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Research Paper

Susceptibility of *Salmonella* Biofilm and Planktonic Bacteria to Common Disinfectant Agents Used in Poultry Processing

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ABSTRACT

Poultry contaminated with *Salmonella enterica* subsp. *enterica* are a major cause of zoonotic foodborne gastroenteritis. *Salmonella* Heidelberg is a common serotype of *Salmonella* that has been implicated as a foodborne pathogen associated with the consumption of improperly prepared chicken. To better understand the effectiveness of common antimicrobial disinfectants (i.e., peroxyacetic acid [PAA], acidified hypochlorite [aCH], and cetylpyridinium chloride [CPC]), environmental isolates of nontyphoidal *Salmonella* were exposed to these agents under temperature, concentration, and contact time conditions consistent with poultry processing. Under simulated processing conditions (i.e., chiller tank and dipping stations), the bacteriostatic and bactericidal effects of each disinfectant were assessed against biofilm and planktonic cultures of each organism in a disinfectant challenge. Log reductions, planktonic MICs, and mean biofilm eradication concentrations were computed. The biofilms of each *Salmonella* isolate were more resistant to the disinfectants than were their planktonic counterparts. Although PAA was bacteriostatic and bactericidal against the biofilm and planktonic *Salmonella* isolates tested at concentrations up to 64 times the concentrations commonly used in a chiller tank during poultry processing, aCH was ineffective against the same isolates under identical conditions. At the simulated 8-s dipping station, CPC was bacteriostatic against all seven and bactericidal against six of the seven *Salmonella* isolates in their biofilm forms at concentrations within the regulatory range. These results indicate that at the current contact times and concentrations, aCH and PAA are not effective against these *Salmonella* isolates in their biofilm state. The use of CPC should be considered as a tool for controlling *Salmonella* biofilms in poultry processing environments.

Key words: Biofilm; Disinfectant; Poultry processing; *Salmonella*

A biofilm is an adaptive mechanism where bacteria are able to adhere to a surface via the secretion of a protective exopolysaccharide matrix *(20)*. The ability of *Salmonella* to form a biofilm on both biotic and abiotic surfaces has previously been established *(9, 25, 26)*. In various food production systems the ability to form biofilms is an important factor in bacterial persistence *(4, 7, 25)*. Specifically, biofilms are recognized as significant contributors to foodborne contamination in food processing environments because of their resistance and persistence in both biotic and abiotic environments *(19)*. Although contamination of poultry skin and feathers with *Salmonella* is well known *(31), Salmonella* biofilms can grow on various types of epithelium, including the intestinal epithelium of poultry *(16)*.

When *Salmonella* biofilms become established in food processing environments, they are difficult to remove and can persist even in the face of cleaning procedures and chemical disinfection, and these persistent strains can promote cross-contamination *(8, 9)*. Bacterial species with the ability to produce biofilms appear to have increased resistance to acidification, desiccation, chlorination, heating, ionizing radiation, and antimicrobial agents *(14, 25)*. This increased tolerance may play a role in the pathogenesis of infections and foster epidemics *(23)*. Although biofilms are more difficult to remove via standard cleaning procedures, biofilm cells are less virulent or pathogenic than their planktonic counterparts *(17)*. The transition of *Salmonella* between its biofilm and planktonic state may contribute to variation with respect to virulence and the overall ability to infect other animals *(15)*.

Antibiotic efficacy, as quantified by MICs and minimum biofilm eradication concentrations (MBECs), is used to evaluate and quantify bacterial susceptibility and resistance *(20)*. However, these tools are typically not utilized with other antimicrobial agents, including disinfectants, to measure their performance. The U.S. Environmental Protection Agency regulates the labeling, handling, and efficacy of sterilants, disinfectants, and sanitizers to validate vendor claims and maintain quality assurance *(2)*. Testing guidelines and standards are developed by AOAC International (Gaithersburg, MD) to determine bactericidal, virucidal, tuberculocidal, fungicidal, and sporicidal activity against American Type Culture Collection (ATCC) organisms, including various *Salmonella* strains. These tests are

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TABLE 1. *Disinfectant concentrations used in the log reduction, MIC, and MBEC assays*[a]

Disinfectant	Concn (ppm)									
CPC	128,000	64,000	32,000	16,000	8,000	4,000	2,000	1,000	500	250
aCH	3,200	1,600	800	400	200	100	50	25	12	6
PAA	14,720	7,360	3,680	1,840	920	460	230	115	57	28

[a] The regulatory ranges in the carcass chiller are 200 to 2,000 ppm for PAA and 20 to 50 ppm for aCH. The regulatory range for CPC at the dipping station is 500 to 8,000 ppm.

composed of three phases, including practical field in-use and kill-time tests on a variety of surfaces *(22)*. Although effective for many purposes, a major limitation of the AOAC tests in a poultry processing environment is the lack of testing on biofilms, whose cells are more ubiquitous, robust, and resistant than their planktonic counterparts in food production environments *(14, 15)*. The recognition that bacteria exist primarily as biofilms rather than in their planktonic state has fundamental implications for food safety and food safety research.

