

Reduction of Experimental *Salmonella* and *Campylobacter* Contamination of Chicken Skin by Application of Lytic Bacteriophages

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Lytic bacteriophages, applied to chicken skin that had been experimentally contaminated with *Salmonella enterica* serovar Enteritidis or *Campylobacter jejuni* at a multiplicity of infection (MOI) of 1, increased in titer and reduced the pathogen numbers by less than 1 log₁₀ unit. Phages applied at a MOI of 100 to 1,000 rapidly reduced the recoverable bacterial numbers by up to 2 log₁₀ units over 48 h. When the level of *Salmonella* contamination was low (< log₁₀ 2 per unit area of skin) and the MOI was 10⁵, no organisms were recovered. By increasing the number of phage particles applied (i.e., MOI of 10⁷), it was also possible to eliminate other *Salmonella* strains that showed high levels of resistance because of restriction but to which the phages were able to attach.

Bacterial food poisoning remains a major worldwide health problem. Much of human *Salmonella* infection originates from poultry, although pigs are thought to be increasing in significance, albeit to an unknown extent (5). Thermophilic *Campylobacter* spp. are the most frequently identified human enteric pathogen in England and Wales (21), with more than 56,000 fecal isolates made in 2000 (4). A recent community-based study on enteric infectious disease estimated an annual incidence of up to 500,000 cases (30). Poultry meat is a major source of *Campylobacter jejuni* infection (3, 16), and up to 90% of flocks appear to be colonized with this organism at the time of slaughter (13; N. J. Stern, J. S. Bailey, N. A. Cox, S. E. Craven, and P. F. Cray, 10th Int. Workshop *Campylobacter*, *Helicobacter* Related Organisms, abstr. CF17, 1999). In *Campylobacter*-positive flocks, almost all the birds are infected and carcasses are frequently contaminated with large numbers of bacteria (10, 31).

Procedures that reduce surface contamination of carcasses by *Salmonella* and other enteric pathogens have therefore been examined over many years. Hygiene improvements within processing plants have been largely based on chlorinated water sprays (19, 29), and although chlorine is no longer allowed under current United Kingdom and European Union legislation, it remains in use in the United States. In Europe, air chilling rather than water chilling has become the standard practice (2).

Spraying carcasses with different salt solutions and antibacterial agents, such as trisodium phosphate, has been investigated (23). However, this presents problems of acceptability and possible deterioration of the organoleptic properties of the

chicken (e.g., taste, texture, and odor). Physical treatment has included steam, dry heat, and UV light, all carried out with varying degrees of success (17), but organoleptic quality may be impaired (15). Hot spray treatment to reduce *Salmonella* numbers on poultry carcasses has been investigated by several researchers, although reduction in numbers was considerably lower than expected (12). Lytic bacteriophages may prove a suitable alternative.

After initial enthusiasm for phage therapy in the early years of the last century, the poor quality of early work—combined with the limited understanding of phages and their relationship with their bacterial hosts—led to the idea's becoming discredited in the West (20, 22, 32). However, they continued to be used in Eastern Europe, although the value of much of this work is difficult to appraise objectively (24, 25).

Lytic phages have been used effectively against *Escherichia coli* septicemia in mice (26), chickens, and calves (6), against neonatal diarrhea caused by enterotoxigenic *E. coli* strains in calves, pigs, and lambs (27, 28), and to control *Pseudomonas aeruginosa* growth on skin (29). This has led to a reappraisal of this approach for the control of a limited number of infections in humans and animals (1, 7, 30). Although such work indicated that phages were able to multiply in vivo, it was also possible to control infection by administration of a sufficiently high phage dose (8), which overwhelmed the pathogen and lysed the bacterial cells, possibly through a nonproliferative lytic mechanism (i.e., lysis from without).

