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Research Note

Chlorine Inactivation of *Salmonella* Kentucky Isolated from Chicken Carcasses: Evaluation of Strain Variation

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ABSTRACT

The current study was undertaken to evaluate chlorine resistance among strains of *Salmonella* Kentucky isolated from chicken carcasses. Selected strains ($n = 8$) were exposed to 30 ppm of chlorine in 10% buffered peptone water (pH 7.4) for 0 to 10 min at 4°C and 150 rpm. The initial level (mean ± SD) of *Salmonella* Kentucky was 6.18 ± 0.09 log CFU/ml and did not differ ($P > 0.05$) among strains. A two-way analysis of variance indicated that the level of *Salmonella* Kentucky in chlorinated water was affected ($P < 0.05$) by a time by strain interaction. Differences among strains increased as a function of chlorine exposure time. After 10 min of chlorine exposure, the most resistant strain (SK145) was 5.63 ± 0.54 log CFU/ml, whereas the least resistant strain (SK275) was 3.07 ± 0.29 log CFU/ml. Significant differences in chlorine resistance were observed for most strain comparisons. Death of *Salmonella* Kentucky was nonlinear over time and fitted well to a power law model with a shape parameter of 0.34 (concave upward). Time (minutes) for a 1-log reduction of *Salmonella* Kentucky differed ($P < 0.05$) among strains: >10 min for SK145, 6.0 min for SK254, 1.5 min for SK179, and 0.3 to 0.65 min for other strains. Results of this study indicate that strain is an important variable to include in models that predict changes in levels of *Salmonella* Kentucky in chlorinated water.

Salmonella is a leading cause of foodborne illness in the United States, with an annual estimate of 1.04 million cases of salmonellosis resulting in 19,336 hospitalizations and 730 deaths *(7)*. Foods of animal origin, especially poultry meat and eggs, have been involved in many outbreaks of human salmonellosis *(7, 12)*. Commercial processing of poultry is a complex process that involves multiple steps: bleed out, scalding, defeathering, evisceration, inspection, washing, chilling, and further processing. Water used to wash and chill poultry can be a significant source of pathogen (e.g., *Salmonella*) cross-contamination among carcasses *(2)*.

Chlorine is routinely used by the poultry industry in the United States in processing water to reduce levels of bacteria and cross-contamination of carcasses with pathogens such as *Salmonella*. Chlorine is highly soluble in water and as a sanitizing agent has broad bactericidal effects. The U.S. Department of Agriculture (USDA) Food Safety and Inspection Service *(11)* requires that processors add 20 to 50 ppm of chlorine to water in the chill tank to prevent cross-contamination of carcasses with pathogens. Carcasses reside in chillers for substantial periods of time (45 to 60 min). Consequently, chillers are considered critical control points in processing operations for reduction of microbiological hazards *(4, 9, 10)*.

Models that predict the level of pathogens in response to process variables are valuable tools in poultry processing for verifying that critical points are in control. Oscar et al. *(4)* modeled in vitro survival of nonresistant and antibiotic-resistant strains of *Salmonella* Typhimurium in chlorinated water and found that time for a 1-log reduction by chlorine was 2.8 to 5.6 min for 16 strains of *Salmonella* Typhimurium. These results suggest that variation among strains of *Salmonella* is an important independent variable to consider when modeling *Salmonella* behavior in chlorinated water.

Isolates used by Oscar et al. *(4)* were the same as those used by Parveen et al. *(5),* who determined prevalence, serotype, and antibiotic resistance profiles of *Salmonella* isolated from chicken carcasses collected before and after chilling in a commercial plant. Predominant *Salmonella* serotypes found by Parveen et al. *(5)* were Kentucky (59.5%), which is rarely isolated from human clinical samples, and Typhimurium (17.8%), which is one of the top human clinical isolates. However, chlorine resistance of *Salmonella* Kentucky strains has not been investigated. Consequently, the current study was conducted to evaluate strains of *Salmonella* Kentucky for their resistance to chlorine. Because it was not possible to conduct these studies in vivo for occupational health and safety reasons,

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an in vitro approach similar to that of Oscar et al. *(4)* was used.