Although much information on the control of planktonic *Salmonella* via various antimicrobial agents has been published, limited information is available on the control of biofilms in a poultry processing environment *(8, 18, 28, 30)*. The ability of *Salmonella* to form biofilms and quantification of biofilm formation on various surfaces consistent with food processing and storage have been documented *(1, 29)*. However, to our knowledge, there is no clear understanding of how to measure the sensitivity of biofilm and planktonic bacteria to specific disinfectants used during poultry processing. Therefore, we explored the efficacy and practicality of using a disinfectant challenge with log reduction assays for planktonic and biofilm bacteria and MBEC and MIC assays to facilitate the characterization of *Salmonella* isolates found in poultry processing food systems. To test this approach, *Salmonella* Heidelberg (SH) planktonic and biofilm isolates were grown under laboratory conditions and tested under simulated poultry processing conditions with respect to contact time, temperature, and the concentration of each disinfectant.

MATERIALS AND METHODS

Bacterial strains and antimicrobial conditions. Five field isolates of SH were acquired from a commercial poultry company. These isolates were tested against a historic field strain of SH isolated in 1992 and against two additional *S. enterica* serovars (Ohio and Senftenberg) also obtained from the same commercial broiler company. A known strong biofilm former (*Pseudomonas aeruginosa* ATCC 53323) and a known weak biofilm former (*Escherichia coli* ATCC 27853) were purchased from the ATCC (Manassas, VA) *(3, 22)*. Isolates were stored at –80°C in glycerol stocks. Planktonic and biofilm cultures were grown in Trypticase soy broth (TSB; BD, Franklin Lakes, NJ), and bacterial counts and growth assessments were performed on Trypticase soy agar (TSA; BD). Stock solutions of 100× concentrated disinfectants were stored away from light at 21°C and diluted to desired concentrations as needed. Disinfectants were tested at the average concentrations used by one commercial poultry broiler company: PAA at 230 ppm (U.S. Department of Agriculture, Food Safety and Inspection Service [FSIS] regulatory range in the carcass chiller, 200 to 2,000 ppm), aCH at 50 ppm (FSIS regulatory range, 20 to 50 ppm), and CPC at 2,000 ppm (FSIS regulatory range, 500 to 8,000 ppm). All disinfectants were also tested at concentrations that were 0.125× to 64× those average concentrations (Table 1).

Planktonic exposure to antimicrobial agents. *Salmonella* cultures were inoculated into 10 mL of TSB from the glycerol stocks and incubated overnight at 37°C. The following day, the cultures were diluted 1:100 in 100 mL of TSB. The diluted culture was placed in a shaker incubator (MaxQ 4000, Thermo Fisher Scientific, Waltham, MA) at 37°C and 100 rpm for 3.5 h until mid-log phase was reached. For each replicate, 20 μL was then serially diluted and plated onto TSA for pretreatment quantification. Five milliliters of each culture was pipetted into a plastic centrifuge tube and challenged with 50 μL of 100× concentrated disinfectant to achieve 230 ppm of PAA, 50 ppm of aCH, and, 2,000 ppm of CPC for exposure at 4°C for 90 min, with a no-treatment control (Table 1). An aliquot (20 μL) from each replicate was used to inoculate a labeled 96-well plate for dilution. The samples were serially diluted and then plated on TSA. All plates were incubated overnight at 37°C, and colony counts were done the following day.

MIC protocol for planktonic cultures. Two 96-well plates were prepared for challenging the mid-log phase planktonic cultures with 200 μL of serially diluted disinfectants at all concentrations listed in Table 1. The first column served as the negative control for TSB sterility, the last column served as the positive control for no treatment, and one row was skipped between each disinfectant series to prevent cross-contamination. For each replicate, 20 μL of each planktonic isolate was then serially diluted and plated onto TSA for pretreatment quantification, and 20 μL of each isolate was pipetted into the challenge wells. After 90 min at 4°C to simulate the time and temperature in the carcass chiller, 30 μL from each well was spot plated onto labeled TSA plates (four drops fit on each plate). The plates were then incubated overnight at 37°C, and growth was assessed as positive or negative for each concentration of disinfectant the following day. The 96-well challenge plates were also incubated overnight at 37°C, and the optical density at 650 nm (OD_{650}) of the exposed cultures was measured with an spectrometer (Epoch, BioTek Instruments, Winooski, VT) using Gen5 2.01 all-in-one microplate reader software (BioTek) to determine the bactericidal concentration of each disinfectant.

