on the results of similarity, we predict that they have the same origin. Among them, however, clusters S79 and S81 (*S.* Kentucky and *S.* Infantis) showed 94.1% similarity, although *S.* Kentucky and *S.* Infantis are from different origin and serotypes (Figure 2).

S. enterica serotype Kentucky is one of the nontyphoidal *Salmonella* species that microbiologists and public health professionals face infrequently. It has been known as the "new superbug" that shows antibiotic resistance that is more difficult to treat. A particular concern of this strains is a large number that are widely distributed in the Middle East and Africa with high resistant to ciprofloxacin, one of the drugs used to treat *Salmonella* disease (Turki et al., 2014). Multinational surveillance is focused on the increased detection frequency of the drug-resistant *S.* Kentucky and its potential for global spread. The PFGE analysis can be used to identify a

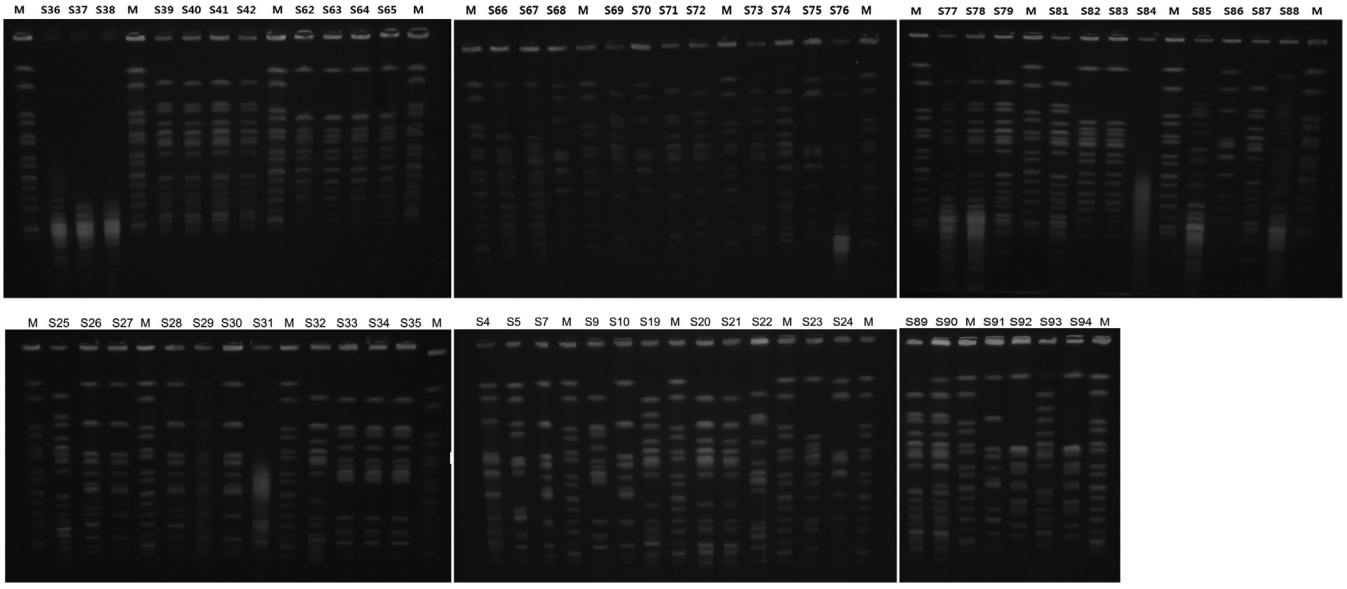

Figure 1. Pulsed-field gel electrophoresis plasmid profiles/fingerprint patterns of 61 *Salmonella* spp.

