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Research Note

The characterization of *Salmonella enterica* serotypes isolated from the scalder tank water of a commercial poultry processing plant: Recovery of a multidrug-resistant Heidelberg strain

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ABSTRACT The recent multistate outbreak of a multidrug-resistant (MDR) *Salmonella* Heidelberg strain from commercial poultry production highlights the need to better understand the reservoirs of these zoonotic pathogens within the commercial poultry production and processing environment. As part of a larger study looking at temporal changes in microbial communities within the major water tanks within a commercial processing facility, this paper identifies and characterizes *Salmonella enterica* isolated from the water in a final scalder tank at 3 times during a typical processing day: prior to the birds entering the tank (start), halfway through the processing day (mid), and after the final birds were scalded (end). Over 3 consecutive processing days, no *Salmonella* were recovered from start-of-day water samples, while a total of 56 *Salmonella* isolates were recovered from the mid-day and end-of-day scalder water samples. Traditional and newer PCR-based serotyping methods eventually identified these isolates as either group C3 *S.* Kentucky (n = 45) and group B *S.* Heidelberg (n = 11). While none of the *S.* Kentucky isolates possessed any resistances to the antimicrobials tested, all *S.* Heidelberg isolates were found to be multidrug resistant to 5 specific antimicrobials representing 3 antimicrobial classes. Due to the potential public health impact of *S.* Heidelberg and the recent nationwide poultry-associated outbreak of multidrug-resistant *S.* Heidelberg, future studies should focus on understanding the transmission and environmental growth dynamics of this serotype within the commercial poultry processing plant environment.

Key words: *S.* Heidelberg, multidrug resistance, scalder water

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INTRODUCTION

Salmonella enterica is one of the most prevalent sources of human gastroenteritis in the United States (Painter et al., 2013) as well as globally, resulting in an estimated 93.8 million infected individuals and 155,000 deaths annually (Majowicz et al., 2010). The infection often results in clinical symptoms such as diarrhea, abdominal pain and vomiting that generally resolve within a week. *Salmonella* is especially concerning among the very young, older adults, and immunocompromised populations, as they are more susceptible to complications such as endocarditis, bacteremia, meningitis, and pneumonia (Arshad et al., 2008; Hohmann, 2001). It has been reported that food is the source of more than 95% of all nontyphoidal *Salmonella* infections (Hohmann, 2001), making it a major food safety issue.

The poultry industry has been frequently implicated in *Salmonella* outbreaks, with reports of human pathogenic *S. enterica* serotypes (e.g., Enteritidis, Heidelberg, Typhimurium) in poultry products representing a major food safety concern for the industry. The link between human illness and *Salmonella* contamination of poultry products remains strong, from the consumption of both eggs (Anonymous, 2004; Currie et al., 2005; Hennessy et al., 2004; Tribe et al., 2002) and broiler meat (Altekruse et al., 2006; Anonymous, 2004; Gallegos-Robles et al., 2008; Mohle-Boetani et al., 2009; Nunes et al., 2003). In addition to the presence of the different serotypes throughout the poultry production and processing spectrum, recent increases

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in the incidence of antimicrobial-resistant *Salmonella* serotypes represent an emerging food safety and public health concern. Treatment of antimicrobial-resistant pathogen infections is typically more complex and expensive (Cosgrove, 2006), with estimated direct medical costs from drug-resistant nontyphoidal *Salmonella* of $365 million annually in the United States alone (CDC, 2013a).

Considering that the commercial poultry processing plant is the most direct link between vector (broiler) and host (consumer), it is imperative to understand the diversity of *Salmonella* serotypes that can exist in multiple reservoirs within the processing plant. One major *Salmonella* reservoir within a processing plant, and one that has the potential to rapidly transmit pathogens between carcasses within the processing plant, is the scalder tank (Buncic and Sofos, 2012; Cason and Hinton, 2006; Cason et al., 2000; Finstad et al., 2012; Yang et al., 2001). Therefore, as part of a larger study observing temporal changes in the microbiology of the major water tanks within a commercial broiler processing plant (Rothrock et al., 2013), this note describes the presence of *Salmonella* within the commercial scalder tank throughout the processing day and characterizes the recovered isolates in terms of serotyping (serological and molecular) and antimicrobial susceptibilities.