Preparation of biofilm. *Salmonella* cultures were inoculated into 10 mL of TSB from the glycerol stocks and incubated overnight at 37°C. As a negative control, pure TSB was also incubated overnight. The following day, the culture samples were diluted 1:100 in 50 mL of TSB. The resultant cultures were then diluted 1:30 in 30 mL of TSB. This dilution served as the inoculum for the MBEC 96-well biofilm inoculator peg plate (Innovotech,

Edmonton, Alberta, Canada), with 150 μL of the inoculum aliquoted into each well of the biofilm plate. The plate was sealed with parafilm to prevent cross-contamination and evaporation and placed on a plate rocker (GeneMate, BioExpress, Kaysville, UT) set to rock at 12° of inclination and five rocks per minute inside a 37°C incubator for 4 days to allow the biofilm to mature, following the steps outlined by Corcoran et al. *(8)*.

Pretreatment quantification of biofilm cultures. Biofilm growth was tracked starting on the second day. The biofilm plate was retrieved from the incubator, and two randomly selected pegs from each replicate were broken off with flame-sterilized pliers. The pegs were placed in 200 μL of TSB inside a labeled 96-well plate for sonication *(20)*. The plate was sealed with adhesive microplate tape under the lid to prevent water leakage. After sonication for 10 min at 35 kHz in an ultrasonic cleaner (model 97043-960, VWR, Radnor, PA), the pegs were removed from their wells with flame-sterilized tweezers, and the OD_{650} of the suspended cultures was measured with a spectrometer (BioTek) to verify biofilm growth. A 20-μL aliquot from each biofilm isolate was used to inoculate a labeled 96-well plate for dilution. The samples were serially diluted onto TSA plates and incubated overnight at 37°C, and colonies were counted the next day and used to determine the dilutions for the next part of the experiment. At the end of the incubation period on the fourth day, the biofilm peg plates were retrieved from the incubator. The peg lid of the biofilm plate was rinsed by immersion in a 96-well plate filled with 200 μL of sterile TSB in each well. After rinsing, four sample pegs were broken off with flame-sterilized pliers and immersed in another 96-well plate with 200 μL of sterile TSB in each well for sonication and plating, as described above.

Log reduction protocol for biofilms. The biofilm peg plate (MBEC peg plate, Innovotech) was challenged with 230 ppm of PAA, 50 ppm of aCH, and 2,000 ppm of CPC for 90 min at 4°C. A similar protocol was followed for the 8-s experiments at room temperature using 1,000 ppm (0.5×), 2,000 ppm (2×), and 4,000 ppm (4×) CPC (Table 1). At the conclusion of the challenge, the pegs were transferred through two 96-well rinse plates, and two pegs from each treatment were broken off and placed into a third 96-well recovery plate for sonication and plating, as described above. All plates were incubated overnight at 37°C. Colonies were counted on the following day.

MBEC protocol. The MBEC assay was done according to the manufacturer's instructions *(13)*. Each individual experiment was done four times in duplicate.

Statistical analysis. Basic descriptive statistical analyses were conducted with Excel (Microsoft, Redmond, WA). Data were further coded and analyzed using R 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). An analysis of variance and a post hoc Tukey test for the different treatments were also done using R 3.2.2 and the R package "ggplot."

RESULTS AND DISCUSSION

Both planktonic bacteria and bacteria in biofilms contribute to chronic microbial contamination of food *(11)*. Because biofilms and planktonic bacteria are biochemically different and hence respond differently to antimicrobial agents *(8, 9)*, the overall goal of this study was to understand differences in how field strains of host-adapted zoonotic *Salmonella* respond to commonly used poultry processing disinfectants, based on the results of log reduction, MIC, and MBEC assays. For each of the assays, PAA, aCH, and CPC concentrations within and above the FSIS regulatory range were assayed to determine whether modifications to the disinfectant concentrations would significantly affect the efficacy of each disinfectant (Tables 2 through 4).

All seven field strains of *Salmonella* were determined to be biofilm formers, which is consistent with previously published information that the vast majority of *Salmonella* strains are capable of forming biofilms *(1)*. However, not all biofilms, even within the same strain, react to environmental stresses in the same way under different laboratory conditions *(19)*. For example, over time the strength and structure of a biofilm can change. Scanning electron microscope images revealed that a biofilm after 168 h was morphologically different, denser, and more adherent to the substratum than the same biofilm at 48 h *(8)*. Therefore, comparing results of our 4-day protocol used to grow the biofilms with results of other studies *(31)* that utilized different procedures for biofilm production may be problematic. Consequently, it is essential to develop consistent growth conditions to facilitate appropriate comparisons with respect to biofilm sensitivity. Development of AOAC-based guidelines for biofilm sensitivity testing would help ensure consistency. However, future research should also focus on the sensitivity of biofilms to disinfectants after different periods of time consistent with poultry production to better understand and characterize this potential source variability.

Log reduction of *Salmonella* in a simulated chiller tank (90 min at 4°C). PAA and aCH were ineffective for producing a significant log reduction ($P < 0.05$) of all seven field strains of *Salmonella* tested at the temperature (4°C), time (90 min), and PAA (230 ppm) and aCH (50 ppm) concentrations used in commercial poultry chiller tanks (Table 2). In contrast, with the same temperature and contact times, CPC at 2,000 ppm did produce a significant reduction ($P < 0.05$) in biofilm and planktonic culture growth (Table 2); CPC at 2,000 ppm killed all *Salmonella* strains tested (Table 2).