MATERIALS AND METHODS

***Salmonella* Kentucky isolates.** Eight *Salmonella* Kentucky isolates recovered from chicken carcasses *(5)* were selected according to their pulsed-field gel electrophoresis (PFGE) profiles *(3)*. Each isolate displayed unique PFGE profiles with the exception of strains SK439 and SK533, which represented distinct strains. Isolates were maintained at −70°C in brain heart infusion (BHI) broth (BBL, Difco, BD, Sparks, MD) that contained 15% (vol/vol) glycerol (Sigma, St. Louis, MO).

Experimental design. A 6 × 8 full factorial design was used to study main effects and interaction of time (0, 0.5, 1, 2, 5, and 10 min) and strain (SK496, SK145, SK439, SK513, SK533, SK275, SK254, and SK179) on survival of *Salmonella* Kentucky in chlorinated water incubated at 4°C and 150 rpm. Three replicate trials were conducted for each strain for a total of 24 trials (three replicates for eight strains).

Chlorinated water. Preliminary experiments (results not shown) indicated that addition of 30 ppm of chlorine (sodium hypochlorite; Intercostal Trading Inc., Secretary, MD) to sterilized spring water (Eastern Springs Water Company, Preston, MD) resulted in death of *Salmonella* Kentucky that was too rapid to measure by spiral plating (i.e., viable counts), whereas addition of 30 ppm of chlorine to buffered peptone water (BPW; BBL, Difco, BD) resulted in no death of *Salmonella* Kentucky in 10 min because BPW acted as an effective stopping solution by rapidly consuming the chlorine. In contrast, 10 ml of BPW plus 90 ml water (10% BPW) with a final pH of 7.4 provided the right conditions for evaluating strain variation in response to 30 ppm chlorine during a 10-min period. After chlorine was added to 10% BPW, the pH was measured, and the chlorine concentration (mean ± SD) of the solution was determined before (29.8 ± 0.53 ppm) and after (26 ± 1.8 ppm) each experiment. Before addition of *Salmonella* Kentucky, a chlorine test kit (model PCT-DR, LaMotte, Chestertown, MD) was used to measure residual chlorine, and a pH meter (pH Spear, Oakton, Instruments, Vernon Hill, IL) was used to measure pH.

Preparation of inoculum. Inoculum was prepared by adding 5 μl of the appropriate stock culture to 5 ml of BHI broth in a 25-ml Erlenmeyer flask. Flasks were sealed with foam plugs and incubated at 30°C for 24 h at 150 rpm. After incubation, cultures were serially diluted (1:10) in BPW, and 10^{-6} and 10^{-7} dilutions were spiral plated (Whitely automated spiral plater, Microbiology International, Frederick, MD) onto BHI agar. Spiral plates were incubated for 24 h at 30°C, and then colonies were counted with an automated counter (Protocol, Microbiology International) to determine *Salmonella* levels in the original cultures.

In vitro trials. One hundred microliters of a 10^{-1} dilution of the appropriate *Salmonella* Kentucky culture was inoculated into 250-ml Erlenmeyer flasks that contained 100 ml of 10% BPW and 30 ppm of chlorine. Flasks were incubated (refrigerated incubator shaker, Innova 4230, New Brunswick Scientific, Edison, NJ) at 4°C and 150 rpm. At 0, 0.5, 1, 2, 5, and 10 min, 4- and 1-ml samples were removed. The 4-ml sample was immediately used to spiral plate 50 μl onto BHI agar, and the 1-ml sample was serially diluted in BPW to 10^{-1} or 10^{-2} and then spiral plated onto BHI agar. Viable counts (log CFU per milliliter) were determined as described above. The lower limit of detection was 2.5 log CFU/ml.