MATERIALS AND METHODS

Experimental Design

Processing water samples were collected from a commercial broiler processing facility that was processing small (approx. 2 kg) Cobb broilers during the time of this study. Broilers were processed at a line speed of 364 birds/min^{-1} for 18 hr each day. Three sterile 1-L plastic Nalgene bottles (Fisher Scientific, Pittsburgh, PA) were used to collect 3 L water from approx. 5 cm below the surface at the turnaround (midpoint) of the final scalder tank of a triple tank counterflow system. Samples were collected from the final scalder water tank at 3 times during the processing shift: 1) prior to the first birds entering the cleaned and disinfected tanks (start), 2) after 9 hours of processing (approx. half of the processing day; mid), and 3) after the last birds left the tank and the waters were considered "dirtiest" (end). Samples were taken from these 3 time points on 3 successive days and placed on ice for transport back to the laboratory for further sample processing and preparation. Each group of 3 water samples from a single time point will henceforth be referred to as a single sample.

Salmonella *Culture Methods*

All water samples were vigorously homogenized. To identify the number of *Salmonella* that were present in each sample, enumeration was done using a 3-tube most probable number (**MPN**) analysis according to Cason and Hinton (2006). In short, triplicate 10 mL processing water samples were added to sterile tubes containing 10 mL 2× buffered peptone water (**BPW**), and triplicate 1 mL processing water samples were added to sterile tubes containing 9 mL 1× BPW. For the final triplicate sample, 1 mL processing water samples were diluted 1:10 in 0.1% peptone water and vortexed, and then 1 mL of that dilution was added to 9 mL 1× BPW. The MPN tubes were incubated 18 to 24 hr at 35°C. After incubation, 0.1 mL from each tube was transferred to 9.9 mL Rappaport-Vassiliadis broth (**RV**; Becton-Dickinson, Sparks, MD) and incubated for 18 to 24 hr at 42°C. After incubation, a loopful (approx. 0.01 mL) from each enrichment tube was struck onto both xylose lysine tergitol-4 (**XLT-4**; Becton-Dickinson) and brilliant green sulfa with novobiocin (**BGS**; Becton-Dickinson) agar plates and incubated for 18 to 24 hr at 35°C. On each plate, 3 *Salmonella*-like colonies were picked and confirmed using triple sugar iron agar (**TSI**; Becton-Dickinson) and lysine iron agar fermentation (**LIA**; Becton-Dickinson) and an incubation period of 18 to 24 hr at 35°C. Final confirmation of suspect TSI/LIA isolates was performed using *Salmonella* polyvalent O antiserum agglutination (Becton-Dickinson), per manufacturer specifications. Positive *Salmonella* were then serogrouped using individual *Salmonella* poly O antisera for O groups A through I, following the Kauffman-White scheme.

Molecular Characterization Methods

For all molecular characterization/serotyping methods described below, single colonies for each isolate were grown in 10 mL brain heart infusion (**BHI**) broth (Difco BD, Franklin Lakes, NJ) at 37°C for 16 h. Bacterial cells were pelleted in a Sorvall RC5B Plus centrifuge at 5,000 × *g* for 15 min in a Sorvall Super-lite SLA 600TC rotor. The DNA from all *Salmonella* isolates was extracted and purified using the PureLink Genomic DNA Mini Kit (Invitrogen, Grand Island, NY). Spectrometer readings of DNA samples were obtained using a NanoDrop 1000 (ThermoScientific, Wilmington, DE) to ensure 260:280 optical density (**OD**) ratios were greater than 1.7 and that DNA concentration was above 20 ng/μL. Standard protocols were used for all molecular characterization methods, and each is shortly described below.