Similar results demonstrating the effectiveness of CPC were also found for the *E. coli* strain tested, which forms weaker biofilms *(22)* (Table 2). In contrast, CPC was not effective against the biofilm or planktonic form of *P. aeruginosa,* which is known as a strong biofilm former *(3)*. Although *P. aeruginosa* is not a common pathogen in poultry, the CPC result illustrates the value of testing newly identified pathogens against each disinfectant to evaluate the efficacy of those disinfectants against the planktonic and biofilm forms.

Although CPC is considered an effective alternative to chlorine-based chemicals, it is not used in the chiller tank in part because of the relative cost *(10)*. However, these experiments were done to test the disinfectants against each other under identical conditions.

The log reduction assay approach has several distinct disadvantages. One significant limitation is that a direct

TABLE 2. *Log reductions for biofilm (B) and planktonic (P) cultures to determine the effectiveness of each disinfectant*

Bacterial strain	Mean (range) reduction (log CFU/mL)[a]			
	Control	230 ppm of PAA	50 ppm of aCH	2,000 ppm of CPC[b]
SH field strain 10				
B	−0.9 (−1.5 to −0.7)	−1.2 (−1.6 to −0.9)	−1.4 (−2.0 to −1.3)	−6.0* (K)
P	+0.9 (−0.5 to +1.5)	−2.7 (−4.4 to −2.1)	−2.9 (−3.8 to −2.3)	−13.0* (K)
SH field strain 18				
B	+2.4 (−0.4 to +2.9)	−1.0 (−1.1 to −1.0)	+0.2 (−1.2 to +0.6)	−8.0* (K)
P	−0.1 (−1.3 to +0.4)	−0.3 (−0.8 to +0.1)	−1.0 (−1.9 to −0.4)	−10.0* (K)
SH field strain 29				
B	−0.2 (−9.4 to +0.3)	−5.3 (−13.3 to −4.7)	−5.4 (−13.2 to −4.7)	−20.0* (K)
P	−0.8 (−1.4 to −0.3)	−1.4 (−1.8 to −1.1)	−1.5 (−1.8 to −1.1)	−10.0* (K)
SH field strain 30				
B	−3.0 (−9.4 to −2.7)	−3.6 (−12.6 to −3.0)	−4.1 (−12.5 to −3.5)	−20.0* (K)
P	−4.7 (−14.0 to −4.0)	−7.8 (−15.1 to −7.1)	−7.4 (−14.7 to −6.7)	−21.0* (K)
SH historic 1992				
B	+5.0 (0 to +5.6)	+6.3 (−1.2 to +6.8)	+3.1 (−1.0 to +3.4)	−7.0* (K)
P	+0.5 (−0.6 to +1.0)	−0.7 (−2.0 to −0.4)	−0.9 (−1.2 to −0.7)	−8.0* (K)
Salmonella Ohio field strain 11				
B	+1.8 (−1.3 to +2.3)	+3.8 (−1.3 to +4.5)	+3.5 (−1.4 to +4.2)	−8.0* (K)
P	+3.4 (−0.8 to +3.9)	+0.5 (−2.7 to +0.9)	+0.2 (−3.3 to +0.7)	−14.0* (K)
Salmonella Senftenberg field strain 65				
B	−0.1 (−6.8 to +0.7)	−3.9 (−6.9 to −3.2)	−1.6 (−6.8 to −1.1)	−14.0* (K)
P	+3.7 (−4.2 to +4.3)	+0.8 (−6.2 to +1.4)	+1.0 (−6.3 to +1.8)	−13.0* (K)
P. aeruginosa				
B	−0.7 (−1.2 to −0.2)	−0.4 (−1.9 to +0.1)	−1.1 (−1.7 to −0.9)	−1.3 (−2.5 to −0.8)
P	+0.1 (−1.2 to +0.6)	−0.8 (−1.7 to −0.4)	−1.0 (−2.2 to −0.4)	−2.8 (−3.7 to −2.4)
E. coli				
B	−0.4 (−0.5 to −0.3)	−1.1 (−1.4 to −0.9)	−0.9 (−1.0 to −0.8)	−5.0* (K)
P	−0.1 (−0.8 to +0.4)	−0.6 (−1.1 to −0.3)	−0.4 (−1.0 to +0.1)	−6.0* (K)

[a] Biofilm and planktonic cultures were exposed to each disinfectant for 90 min at 4°C, simulating the contact conditions of a typical chiller in a poultry processing plant. The values reflect the negative or positive log reduction. CPC is not typically used in a chiller tank but was included in this experiment to understand its potential efficacy relative to the other disinfectants. Two repetitions of triplicate runs were performed with each strain using PAA, aCH, and CPC at concentrations within regulatory ranges.

