Use of a Specific Bacteriophage Treatment to Reduce *Salmonella* in Poultry Products[1,2]

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ABSTRACT Bacteriophages represent a group of viruses that specifically infect and replicate in bacteria and could potentially be used to reduce recovery of *Salmonella* from poultry carcasses. Bacteriophages were isolated from municipal wastewater in the presence of *Salmonella enteritidis* phage type 13A (*SE*). In the first 2 experiments, commercially processed broiler carcass rinse water was pooled and divided. The addition of 10^{10} pfu/mL of a single bacteriophage (PHL 4) with selected concentrations of *SE* reduced ($P < 0.05$) frequency of *SE* recovered as compared with the control rinse water sample. In experiments 3 and 4, broiler carcasses were intentionally inoculated with *SE*, sprayed with selected concentrations of PHL 4, and rinsed for *SE* enrichment and isolation. Application of 5.5 mL of 10^8 or 10^{10} pfu/mL of PHL 4 reduced ($P < 0.05$) the frequency of *SE* recovery as compared with controls. In experiments 5 and 6, commercially processed turkeys were rinsed with water containing 72 wild-type bacteriophages isolated against *SE*, which were amplified in *SE*, or the *Salmonella* isolated antemortem from drag swabs from the flock selected for in-plant treatment, or a combination of bacteriophages amplified by each bacterial host. All bacteriophage treatments reduced ($P < 0.05$) frequency of *Salmonella* recovery as compared with controls. Sufficient concentrations of an appropriate bacteriophage, or a bacteriophage mixture, can significantly reduce recoverable *Salmonella* from carcass rinses.

(*Key words*: *Salmonella*, bacteriophage, carcass rinse, poultry)

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INTRODUCTION

Bacteriophages can attach to a bacterium with the virion tail and penetrate the bacterial cell wall, followed by injection of the necessary components for bacteriophage replication (Voyles, 1993). Alternatively, the bacteriophage may completely enter the bacterium and initiate replication of bacteriophage particles. The bacteriophage particles then are assembled and released, usually causing lysis of the bacterium. In some cases bacteriophages are released without causing lysis of the bacterium (Ellis, 1966; Pratt et al., 1966). F. W. Twort (1915) and F. d'Herelle (1917) independently discovered the bacteriophage (Duckworth, 1976), and for most of the last century clinical evaluations of bacteriophages as therapeutic agents progressed with little knowledge of the biology of these viruses. Bacteriophages were largely ignored as some clinical trials (principally involving *Vibrio cholera*) failed, and broad-spectrum, highly efficacious antimicrobial chemotherapy became available (Marcuk et al., 1971). However, recent successes involving the use of bacteriophages for controlling *Staphylococcus* and *Escherichia coli* infections have renewed interest in bacteriophage therapy against additional bacterial species (Smith and Huggins, 1982, 1983; Slopek et al., 1985; Smith et al., 1987; Huff et al., 2005). Recently, O'Flynn et al. (2004) found that application of selected bacteriophages to beef is effective for elimination of *E. coli* O157:H7 in 7 out of 9 samples. The present experiments represent an initial evaluation of the potential of selected wild-type bacteriophages to reduce the recovery of *Salmonella* from poultry carcass rinse samples.

MATERIALS AND METHODS

Bacteriophage Isolation

Samples were obtained from a local municipal wastewater treatment plant and filtered through a 0.2 μm filter.[4]

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[2]Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee of a warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

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[4]Catalog number 211043, Becton Dickinson, Sparks, MD.

Abbreviation Key: BGA = brilliant green agar; NA = naladixic acid; NO = novobiocin; *SE* = *Salmonella enteritidis* phage type 13A; TSA = tryptic soy agar; TSB = tryptic soy broth.

A combination of 100 μL of 10^7 cfu/mL *Salmonella enteritidis* PT 13A (*SE*) and 1 mL of the wastewater sample filtrate was added to 1.5 mL of soft tryptic soy agar[5] (TSA) and poured over a warm TSA plate. Plates were incubated overnight at 37°C. Plates with confluent lysis were flooded with 15 mL of sterile saline containing 0.9% NaCl. The fluid was then poured off the plate and filtered through a 0.2 μm filter, and dilutions were made in sterile saline. Plates were poured as described above using 1 mL of each bacteriophage dilution. Individual distinct plaques resulting from this plating were then differentiated on the basis of plaque morphology, and different plaques were sequentially passed on TSA plates at least 3 subsequent times to purify the isolate.