Pulsed-field Gel Electrophoresis. Bacterial isolates were propagated, prepared, and analyzed as previously described (Matushek et al., 1996; Ribot et al., 2006). *Salmonella* ser. Braenderup H9812 (ATCC BAA-664) restricted with *XbaI* (Hunter et al., 2005) was used as a control and as the DNA size standard. Restriction fragments were separated by electrophoresis in 0.5M Tris borate–EDTA buffer at 14°C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times ranging between 2.16 and 54.17 s. Interpretation of DNA fingerprint patterns was accomplished using Bionumerics 4.0 software (Applied Maths, Austin, TX) and comparison to the PulseNet Database. The banding patterns were compared using

Table 1. Serological and molecular serotyping results for *Salmonella enterica* isolates recovered throughout the processing day from the scalder water at a commercial poultry processing plant.

	Kaufmann-White		PFGE		*dkgB*-ISR PCR	
Sampling time during processing day[1]	Serotype	No. of isolates	Closest serotype match[2]	No. of isolates	Serotype	No. of isolates
Mid (19 isolates)	B	15	Kentucky (0001 ARS)	15	Kentucky	15
	C3	4	Heidelberg (0015 ARS)	4	Heidelberg	4
End (37 isolates)	B	30	Kentucky (0001 ARS)	30	Kentucky	30
	C3	7	Heidelberg (0015 ARS)	7	Heidelberg	7

[1]No *Salmonella* isolates were ever recovered from the start sampling time.

[2]Code in parenthesis represents the closest serotype match identifier from the PulseNet database.

Dice coefficients with a 1.5% band position tolerance. Patterns with no noticeable differences were considered indistinguishable and were assigned the same pulsed-field gel electrophoresis (**PFGE**) pattern designation.

dkgB-linked Intergenic Space Region PCR. The PCR protocol and primers targeting the *dkgB*-linked intergenic space region (**dkgB-ISR**) (including the entire 5s ribosomal gene) have been described previously (Morales et al., 2006). To determine serotype, an amplicon sequence trimmed to the aforementioned ISR was aligned to reference sequences deposited at the National Center for Biotechnology Information (NCBI) by DNASTAR Lasergene SeqMan Version 8.0.2 using default project assembling parameters except as follows: minimum match percentage 100, minimum sequence length 100. Only perfect matches can be used to call serotype. ISR reference sequences that define serotype have GenBank accession numbers JN105119-JN105125 and JN092293-JN092328.

Antimicrobial Susceptibility Testing

Recovered isolates were subcultured on blood agar plates (**BAP**) overnight at 36 ± 1°C. One to 2 colonies were used to inoculate 5 mL demineralized water to achieve a 0.5 McFarland equivalent using the Sensititre nephelometer (ThermoScientific, TREK Diagnostics, Inc., Cleveland, OH). Following vortexing, 10 μL cell suspension was transferred to 11 mL Sensititre cation adjusted Mueller-Hinton broth with TES buffer, followed by thorough vortexing. Into each well of the Sensititre NARMS Gram-Negative Format CMV2AGNF plate (Trek Diagnostic Systems) was transferred 50 μL inoculum. These AST plates contained varying concentrations of the following antimicrobials: cefoxitin, azithromycin, chloramphenicol, tetracycline, ceftriaxone, amoxicillin/clavulanic acid (2:1), ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, sulfisoxazole, trimethoprim/sulfamethoxazole, kanamycin, ampicillin, and streptomycin. Plates were sealed with a porous cover and incubated at 36 ± 1°C for 18 h. Quality control strain (*Escherichia coli* ATCC 25922), obtained from the ATCC, was included in susceptibility tests as a positive control (Clinical and Laboratory Standards Institute (CLSI), 2010).