[b] * $P < 0.05$, significant differences were observed for each strain when comparing each disinfectant against each other disinfectant for the same strain. K, disinfectant killed all bacteria.

comparison between the biofilm and planktonic samples cannot be made because the biofilm and planktonic controls were separate (Table 2). Another significant difficulty with the log reduction assay is the wide range (up to 10 log CFU/mL) in both planktonic and biofilm log reductions (Table 2). The repeatability of each log reduction assay in which duplicate samples were evaluated in triplicate created a batch effect, where the repeatability of each experiment was not consistent with respect to the numerical log reduction. When the data were stratified based on the batch effect and analyzed as three different experiments, there was no significant difference between the controls and the PAA and aCH treatments (Table 2) because of the small sample size ($n = 2$) for each experiment and the wide variance. However, although no significant difference was found between the controls and the PAA and aCH treatments, log reductions of >1 log CFU/mL were observed for many of the isolates (Table 2). In summary, although the log reduction experiment is a relatively simple yet time-consuming and labor-intensive method to quantitatively define the efficacy of a disinfectant, the repeatability of the assay should be questioned even when starting with the same relative level of bacteria for each experiment.

MIC and MBEC of *Salmonella* in a simulated chiller tank (90 min at 4°C). The biofilm form of each isolate was more resistant than the corresponding planktonic form (Table 3), demonstrating why it is essential to test both the planktonic and biofilm forms of any bacterial strain of interest. Based on the lowest effective concentration, PAA was most effective against planktonic *E. coli* because the

TABLE 3. *Bacteriostatic and bactericidal concentrations of each disinfectant against planktonic (P) and biofilm (B) cultures*[a]

	PAA (ppm)		aCH (ppm)		CPC (ppm)	
Bacterial strain	Static	Cidal	Static	Cidal	Static	Cidal
SH field strain 10						
B	14,700	14,700	**>3,200**	**>3,200**	400	2,800
P	**900**	**3,700**	**>3,200**	**>3,200**	**300**	2,100
SH field strain 18						
B	**7,400**	8,300	**>3,200**	>3,200	**300**	2,400
P	**900**	**3,700**	**>3,200**	**>3,200**	**300**	400
SH field strain 29						
B	**14,700**	14,700	**>3,200**	**>3,200**	300	2,500
P	**900**	4,600	**>3,200**	**>3,200**	**300**	600
SH field strain 30						
B	11,000	**14,700**	**>3,200**	**>3,200**	**300**	1,100
P	700	5,500	**>3,200**	**>3,200**	**300**	**300**
SH historic 1992						
B	**14,700**	14,700	>3,200	>3,200	800	**1,000**
P	**900**	**3,700**	**>3,200**	**>3,200**	**300**	300
Salmonella Ohio field strain 11						
B	**>14,700**	**>14,700**	**>3,200**	**>3,200**	**300**	**300**
P	**900**	6,400	**>3,200**	**>3,200**	**300**	600
Salmonella Senftenberg field strain 65						
B	**14,700**	**14,700**	**>3,200**	**>3,200**	**500**	500
P	**1,800**	**7,400**	**>3,200**	**>3,200**	**300**	600
P. aeruginosa						
B	5,500	**7,400**	**>3,200**	**>3,200**	128,000	**>128,000**
P	**500**	**1,800**	**>3,200**	**>3,200**	**500**	**128,000**
E. coli						
B	**>14,700**	**>14,700**	**>3,200**	**>3,200**	**300**	**300**
P	**500**	**700**	**>3,200**	**>3,200**	**300**	**300**

[a] Cultures were exposed to each disinfectant for 90 min at 4°C. Values are based on MIC and minimum biofilm eliminating concentration assays. Two repetitions of triplicate runs were performed on each strain using PAA, aCH, and CPC at concentrations commonly utilized in poultry processing facilities (Table 1). Values consistent between all trials are shown in bold.

lowest concentrations of PAA were required to obtain bactericidal and bacteriostatic effects (Table 3). However, PAA was ineffective against the biofilm form of *E. coli* at all PAA concentrations tested (Table 3). This finding is consistent with published reports in which biofilms had increased tolerance to disinfectants and antibiotics compared with the same bacteria in the planktonic state *(6, 21)*. Consequently, development of AOAC-based guidelines for biofilm sensitivity testing would help food producers, including poultry producers, identify environmental isolates that they wish to characterize.

For all the planktonic *Salmonella* isolates tested, PAA was bacteriostatic at 900 to 1,800 ppm and bactericidal at 3,700 to 7,400 ppm (Table 3). For six of the seven strains of *Salmonella* biofilms, PAA was bacteriostatic at 7,400 to 14,700 ppm and bactericidal at 8,300 to 14,700 ppm (Table 3). The only *Salmonella* biofilm that was resistant to PAA was that of *Salmonella* Ohio. The MIC of PAA was higher for *Salmonella* Ohio than for all the other *Salmonella* strains tested (Table 3).