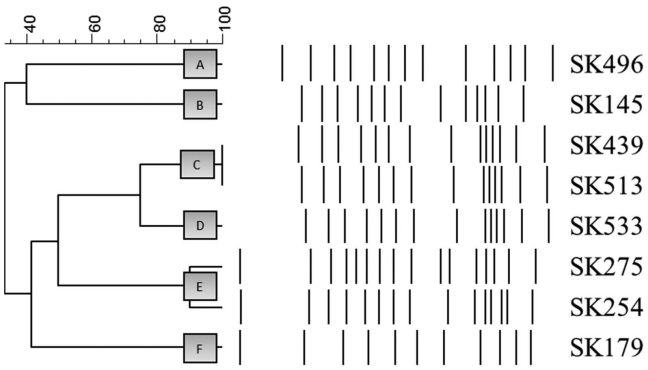


FIGURE 1. *Dendrogram of pulsed-field gel electrophoresis profiles of* Salmonella *Kentucky isolated from chicken carcasses. Similarity index is indicated on the left axis. Letters in boxes (A through F) indicate clusters.*

Statistical analysis. A two-way analysis of variance (ANOVA) was used to determine main effects and the interaction of time and strain on level of *Salmonella* Kentucky in chlorinated water. When a significant main effect or interaction was observed ($P < 0.05$), means were compared using Tukey's multiple comparison tests. Statistical analysis was performed using version 6.2 of GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Nonlinear regression (GraphPad Prism) was used to fit viable count data (log CFU per milliliter) as a function of time to the power law model *(6)*:

$$N(t) = N_0 - \left[\left(\frac{t}{d}\right)^p\right]$$

where $N(t)$ is the level of *Salmonella* Kentucky at time t (minutes), N_0 is the initial level of *Salmonella* Kentucky, d is the time (minutes) needed for a 1-log reduction of *Salmonella* Kentucky, and p is the shape parameter, which is 1 for a linear line, <1 for a concave upward line, and >1 for a concave downward line. After an initial round of nonlinear regression, N_0 and p were fixed to their average values among strains (6.18 log CFU/ml for N_0 and 0.34 for p) to facilitate comparison of d among strains using an F test in GraphPad Prism.

RESULTS AND DISCUSSION

Although chlorine is widely used in the poultry industry in the United States, it can react with organic compounds in poultry meat and eggs, leading to the formation of potentially carcinogenic and teratogenic trihalomethanes and haloacetic acids *(9)*. Consequently, in Europe, chlorine is not used in poultry processing; air chilling rather than immersion chilling in chlorinated water is the primary method for chilling chickens to prevent growth of spoilage and pathogenic bacteria.

In an initial experiment, chlorinated (30 ppm) spring water with a pH of 7.4 resulted in no viable counts of *Salmonella* Kentucky after 0.5 min of incubation at 4°C (results not shown) because the chlorine was very active. As organic material builds up in the chill tank during a process run in a commercial poultry processing plant, it binds chlorine and reduces its ability to kill bacteria in process water *(1)*. Thus, chlorine must constantly be supplied to comply with USDA guidelines for maintenance of a level between 20 and 50 ppm *(11)*.