By using this technique, 72 bacteriophages were isolated from 4 wastewater samples, each collected on separate days. These bacteriophage isolates were arbitrarily designated PHL 1 to PHL 72 for use in these studies. Although several different plaque morphologies were noted, and isolates were obtained from 4 different wastewater collections, no attempt was made to differentiate bacteriophages based on molecular or physiological characteristics. The 72 bacteriophage isolates used in these experiments, therefore, may have a certain degree of redundancy.

SE Amplification

A primary poultry isolate of *Salmonella enteritidis*, bacteriophage type 13A (*SE*), was obtained from the USDA National Veterinary Services Laboratory. This isolate was resistant to novobiocin[6] (NO) (25 μg/mL) and was selected for resistance to naladixic acid[7] (NA) (20 μg/mL) in our laboratory. For these studies *SE* was grown overnight in tryptic soy broth[8] (TSB) at 37°C. Cells were washed 3 times in sterile saline by centrifugation at 1,864 $\times$ *g*, and the concentration was estimated with a spectrophotometer using a previously generated standard curve, to approximately 10^9 cfu/mL in sterile saline and then diluted to inoculated concentrations as described below. Concentrations of *SE* were retrospectively determined by spread plating on brilliant green agar[9] (BGA) plates containing NO (25 μg/mL) and NA (20 μg/mL), followed by enumeration for each experiment. Actual determined colony-forming units for each experiment are reported.

Bacteriophage Amplification

All bacteriophages used in these experiments were amplified in broth using a ratio of 1:3:5 (bacteriophage: turbid *SE* culture in TSB: fresh TSB) and were incubated at 37°C for 1.5 h. Briefly, an agar plug containing bacteriophage was obtained by pushing a sterile Pasteur pipette into the center of bacteriophage plaque on a TSA plate where *SE* were lysed. This bacteriophage plug was then resuspended in 1 mL of sterile saline per plug and then filtered through a 0.2 μm filter. Turbid *SE* was obtained from an overnight culture of TSB inoculated with *SE* and incubated at 37°C. After 1.5 h of incubation of the host and bacteriophage, bacteria were removed by sterile filtration, and bacteriophage was quantified as described above.

Carcass Rinse Water Samples (Experiments 1 and 2)

Carcass rinse samples were obtained using previously published procedures (Cox et al., 1981). Briefly, each carcass was removed from the processing line prior to chill tank immersion and placed in an individual autoclave bag (25 $\times$ 35 cm; n = 9). One hundred milliliters of sterile water was then added to the abdominothoracic cavity of each carcass, the bag was sealed with a cable tie, and the contents were vigorously shaken by hand for 30 s. The rinse fluid was pooled from all carcasses in a 1-L bottle and divided into 4-mL aliquots for experiments 1 and 2.

SE and Bacteriophage Inoculation of Rinse Water (Experiments 1 and 2)

For experiment 1, 120 aliquots (4 mL each) of pooled rinse water samples were prepared as described above. *SE* was added in a 0.5-mL volume to each rinse water sample to achieve final concentrations of 0.92×10^0, 10^1, 10^2, and 10^3 cfu/mL (n = 30 per treatment). Control rinse samples received an additional 0.5 mL of sterile saline (n = 40). A single bacteriophage isolate (PHL 4) was amplified and sterile filtered as described above, and 0.5 mL was added to principle rinse water samples to achieve estimated final concentration of 10^6 or 10^{10} pfu/mL (n = 40 per treatment). All tubes were held on wet ice for 24 h prior to enrichment to simulate shipping.

For experiment 2, rinse water was collected, and *SE* was amplified as described above. Tubes containing rinse water were inoculated with final concentrations of 10^2 or 10^3 cfu/mL of *SE* (n = 60 per treatment) and 0, 10^2, 10^4, 10^6, 10^8, or 10^{10} pfu/mL of the bacteriophage PHL 4 (n = 20 per treatment) as described for experiment 1.

Enrichment and SE Recovery

For experiments 1 and 2, we added 5 mL of concentrated (2$\times$) tetrathionate broth[10] to each rinse water sample and incubated the samples at 37°C overnight. Samples were streaked for isolation on BGA plates containing NO (25 μg/mL) and NA (20 μg/mL) and were incubated overnight at 37°C. Each plate was evaluated for the presence or absence of lactose negative colonies with morphology consistent with the antibiotic-resistant *SE*.

[5]Catalog number 2004-08, Pall-Gelman Laboratory, Ann Arbor, MI.
[6]Catalog number N-1628, Sigma, St. Louis, MO.
[7]Catalog number N-4382, Sigma.
[8]Catalog number 211822, Becton Dickinson.
[9]Catalog number 228530, Becton Dickinson.
[10]Catalog number 210420, Becton Dickinson.