RESULTS AND DISCUSSION

While *Salmonella* spp. were not recovered from the scalder water samples taken prior to the first line of carcasses, low concentrations were isolated from the mid- and end sampling times (0.198 and 0.125 cfu/mL^{-1}, respectively). While the final scalder tank is typically kept at a temperature adequate to kill many organisms (approx. 58°C), *Salmonella* spp. have been isolated from commercial scalder tanks previously (Buncic and Sofos, 2012; Finstad et al., 2012; Liu et al., 2001). A total of 56 *Salmonella* spp. isolates were recovered from these samples, and each was characterized using a panel of traditional and molecular typing techniques to determine the potential diversity and/or clonality of these isolates.

Serotyping results for the 56 isolates are shown in Table 1. The traditional serological typing scheme (Kaufmann-White) showed that approx. 80% of the isolates matched serogroup C3, and the remaining approx. 20% matched serogroup B, but further molecular characterization was required to identify the specific serotype of each isolate. To initially predict the serotype of these environmental isolates, PFGE was performed to determine the genetic similarity of these environmental isolates to known *Salmonella* serotypes (Zou et al., 2010, Zou et al., 2013). These results supported the serological findings, with the isolates falling into 2 groups based on their matches to serotypes in the PulseNet database: Kentucky (0001 ARS) and Heidelberg (0015 ARS). To genetically confirm the PFGE-predicted serotype designations, the *dkgB*-ISR PCR method (Morales et al., 2006) was used, and the serotypes of these 2 groups were confirmed as *S.* Kentucky and *S.* Heidelberg. Over the past several decades, *S.* Heidelberg and *S.* Kentucky have become 2 of the most commonly detected serotypes in meat and poultry products (Foley et al., 2011; USDA, 2013) and have been repeatedly found in poultry processing plants and on retail poultry in the United States (Logue et al., 2003; Parveen et al., 2007; Zhao et al., 2006); therefore, their recovery from this commercial scalder tank was not unexpected.

Given the increased focus on antibiotic resistance among *Salmonella* spp., the antimicrobial susceptibility of each isolate was further characterized using the FDA NARMS (National Antimicrobial Resistance

Table 2. Antimicrobial resistances of *S.* Heidelberg isolates recovered from scalder tank water at two time points during the processing day at a commercial processing plant.[1,2,3]

Isolate ID	Sampling time	Cephems			β-lactam/β-lactamase inhibitor combinations	Penicillins
		Cefoxitin	Cetiofur	Ceftriaxone	Amoxicillin-clavulanic acid	Ampicillin
C2–8	Mid	32 (R)	8 (R)	8 (R)	32/16 (R)	32 (R)
C2–9	Mid	32 (R)	8 (R)	16 (R)	32/16 (R)	32 (R)
C2–10	Mid	32 (R)	8 (R)	16 (R)	32/16 (R)	32 (R)
C2–11	Mid	32 (R)	8 (R)	8 (R)	32/16 (R)	32 (R)
A3–9	End	32 (R)	8 (R)	16 (R)	32/16 (R)	32 (R)
A3–10	End	32 (R)	8 (R)	8 (R)	32/16 (R)	32 (R)
A3–16	End	32 (R)	8 (R)	8 (R)	32/16 (R)	32 (R)
A3–17	End	32 (R)	8 (R)	8 (R)	32/16 (R)	32 (R)
A3–18	End	16 (I)	8 (R)	8 (R)	32/16 (R)	32 (R)
A3–19	End	16 (I)	8 (R)	8 (R)	32/16 (R)	32 (R)
A3–20	End	16 (I)	8 (R)	8 (R)	32/16 (R)	32 (R)

[1]All isolates were determined to be susceptible to all other antimicrobials tested for on the CMV2AGNF Sensititre plate.

[2]Values represent highest antimicrobial concentration (mg/L) where growth was observed.

[3]Letter in parentheses indicates the susceptibility toward that antimicrobial: R = resistant, I = Intermediate.