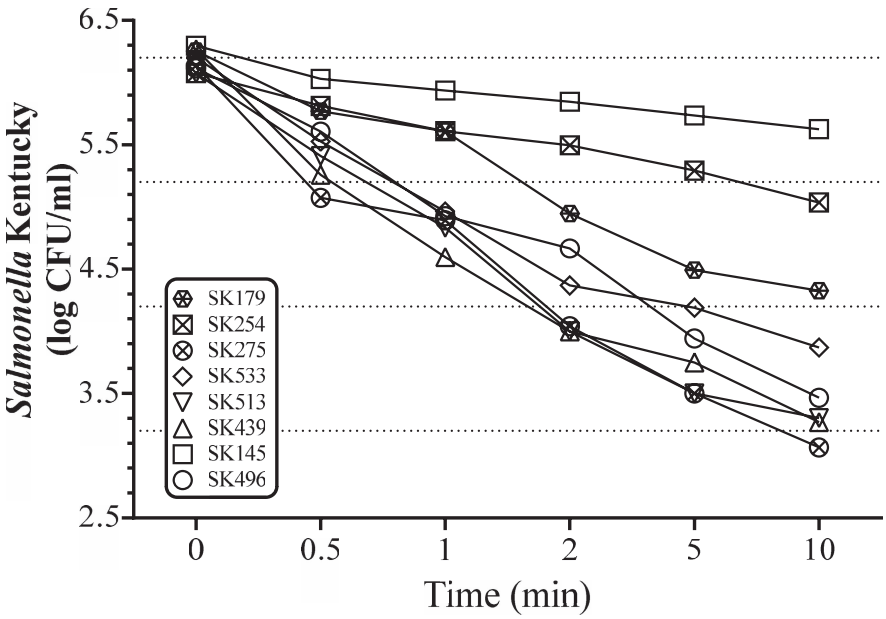


FIGURE 2. *Effect of time and strain on level of* Salmonella *Kentucky in chlorinated 10% buffered peptone water (pH 7.4) incubated at 4°C and 150 rpm.*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	13.80	35	0.3942	F (35, 96) = 5.790	P < 0.0001
Time	80.01	5	16.00	F (5, 96) = 235.0	P < 0.0001
Strain	36.27	7	5.181	F (7, 96) = 76.10	P < 0.0001
Residual	6.536	96	0.06808		

One way to control the death rate of *Salmonella* in response to a target level of chlorine (e.g., 30 ppm) in an in vitro water system is to manipulate the amount of organic material. Consequently, a series of test runs with different concentrations of organic material in the form of peptone from buffered peptone water (BPW) were conducted. When the level of BPW was more than 10% at pH 7.4, no death of *Salmonella* Kentucky was observed over the targeted period of 10 min (results not shown). Therefore, by trial and error, 10% BPW was identified as an appropriate level of organic material to use in this study to evaluate variation in chlorine resistance among strains of *Salmonella* Kentucky exposed to 30 ppm of chlorine for 10 min.

Isolates used in this study were the same as those used in a previous study *(5)* of prevalence, serotype, and antibiotic resistance of *Salmonella* isolated from chicken carcasses sampled before and after chilling in a commercial plant. Selection of isolates in the present study was primarily based on their PFGE profiles. Seven PFGE banding patterns were found among the eight *Salmonella* Kentucky isolates selected (Fig. 1). Based on the PFGE results, isolates were grouped into six clusters (A through F) with 90% pattern similarity: SK496 (A), SK145 (B), SK439 and SK513 (C), SK533 (D), SK275 and SK254 (E), and SK179 (F). Isolate SK145 was designated as the reference isolate because it was the isolate most resistant to chlorine (Figs. 2 through 5). PFGE pattern analysis revealed 40% similarity of this isolate with isolate SK496 and less than 40% similarity with the other six isolates.

Strain selection is an important consideration when developing models that predict the behavior of pathogens in unit operations of the food production chain. Having strains isolated from the unit operation of interest is a good first step. A good second step is to evaluate those strains for their behavior under conditions found in the unit operation. Because it was not possible to evaluate the selected strains of *Salmonella* Kentucky for their behavior in a commercial chill tank because of occupational health and safety concerns, an in vitro approach similar to that used by Oscar et al. *(4)* with strains of *Salmonella* Typhimurium was used with strains used by Parveen et al. *(5)*. Chlorinated water prepared in the laboratory (i.e., 10% BPW with 30 ppm of chlorine) rather than chlorinated water from a commercial chill tank was used because of lack of steady access to the commercial water supply and to provide better control of the concentration of chlorine and organic material in the water