Inoculation, Treatment, and Culture of Broiler Carcasses (Experiments 3 and 4)

For experiments 3 and 4, commercially processed broiler carcasses were removed from the processing line immediately prior to chill tank immersion. Each carcass was placed in an individual autoclave bag (25 × 35 cm), sealed with a cable tie, and randomly assigned to treatment groups. Carcasses were transported to the laboratory, and each carcass was inoculated with *SE* using previously described methods (Cox et al., 1980). Briefly, a sterile bent glass rod was used to thoroughly spread 100 μL of inoculum over the entire breast area. The inoculum contained 31 or 20 cfu of *SE* for experiments 3 and 4, respectively. Each broiler carcass was then placed back into its original autoclave bag and stored at 4°C (2 h). Carcasses were then individually removed from each bag and sprayed with 5.5 mL of sterile saline alone (controls) or saline containing PHL 4, amplified as described above. Hand-held garden sprayers were calibrated to apply a fine mist of treatment over the breast area (5.5 mL/carcass). In experiment 3, carcasses were sprayed with sterile saline (control: n = 19) or 10^{10} pfu/mL of PHL 4 (n = 21). Carcasses in experiment 4 were sprayed with sterile saline (controls) or 0.53×10^4, 10^6, 10^8, or 10^{10} pfu/mL of PHL 4 (n = 15/treatment). One hundred milliliters of sterile water was then added to the abdominothoracic cavity of each carcass, the bag was sealed with a cable tie, and the contents were vigorously shaken by hand for 30 s. The rinse fluid was aseptically collected in sterile sealed containers[11] and held on wet ice (24 h). An equal volume of concentrated (2×) tetrathionate broth base was added to the rinse fluid of each sample, and samples were enriched and plated for *SE* recovery as described above.

Treatment/Culture of Contaminated Carcasses at Processing (Experiments 5 and 6)

In experiments 5 and 6, *Salmonella*-positive commercial turkey flocks were identified by antemortem isolation of *Salmonella* from drag swab environmental sampling (Caldwell et al., 1995). *Salmonella* isolates were confirmed biochemically and serotyped (type B from each of the flocks selected for experiments 5 and 6) prior to processing. In each experiment, a single colony of the field isolate was grown in 500 mL of TSB at 37°C overnight. An agar plug containing each bacteriophage (PHL 1 to PHL 72) was removed from plaques on TSA plates using the original host *SE* as described above, resuspended in 72 mL of sterile saline, and sterile filtered through a 0.2 μm filter. For each experiment, the phage mixture was amplified in broth culture by maintaining the ratio of bacteriophage: turbid *Salmonella*: fresh medium volume as described above. The bacteriophage mixture was amplified once in the presence of *SE* or 1 of 2 selected field isolates of *Salmonella*. These 2 cultures were serially passed 3 times, and by the third passage the medium was markedly clarified, suggesting substantial amplification of bacteriophages. All 3 cultures were then sterile filtered, and plaque-forming units were determined.

Commercially processed turkeys were obtained prior to chill tank immersion, individually placed in rinse bags,[12] and randomly assigned to treatment groups. In experiment 5, treatments consisted of 100 mL sterile saline alone (control), 100 mL sterile saline with bacteriophages amplified in the *SE* host (1.6×10^8 pfu/mL final concentration), bacteriophages amplified with the field isolate of *Salmonella* (1.8×10^7 pfu/mL final concentration), or a combination of bacteriophages amplified by each of the 2 hosts (8.9×10^7 pfu/mL final concentration). Each bag was sealed with a cable tie, and the contents were shaken by hand for 60 s (n = 30/treatment group). The rinse fluid was aseptically collected in sterile sealed containers[13] and held on wet ice for transport to the laboratory (3 h). In experiment 6, 200 mL of sterile saline was added to each carcass for rinsing. Treatments consisted of no bacteriophages (control) or saline with bacteriophages amplified in the *SE* host (8.0×10^7 pfu/mL final concentration), bacteriophages amplified with the field isolate of *Salmonella* (9.0×10^6 pfu/mL final concentration), or a combination of bacteriophages amplified by each of the 2 hosts (4.5×10^7 pfu/mL final concentration). Each bag was sealed with a cable tie and the contents were shaken by hand for 60 s (n = 30/treatment group). The rinse fluid was aseptically collected in sterile sealed containers and held on wet ice for transport to the laboratory (3 h). Upon arrival at the laboratory, an equal volume of concentrated (2×) tetrathionate broth base was added to the rinse fluid of each sample. Samples were then incubated, and *Salmonella* was isolated as described above with no antibiotics added to the BGA.