The regulatory range of PAA in the finishing chiller is 200 and 2,000 ppm. However, for biofilm control the effective concentrations were above the maximum currently allowed by the FSIS *(27)*. In contrast, for bacteriostatic control of planktonic bacteria the effective concentration was within the FSIS regulatory range (Table 3). Therefore, at the maximum allowed regulatory level of 2,000 ppm, PAA would not have been bacteriostatic or bactericidal against any of the *Salmonella* biofilms tested at 4°C for 90 min (Table 3).

Although PAA had limited efficacy in these experiments, aCH at all tested concentrations was ineffective against all bacteria tested (Table 3). Because resistance to aCH is a predictor of resistance to antibiotics *(5),* these results further support the need to test the efficacy of disinfectants against known pathogens commonly isolated in poultry processing facilities. Disinfectant efficacy testing

TABLE 4. *Bacteriostatic and bactericidal concentrations of CPC against biofilm cultures*[a]

Bacterial strain	CPC MBEC (ppm)	
	Static	Cidal
SH field strain 10	**3,400**	8,000
SH field strain 18	**1,000**	8,000
SH field strain 29	2,400	6,400
SH field strain 30	**3,200**	6,770
SH historic 1992	**1,600**	>12,800
Salmonella Ohio field strain 11	1,200	4,000
Salmonella Senftenberg field strain 65	**6,400**	>12,800
P. aeruginosa	12,800	**>12,800**
E. coli	**4,000**	11,200

[a] Cultures were exposed for 8 s at 21°C. Values are based on minimum biofilm eliminating concentration (MBEC) assays. Two repetitions of triplicate assays were performed on each strain using CPC at the range of concentrations identified in Table 1. Bactericidal concentrations were based on growth, and bacteriostatic concentrations were based on optical density at 650 nm. Values with no statistical variance are shown in bold.

should be viewed as an integral component of efforts to mitigate antimicrobial resistance.

Although CPC is not used in chiller tanks and hence is not in contact with the broiler carcasses for 90 min, it was nevertheless tested under chiller tank conditions to explore the efficacy of CPC compared with PAA and aCH. For both planktonic and biofilm *Salmonella* isolates, CPC was bacteriostatic at 300 and 500 ppm and bactericidal at 300 to 2,800 ppm (Table 3). CPC was not bactericidal for *P. aeruginosa* and was bacteriostatic at only the highest concentration tested (Table 3).

A review of the literature revealed that MIC- and MBEC-based assays are not used to study the efficacy of disinfectants against commonly isolated poultry pathogens. However, the overall advantage of these assays is that they more easily allow for the identification of effective bacteriostatic and bactericidal concentrations for the planktonic and biofilm forms of each organism. The MIC and MBEC assays also allow direct comparisons between planktonic and biofilm cultures to determine the differences between them with respect to the different disinfectants (Table 3). In contrast to the log reduction assay, the MIC and MBEC results were consistent between trials (see bold MIC and MBEC values without variance, Tables 3 and 4). One advantage of the log reduction assay is the ability to further refine the effect of the disinfectant based on the MIC and MBEC results. The MIC and MBEC assays could be used to identify the effective bacteriostatic concentrations, and the log reduction assay could be used to determine the specific log reductions (either CFU per milliliter or percentage) at the effective bacteriostatic concentrations. This information could help identify bacteria that are challenged but not killed, which appear to play a role in resistance, hypervirulence, and antimicrobial resistance *(12, 21, 24)*.

Eradication of *Salmonella* biofilm in a simulated dipping station (8 s at 21°C). To determine the efficacy of CPC under time conditions more consistent with its use in poultry dipping stations during second processing (i.e., postchiller), MBEC experiments were done with CPC in contact with the bacteria for 8 s (Table 4). After 8 s of contact time, the CPC was bacteriostatic at 1,200 and 6,400 ppm for all strains of *Salmonella* tested (Table 4). In contrast, CPC was bactericidal at 4,000 to 8,000 ppm for five of the seven *Salmonella* strains tested (Table 4). No CPC concentration was effective against the remaining two *Salmonella* strains tested, including the *Salmonella* Senftenberg field strain and the 1992 SH field strain (Table 4)

The 1992 SH field strain was included in this study because it could be used to help understand the potential for increased resistance between a historic strain of SH and SH isolates found more recently. Overall when comparing the historic 1992 SH strain to other *Salmonella* serovars and other SH strains tested in this study, there was no specific difference with respect to disinfectant sensitivity in all the 90-min MIC and MBEC experiments (Table 3). However, the log reduction and the 8-s MBEC results revealed that the historic SH 1992 strain was the most resistant of the SH strains tested against PAA and aCH in the log reduction experiment and against CPC in the 8-s experiment with respect to the disinfectant's bactericidal effect (Tables 2 and 4). The bacteriostatic concentration of CPC against the SH 1992 strain was on average lower (1,600 ppm) than that against three of the remaining four SH strains tested (Table 4). Although any sensitivity differences to disinfectants are likely the result of genotypic differences between the different SH strains rather than differences based on when the isolates were obtained historically, these results provide limited evidence that newer isolates are not significantly more resistant to disinfectants as a result of selective pressure.