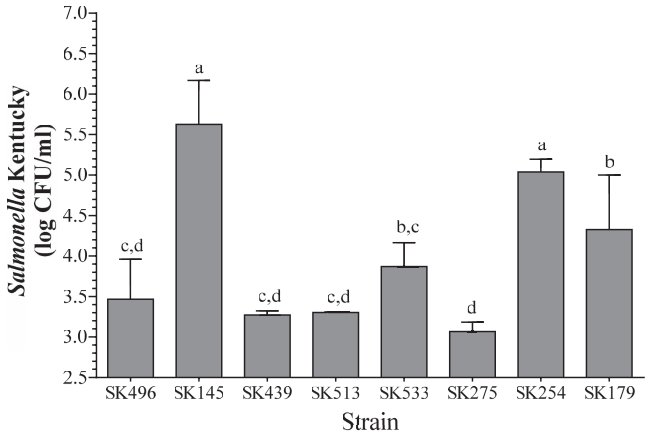


FIGURE 3. *Levels of eight strains of* Salmonella *Kentucky in chlorinated (30 ppm) 10% buffered peptone water (pH 7.4) at 10 min of incubation at 4°C and 150 rpm. Bars with different lowercase letters are significantly different at* $P < 0.05$.

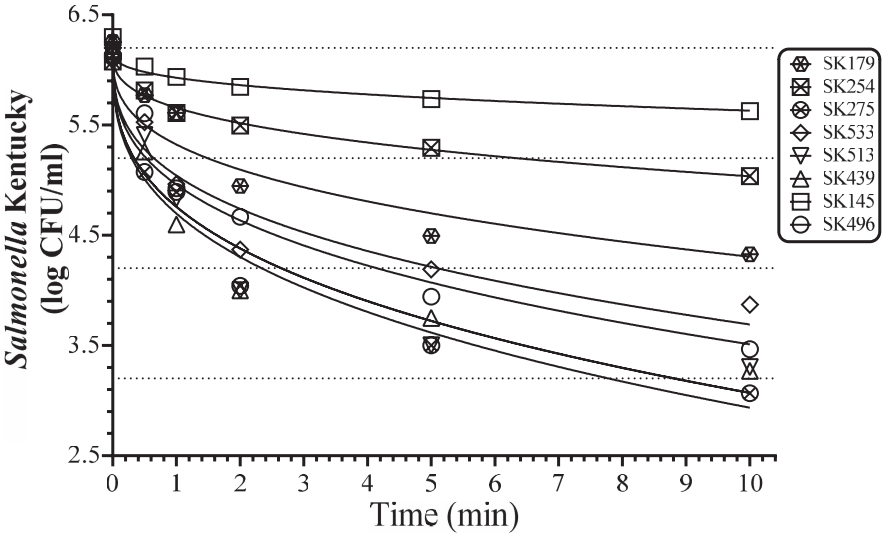
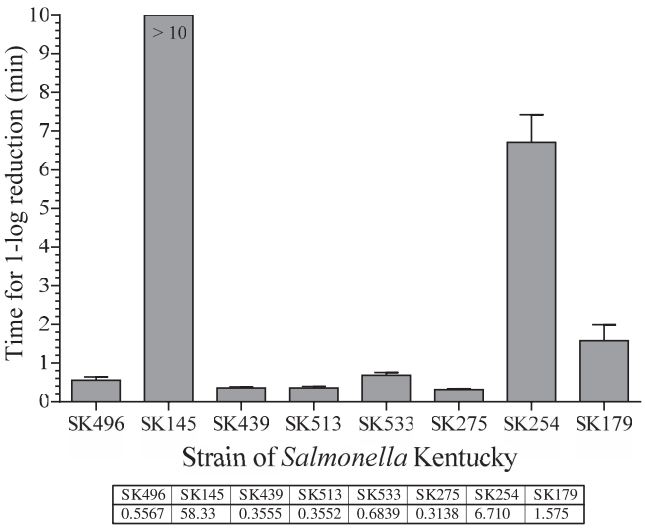


FIGURE 4. *Power law model fits to kinetic data for death of* Salmonella *Kentucky in chlorinated water. Symbols are the mean observed values of three replicate experiments for each strain, and lines are best-fit lines.*

and thus a better (i.e., more controlled) experimental system to evaluate strain variation.