Statistical Analysis

All possible combinations of incidence of *Salmonella* recovery within experiments were compared using the chi-squared test of independence (Zar, 1984) to determine significant ($P < 0.05$) differences.

RESULTS

In experiment 1, the highest concentration of bacteriophage PHL 4 applied (10^{10} pfu/mL) significantly ($P < 0.05$) reduced *SE* recovery between 50 and 100% as compared with the controls at all levels of contamination applied (Table 1). Similarly in experiment 2, PHL 4 added at a final concentration of 10^8 or 10^{10} pfu/mL significantly ($P < 0.05$) reduced *SE* recovery by 70% as compared with the controls, regardless of the *SE* inoculum used (Table 1).

Data from experiment 3 demonstrate that treatment of inoculated (31 cfu) broiler carcasses with 5.5×10^{10} pfu

[11]Catalog number TR-B-3037, International BioProducts Inc., Bothell, WA.
[12]Catalog number 24384-148, VWR International, South Plainfield, NJ.
[13]Catalog number 15704-016, VWR International.

TABLE 1. Recovery of *Salmonella enteritidis* (SE) from carcass rinse samples contaminated with *SE* and then treated with 2 concentrations (experiment 1) or 5 concentrations (experiment 2) of a single bacteriophage isolate

Bacteriophage (pfu/mL)	*Salmonella enteritidis* final concentration (cfu/mL)			
	10^0	10^1	10^2	10^3
Experiment 1				
Control	6/10 (60%)[a]	10/10 (100%)[a]	10/10 (100%)[a]	10/10 (100%)[a]
10^6	9/10 (90%)[a]	7/10 (70%)[a]	6/10 (60%)[b]	10/10 (100%)[a]
10^{10}	1/10 (10%)[b]	1/10 (10%)[b]	0/10 (0%)[c]	2/10 (20%)[b]
Experiment 2				
Control	10/10 (100%)[a]	ND[1]	10/10 (100%)[a]	ND
10^2	10/10 (100%)[a]	ND	10/10 (100%)[a]	ND
10^4	10/10 (100%)[a]	ND	10/10 (100%)[a]	ND
10^8	3/10 (30%)[b]	ND	3/10 (30%)[b]	ND
10^{10}	3/10 (30%)[b]	ND	3/10 (30%)[b]	ND

[a–c]Values within columns and within experiments with different superscripts are significantly ($P < 0.05$) different.

[1]Not determined.

of PHL 4 caused an 85% reduction in the frequency of *SE* recovery as compared with controls (Table 2). Similarly, bacteriophage treatment of inoculated (20 cfu) broiler carcasses with 5.5×10^8 or 10^{10} pfu of PHL 4 caused a 93% reduction in *SE* recovery as compared with controls in experiment 4 (Table 2). At processing, treatment of naturally contaminated turkey carcasses with amplified bacteriophages (experiments 5 and 6) significantly ($P < 0.05$) reduced *Salmonella* recovery by 55 to 58% in experiment 5 and 50 to 60% in experiment 6, as compared with the controls (Table 3).

DISCUSSION

The data from experiments 1 to 4 indicate a large number of selected bacteriophage must be applied to markedly reduce *SE* recovery from inoculated carcass rinse samples or carcasses. In these experiments, by using a single bacteriophage isolate, application of up to 10^6 pfu of this isolate did not markedly affect *SE* recovery, whereas application of 10^8 pfu or more greatly reduced our ability to recover *SE* from these samples (Tables 1 and 2). These studies suggest that treatment with large numbers of bacteriophages is desirable, and at this time there is no evidence to suggest that the highest possible concentrations should not be used.

TABLE 2. Recovery of *Salmonella enteritidis* (*SE*) from broiler carcasses inoculated with 31 (experiment 3) or 20 cfu (experiment 4) of *SE*, and sprayed with 5.5 mL of a single bacteriophage isolate at selected concentrations

Bacteriophage (pfu/mL)	Number positive *SE* / total (%)	
	Experiment 3	Experiment 4
Control	18/19 (95)[a]	15/15 (100)[a]
10^4	ND[1]	15/15 (100)[a]
10^6	ND	15/15 (100)[a]
10^8	ND	1/15 (7)[b]
10^{10}	2/21 (10)[b]	1/15 (7)[b]

[a,b]Values within columns and within experiments with different superscripts are significantly ($P < 0.05$) different.

[1]Not determined.