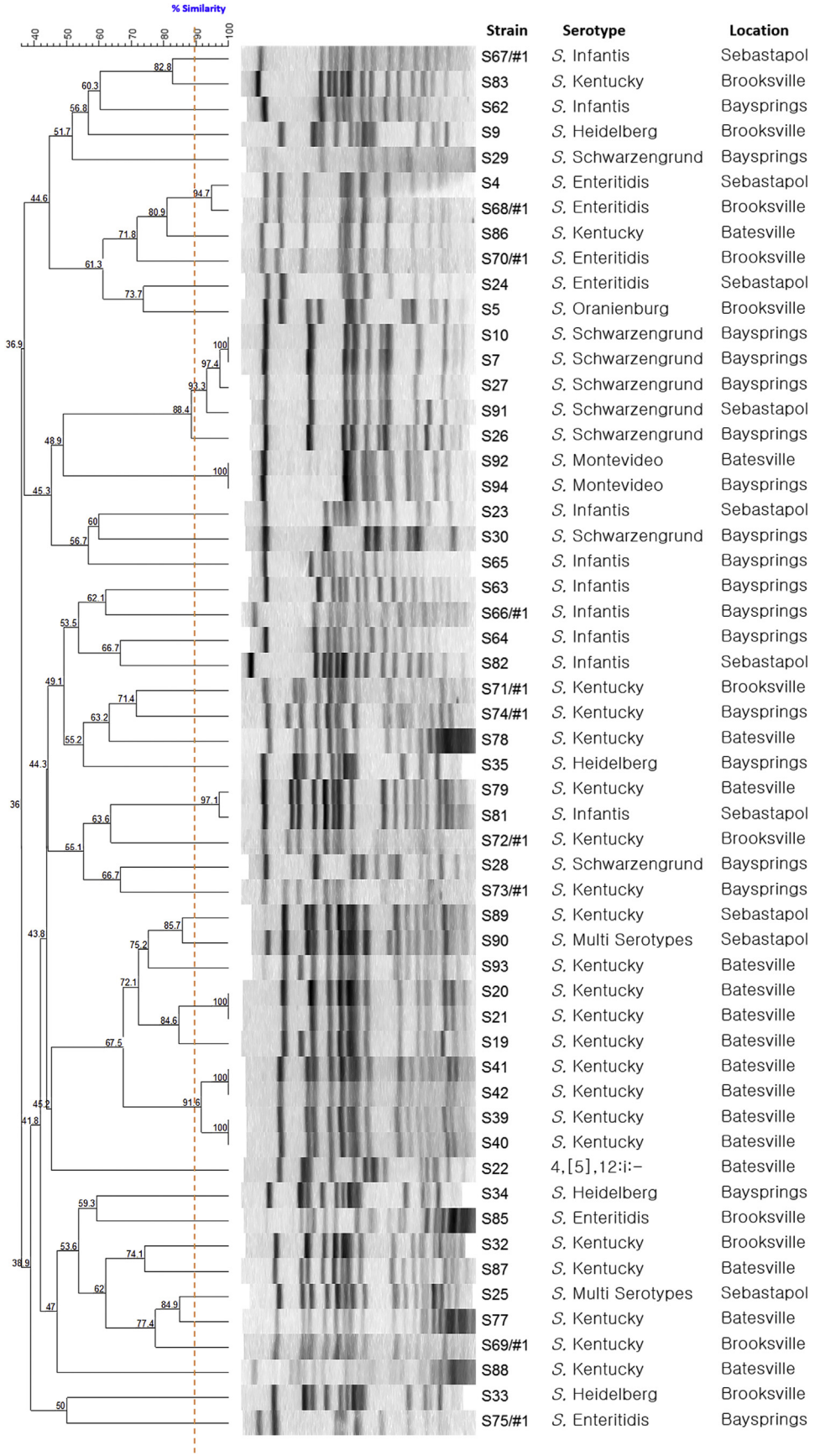

Figure 2. Pulsed-field gel electrophoresis dendrogram analysis for 55 samples with the locations of isolation.

possible path of contamination for several of the isolated strains. Moreover, the PFGE results can signify the genetic similarity between samples isolated from diverse locations on a farm (Melendez et al., 2010). Plasmid DNA profile analysis is a fast, simple, and cheap molecular method used to classify epidemics. It has been used for several yr to separate serovars belonging to *Salmonella* spp. and subtypes within the serovar. This method demonstrates an efficient separation, mainly in serotype typing. *Salmonella* spp. are able to transfer or gain plasmids from other bacteria over time and serve as an antibiotic-resistant bacteria (Ozdemir and Acar, 2014).

In summary, PFGE is a rapid laboratory technique that is mostly used to determine genetic relatedness among *Salmonella* species. The PFGE analysis of *Salmonella* fingerprints in the present study indicates a $\geq$90% genetic similarity. Therefore, PFGE is a suitable tool to determine chromosomal similarities among isolates linked to epidemiologic investigations of *Salmonella* outbreaks, especially those linked to poultry. It can also be used to trace the source of the outbreak and prevent illness, as demonstrated by the Centers for Disease Control and Prevention PulseNet.

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DISCLOSURES

The authors declare no conflicts of interest.

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