Monitoring System) method. While all 45 Kentucky scalder isolates were found to be susceptible to the entire panel of antimicrobials used in this assay, each of the 11 Heidelberg isolates was found to be resistant to 5 different antimicrobials (cefoxitin, cetiofur, ceftriazone, amoxicillin-clavulanic acid, ampicillin) representing 3 different classes of antimicrobials (cephems, β-lactam/β-lactamase inhibitor combinations, penicillins; Table 2). This classifies these Heidelberg scalder isolates as multidrug resistant (**MDR**) strains, but it should be noted that co-resistances to these classes of antimicrobials (especially cephems and β-lactams) have been previously seen in *Salmonella enterica* isolated from agricultural animals, typically associated with a plasmid-encoded AmpC β-lactamase (bla_{CMY}) gene (Gray et al., 2004; Liebana et al., 2004).

The recovery of MDR *S.* Heidelberg from within the final scalder tank at this commercial poultry processing facility is the major discovery of this research note, especially given the recent public health issues related to MDR Heidelberg from poultry products. In mid-2012, an outbreak of salmonellosis cases reported among 134 people was traced back to a single chicken producer (CDC, 2013b); as of July 2014, there have been 634 reported illnesses in 29 states related to this MDR *S.* Heidelberg strain (CDC, 2014). Even though only a single salmonellosis case from this outbreak has been found in Georgia, the isolation of MDR Heidelberg from a commercial processing facility within this state (the largest poultry producing state in the United States) indicates that these types of MDR *Salmonella* may be more widespread within the commercial poultry production and processing industry than initially considered.

It should be noted that MDR Heidelberg isolates were recovered over multiple processing days and at different times during each processing day during May 2012 (around the time of the beginning of the multistate outbreak). The broilers, and specifically the broiler farms themselves, are the most likely source of this *Salmonella* that has entered the poultry processing plant environment (Berghaus et al., 2014), and approx. 1.2 million birds were processed from numerous broiler farms serviced by this commercial processing plant during the time of this sampling. Also of note is that no *Salmonella* were recovered from the final scalder water tanks at the start of any of the processing days. These observations suggest that MDR Heidelberg contamination within the final scalder water tank originated from multiple flocks/farms rather than from a single incidence.

Although the Kentucky scalder isolates were susceptible to all antimicrobials tested, MDR *S.* Kentucky is a growing public health concern (Hedberg, 2011; Le Hello et al., 2011) and has been found within the poultry production spectrum (FDA, 2010). Research has shown that concentrations of *Salmonella* (as well as other foodborne pathogens) can be significantly higher in the first 2 tanks of the triple scalder tank system (Cason and Hinton, 2006; Cason et al., 2000), indicating that these serotypes may be more prevalent in earlier scalder tank waters. Carcass rinse samples were not obtained as part of the larger study of this commercial processing plant, but genetic signatures specific to *Salmonella* spp. were found in the downstream chiller tank at these same sampling times using 2 different PCR-based methods (Rothrock et al., 2013). While these PCR assays did not specifically target MDR Heidelberg, the recovery of these MDR isolates indicates the possibility for further transmission throughout the processing plant environment and, more importantly, a potential food safety issue with public health (both worker and consumer) implications.

Considering these isolates were recovered from a single commercial processing plant over 3 consecutive processing days, the broader applicability of these limited findings is hard to ascertain. While the recovery of MDR *Salmonella* was not the focus of this specific study, future studies could be designed to specifically isolate *Salmonella* from final scalder tanks from a variety of commercial processing facilities over longer

periods of times and determine their serotypes and AST patterns. These data would allow for an expanded understanding of the presence and distribution of MDR *Salmonella* within these processing environments. But even with this limited dataset, the fact that MDR Heidelberg was recovered from a reservoir that comes in contact with all carcasses being processed throughout the processing day highlights a potential emerging poultry food safety concern for the industry that may be more widespread than initially considered.

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