Whether or not the *Salmonella* were immediately killed by bacteriophage treatment was not determined in these studies. It is possible that bacteria were infected prior to enrichment or, alternatively, were infected by the associated bacteriophage during the rinsing, storage, or enrichment steps. Generally, it is only after cross-contamination and temperature/time abuse of food media that *Salmonella* can reach numbers sufficient to cause infections in humans (Hargis et al., 2001). Even in the worst possible scenario, the inability to recover *Salmonella* during these experiments following enrichment suggests that bacteriophage treatment is likely to greatly increase the time/temperature abuse potential of foods that might be contaminated with *Salmonella* from fresh poultry carcasses. As the lytic bacteriophage life cycle is usually less than 30 min, it is reasonable to postulate that the *Salmonella* contacted sufficient bacteriophage numbers to eventually cause lysis and were at least infected prior to rinsing and enrichment. As bacteriophages have been shown to effectively lyse other host bacteria over time even at cold temperatures (Kudva et al., 1999), there is the possibility that many of the contaminating *Salmonella* are truly neu-

TABLE 3. Recovery of *Salmonella* from commercial turkey carcasses rinsed with saline (control), saline containing 72 bacteriophage isolates amplified in the original *Salmonella enteritidis* host (*SE*) or in the *Salmonella* field isolate (S9, experiment 5; S14, experiment 6) obtained from the flock antemortem

Salmonella host	Bacteriophage (pfu/mL)	Number *Salmonella* positive/total (%)
Experiment 5		
Control	0	24/30 (80)[a]
SE	1.6×10^8	7/28 (25)[b]
S9	1.8×10^7	7/30 (23)[b]
SE + S9	8.9×10^7	8/32 (25)[b]
Experiment 6		
Control	0	20/30 (67)[a]
SE	8.0×10^7	5/30 (17)[b]
S14	9.0×10^6	3/30 (10)[b]
SE + S14	4.5×10^7	2/30 (7)[b]

[a,b]Values within columns and within experiments with different superscripts are significantly ($P < 0.05$) different.

tralized by lytic bacteriophage infection prior to potential exposure of consumers. Nevertheless, this question remains essentially unanswered by these studies.

The relative host specificity of *Salmonella* bacteriophages and the relatively large number of potential *Salmonella* isolates from poultry carcasses present a potential problem for development of a bacteriophage cocktail that would effectively reduce natural contamination of poultry during processing. The results of experiments 5 and 6 are encouraging, although preliminary, in this regard. The relatively small library of 72 bacteriophages originally isolated using *SE* as the host was effective for treatment of natural contamination of turkey carcasses (Table 3), regardless of whether the library was amplified in the original host (*SE*) or in the field isolate of *Salmonella* most likely to contaminate the carcasses (Table 3). As each of the antemortem *Salmonella* isolates from the natural infections were of a different serogroup (B) than the original *SE* host bacterium, these data suggest that there is considerable nonspecificity among some of these bacteriophage isolates. If this holds true, it seems likely that a bacteriophage cocktail that would effectively reduce *Salmonella* recovery from many, if not most, field *Salmonella* isolates could be developed. In support of this hypothesis, at least some of these 72 bacteriophages were able to cause lysis (plaques) in 13 of 14 field *Salmonella* isolates recently tested (data not shown).

Another potential issue facing the use of bacteriophage for treatment of foods is the possible inclusion of bacteriophage capable of producing a prophage. It is conceivable that lysogenic bacteriophage could provide genetic information to the host bacterium, encoding for increased bacterial virulence or antibiotic resistance. At this time, it is not clear what percentage, if any, of lytic bacteriophage are capable of inducing lysogeny. To date, unpublished attempts to induce expression of a latent prophage by exposure to mitomycin-C in *SE* surviving bacteriophage treatment and resistant to subsequent exposure to the same bacteriophage have failed (data not shown), a phenomenon previously observed by other investigators (Klieve et al., 1991). Nevertheless, some questions remain regarding the appropriate evaluation of such bacteriophages for use on food products.

In addition to these concerns, public misconceptions about the safety of treating food products with viruses could become an issue in commercial application of this technology. Although bacteriophages are ubiquitous in the environment and have no potential to infect vertebrate animals, misconceptions similar to those surrounding irradiation of foods may present an issue for bacteriophage-treatment of food products.

Although a number of questions surround the appropriateness of bacteriophage treatment of food products, the results of the present experiments are encouraging. The ability to markedly reduce recoverable *Salmonella* from intentionally or naturally contaminated poultry carcasses in the present experiments provides new possibilities for improving food safety, not only as related to *Salmonella* but to other bacterial food borne pathogens as well. Future research must address the several remaining issues prior to commercial implementation of bacteriophage treatment of poultry or other food products.

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