The in vitro system of chlorinated water was inoculated with individual strains of *Salmonella* Kentucky, and viable counts were determined over time on a nonselective agar medium (BHI agar) to ensure detection of injured and healthy pathogen cells and rapid inactivation of residual chlorine. Experimental conditions were selected to allow accurate measurement and detection of differences among strains for sensitivity to chlorine in chilled water. The results of the two-way ANOVA indicated that the level of *Salmonella* Kentucky in chlorinated water was affected ($P < 0.05$) by a time by strain interaction (Fig. 2). The initial level of *Salmonella* Kentucky (6.18 $\pm$ 0.09 log CFU/ml) did not differ ($P > 0.05$) among strains. Differences among strains increased as a function of time (Fig. 2).

At 10 min of exposure to chlorine (Fig. 3), the most resistant strain (SK145) was 5.63 $\pm$ 0.54 log CFU/ml and the least resistant strain (SK275) was 3.07 $\pm$ 0.29 log CFU/ml. Significant differences in chlorine resistance were observed for many strain comparisons at 10 min of chlorine exposure (Fig. 3). Chlorine resistance from highest to lowest was SK145 = SK254 > SK179 = SK533 $\geq$ SK496 = SK513 = SK439 $\geq$ SK275. Average reductions in *Salmonella* Kentucky at 10 min of exposure to 30 ppm of chlorine was <1 log CFU/ml for strain SK145, 1 to 2 log CFU/ml for strains SK254 and SK179, 2 to 3 log CFU/ml for strains SK533, SK496, SK513, and SK439, and >3 log CFU/ml for strain SK275.



SK496	SK145	SK439	SK513	SK533	SK275	SK254	SK179
0.5567	58.33	0.3555	0.3552	0.6839	0.3138	6.710	1.575

FIGURE 5. *Effect of strain of* Salmonella *Kentucky on time (minutes) for a 1-log reduction of viable counts in chlorinated water. Bars are best-fit values, and error bars are the standard error of best-fit values for replicate data from three challenge trials.*

Results of this study agree in principle with those of previous studies; chlorine usage in chiller water decreases levels of *Salmonella* and other bacteria on chicken carcasses by reducing cross-contamination *(10, 13)*. Smith et al. *(8)* concluded that immersion chilling is effective for reducing levels of bacteria, including pathogens, on carcasses. However, immersion chilling alone is insufficient to prevent cross-contamination of carcasses by *Salmonella*. Chlorine should be incorporated into the chiller water because it can reduce cross-contamination by killing bacteria suspended in the water.

The death of *Salmonella* Kentucky followed a nonlinear pattern over time (Fig. 4) and fitted well to a power law model with a shape parameter of 0.34 (concave upward). Time (minutes) for a 1-log reduction of *Salmonella* Kentucky differed (F test; $P < 0.05$) among strains: >10 min for SK145, 6.0 min for SK254, 1.5 min for SK179, and 0.3 to 0.65 min for other strains (Fig. 5). Yang et al. *(13)* reported times of 3.4 and 5.8 min for a 1-log reduction of *Salmonella* Typhimurium in chilled chlorinated water collected at time 0 and after 8 h of processing in a commercial chicken processing plant, respectively. Oscar et al. *(4),* using a similar in vitro chlorinated water model (30 ppm of chlorine, 4°C, 150 rpm) but with strains of *Salmonella* Typhimurium and with lower BPW (2%) and lower pH (6), reported a shape parameter (p) of 1.37 (concave downward) and d values from power model fits of 2.9 to 5.6 min. Differences in serotype, concentration of BPW, and pH between the present study and that of Oscar et al. *(4)* make it difficult to explain differences in the shapes

of the death curves, which were concave upward ($p = 0.34$) in the present study and concave downward ($p = 1.37$) in the study of Oscar et al. Nonetheless, results of this study in general agree with those of Oscar et al. and indicate that the strain of *Salmonella* is an important independent variable that should be included in models for predicting changes in the level of *Salmonella* in chlorinated water.

ACKNOWLEDGMENTS

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