The complexity of environment-host-pathogen relationships in commercial poultry production systems presents a challenge for disease control and represents a risk to human food safety *(25)*. In the poultry processing plant, consideration must be given to management strategies that reduce potential foodborne contaminants to safeguard public health. Control of *Salmonella* in food processing environments is a significant challenge. Biofilm formation is one aspect of this problem, and thus the ability to assess the efficacy of disinfectants with respect to the eradication of *Salmonella* biofilms is of considerable pragmatic importance. In our experiments, we utilized laboratory conditions to grow the planktonic and biofilm SH strains, and the morphology and biochemistry of *Salmonella* biofilms changes based upon experimental setup and environmental conditions *(25)*. The results were obtained after static exposure to the three disinfectants exposed individually to each isolate and, in this regard, do not address the potential summation effect of utilizing multiple disinfectants in concert or the effect of organic matter on disinfectant efficacy *(31)*. The data also do not reflect potential differences in effects on mixed-species biofilms in the presence of food residues, a situation that

more closely represent the reality of food processing environments *(8)*.

In a simulated chiller tank, for the bactericidal control of the *Salmonella* biofilm isolates tested PAA and aCH were either ineffective or were effective only above the current FSIS regulatory ranges (Tables 2 and 3). Although CPC was bactericidal at the concentration used, this chemical is not currently used in chiller tanks in poultry processing. In the simulated dipping station, CPC was bacteriostatic within the FSIS regulatory concentration ranges for five of the seven *Salmonella* strains tested (Table 4). However, the effective bactericidal concentrations were above the FSIS regulatory ranges. Consequently, PAA, aCH, and CPC should not be considered good substitutes for good sanitation, proper biosecurity, and hazard analysis critical control point practices with respect to bactericidal control of the *Salmonella* biofilm isolates tested. Although all planktonic *Salmonella* isolates were static in response to PAA, previous work indicates that biofilms are more common in food environments than are planktonic bacteria *(14, 15)*. The difficulty of eradication of established *Salmonella* biofilms emphasizes the priority of preventing *Salmonella* colonization of food production facilities. We propose that when poultry processing companies consider using new disinfectants or find new strains of bacteria that must be controlled, managers should consider testing for bacterial inhibition utilizing both the MIC and MBEC assays. When companies test only planktonic bacteria, they may obtain results that are not accurate in actual food processing environments, where biofilms are more prevalent *(14, 15)*. MIC and MBEC assays in concert with log reduction assays should be considered as a method for determining the efficacy of disinfectants against isolated pathogens.

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REFERENCES

1. Agarwal, R., S. Singh, K. Bhilegaonkar, and V. Singh. 2011. Optimization of microtitre plate assay for the testing of biofilm formation ability in different *Salmonella* serotypes. *Int. Food Res. J.* 18:1493–1498.
2. Bolder, N. 1998. The microbiology of the slaughter and processing of poultry, p. 158–173. *In* A. Davies and R. Board (ed.), The microbiology of meat and poultry. Blackie Academic, London.
3. Brooun, A., S. Liu, and K. Lewis. 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother*. 44:640–646.
4. Burmølle, M., J. S. Webb, D. Rao, L. H. Hansen, S. J. Sørensen, and S. Kjelleberg. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl. Environ. Microbiol.* 72:3916–3923.
5. Capita, R. 2007. Variation in *Salmonella* resistance to poultry chemical decontaminants, based on serotype, phage type, and antibiotic resistance patterns. *J. Food Prot.* 70:1835–1843.
6. Ceri, H., M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret. 1999. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* 37:1771–1776.
7. Chelvam, K. K., L. C. Chai, and K. L. Thong. 2014. Variations in motility and biofilm formation of *Salmonella enterica* serovar Typhi. *Gut Pathog*. 6:1–10.
8. Corcoran, M., D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery, and M. Cormican. 2014. Commonly used disinfectants fail to eradicate *Salmonella enterica* biofilms from food contact surface materials. *Appl. Environ. Microbiol.* 80:1507–1514.
9. De Oliveira, D. C. V., A. Fernandes, Jr., R. Kaneno, M. G. Silva, J. P. Araújo, Jr., N. C. C. Silva, and V. L. M. Rall. 2014. Ability of *Salmonella* spp. to produce biofilm is dependent on temperature and surface material. *Foodborne Pathog. Dis.* 11:478–483.
10. Food and Agriculture Organization of the United Nations. 2009. Benefits and risks of the use of chlorine-containing disinfectants in food production and food processing. Available at: www.fao.org/docrep/012/i1357e/i1357e01.pdf. Accessed 1 August 2016.
11. Gast, R. K. 2007. Serotype-specific and serotype-independent strategies for preharvest control of food-borne *Salmonella* in poultry. *Avian Dis.* 51:817–828.
12. Harbottle, H., S. Thakur, S. Zhao, and D. G. White. 2006. Genetics of antimicrobial resistance. *Anim. Biotechnol.* 17:111–124.
13. Harrison, J. J. 2012. The MBEC high-throughput (HTP) assay. Instructions for antimicrobial susceptibility testing of biofilms. Innovotech, Edmonton, Alberta, Canada.
14. Hasegawa, A., Y. Hara-Kudo, and S. Kumagai. 2011. Survival of *Salmonella* strains differing in their biofilm-formation capability upon exposure to hydrochloric and acetic acid and to high salt. *J. Vet. Med. Sci.* 73:1163–1168.
15. Heithoff, D. M., W. R. Shimp, J. K. House, Y. Xie, B. C. Weimer, R. L. Sinsheimer, and M. J. Mahan. 2012. Intraspecies variation in the emergence of hyperinfectious bacterial strains in nature. *PLoS Pathog*. 8:e1002647.
16. Ledeboer, N. A., J. G. Frye, M. McClelland, and B. D. Jones. 2006. *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. *Infect. Immun.* 74:3156–3169.
17. MacKenzie, K. D., Y. Wang, D. J. Shivak, C. S. Wong, L. J. L. Hoffman, S. Lam, C. Kröger, A. D. S. Cameron, H. G. G. Townsend, W. Köster, and A. P. White. 2015. Bistable expression of CsgD in *Salmonella enterica* serovar Typhimurium connects virulence to persistence. *Infect. Immun.* 83:2312–2326.
18. Møretrø, T., L. Vestby, L. Nesse, S. Storheim, K. Kotlarz, and S. Langsrud. 2009. Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. *J. Appl. Microbiol.* 106:1005–1012.
19. Nagel, G. M., L. Bauermeister, C. Bratcher, M. Singh, and S. McKee. 2013. *Salmonella* and *Campylobacter* reduction and quality characteristics of poultry carcasses treated with various antimicrobials in a post-chill immersion tank. *Int. J. Food Microbiol.* 165:281–286.
20. Olson, M. E., H. Ceri, D. W. Morck, A. G. Buret, and R. R. Read. 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can. J. Vet. Res.* 66:86–92.
21. Parveen, S., M. Taabodi, J. G. Schwarz, T. P. Oscar, J. Harter-Dennis, and D. G. White. 2007. Prevalence and antimicrobial resistance of *Salmonella* recovered from processed poultry. *J. Food Prot.* 70:2466–2472.
22. Reybrouck, G. 1998. The testing of disinfectants. *Int. Biodeterior. Biodegrad.* 41:269–272.
23. Shah, D. H. 2014. RNA Sequencing reveals differences between the global transcriptomes of *Salmonella enterica* serovar Enteritidis strains with high and low pathogenicities. *Appl. Environ. Microbiol.* 80:896–906.
24. Shah, J. N., P. T. Desai, and B. C. Weimer. 2014. Genetic mechanisms underlying the pathogenicity of cold-stressed *Salmonella enterica* serovar Typhimurium in cultured intestinal epithelial cells. *Appl. Environ. Microbiol.* 80:6943–6953.
25. Steenackers, H., K. Hermans, J. Vanderleyden, and S. C. De Keersmaecker. 2012. *Salmonella* biofilms: an overview on occurrence, structure, regulation and eradication. *Food Res. Int.* 45:502–531.

26. Stepanović, S., I. Ćirković, and L. Ranin. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett. Appl. Microbiol.* 38:428–432.
27. U.S. Department of Agriculture, Food Safety and Inspection Service. 2016. FSIS directive: safe and suitable ingredients used in the production of meat, poultry and egg products. 7120.1 Rev. 36. U.S. Department of Agriculture, Food Safety and Inspection Service, Washington, DC.
28. Vestby, L., J. Lönn-Stensrud, T. Møretrø, S. Langsrud, A. Aamdal-Scheie, T. Benneche, and L. Nesse. 2010. A synthetic furanone potentiates the effect of disinfectants on *Salmonella* in biofilm. *J. Appl. Microbiol.* 108:771–778.
29. Wang, H. H., S. J. Ding, Y. Dong, K. P. Ye, X. L. Xu, and G. H. Zhou. 2013. Biofilm formation of *Salmonella* serotypes in simulated meat processing environments and its relationship to cell characteristics. *J. Food Prot.* 76:1784–1789.
30. Wong, H. S., K. M. Townsend, S. G. Fenwick, G. Maker, R. D. Trengove, and R. M. O'Handley. 2010. Comparative susceptibility of *Salmonella* Typhimurium biofilms of different ages to disinfectants. *Biofouling* 26:859–864.
31. Zhao, T., P. Zhao, J. L. Cannon, and M. P. Doyle. 2011. Inactivation of *Salmonella* in biofilms and on chicken cages and preharvest poultry by levulinic acid and sodium dodecyl sulfate. *J. Food Prot.* 74:2024–2030.