One of the criteria for the successful application of phages is that recycling of the phages in a reservoir of the pathogen should be prevented in order to reduce the rate of development of resistance in the pathogen to the phage used. Phages are thus unlikely to be applicable for the reduction of enteric pathogens in the intestine of poultry on rearing farms, since fecal shedding of both pathogen and phages would rapidly lead to resistance. Success is most likely for scenarios in which the

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TABLE 1. Effect of application of bacteriophage at low MOI on *S. enterica* serovar Enteritidis and *C. jejuni*

Time (h)	Viable counts ^a of organisms in:					
	Experiment 1			Experiment 2		
	<i>S. enterica</i> serovar Enteritidis	Uninfected control	Phage	Uninfected control	<i>C. jejuni</i>	Uninfected control
0	3.91 ± 0.31	3.92 ± 0.28	3.0 ± 0.17	3.04 ± 0.19	4.05 ± 0.25	4.05 ± 0.25
24	3.27 ± 0.42 (22%) ^b	3.71 ± 0.15 (60%)			1.74 ± 0.34 (5%) ^d	2.99 ± 0.27 (10%)
48	2.96 ± 0.21 (11%) ^b	3.70 ± 0.21 (60%)	3.49 ± 0.21 ^c	2.11 ± 0.08		

^a Values represent mean ± standard error. Percentages in parentheses signify ratio of original count remaining.

^b $P < 0.01$.

^c $0.02 < P < 0.01$.

^d $P = 0.04$.

patient (or animal) treated is an epidemiological endpoint. This applies wherever the patient or animal can be isolated or in situations in which the treated animal does not return to its rearing environment. This does occur in the abattoir during slaughter, since animals and carcasses move in one direction. This opens the possibility that phages might be used to reduce bacterial carcass contamination, since the phages would be unlikely to find their way back in significant numbers to the farms from which the animals were derived.

We therefore explored in a preliminary way whether phages might be used to reduce experimental contamination of poultry skin with *Salmonella* and *Campylobacter* spp. To do this, we adopted a laboratory approach, using chicken skin experimentally contaminated with these two organisms. Chicken skin was removed aseptically from fresh chicken portions and frozen at -20°C to facilitate cutting 4- and 60-cm² squares from it while frozen. These were stored (individually wrapped in cling film) until required. They were defrosted and used immediately, experiments then being carried out at ambient temperature (i.e., 18 to 22°C).

The target bacterial strains were used as spontaneous mutants, resistant to nalidixic acid, to facilitate enumeration. *Salmonella* strains were cultured in 10-ml volumes of Luria-Bertani (LB) broth and subjected to shaking at 150 rpm for 24 h at 37°C, and *C. jejuni* was cultured statically in a microaerobic environment in 10-ml volumes of Mueller-Hinton broth for 48 h at 42°C.

Phage preparations were cultured on the target organisms as described previously (8), centrifuged, and filtered (0.22- μm -pore-size) with a filtration unit (Nalgene, Rochester, N.Y.), which provided storage for volumes of 100-ml preparation. These were stored at 4°C until used.

The application and enumeration of bacteria and bacteriophages was done in two ways: using a low multiplicity of infection (MOI) and a high MOI.

Application of phages at low MOI. Attempts were first made to determine whether phages, applied at a low MOI, would reduce *Salmonella* and *C. jejuni* counts on chicken skin. Large pieces of skin (60 cm² in area) in triplicate were inoculated with *S. enterica* serovar Enteritidis phage type 4 strain P125589 (6) using a pipette, and the inoculum was distributed over the surface using a glass spreader to produce a density of approximately 10³ CFU cm⁻². The same technique was then used to inoculate half of the chicken skin portions with *Salmonella* typing phage 12, obtained from the phage-typing unit in the

Laboratory of Enteric Pathogens at the Central Public Health Laboratory, Colindale, London, United Kingdom, at an approximate density of 10³ PFU/cm². The samples were then incubated at 4°C in plastic containers. Cotton wool swab samples were taken from three separate 10-cm² areas of each chicken portion before phage treatment and after 24 and 48 h. These were eluted in 2 ml of phosphate-buffered saline, and the target bacteria were quantified by serial dilution spread plates on selective media (Brilliant Green agar, CM263; Oxoid, Basingstoke, United Kingdom) containing 20 μg of sodium nalidixate per ml and 1 μg of novobiocin per ml. The number of viable bacteria per cm² was estimated for each time as an average of the three swab sample results, and the experiment was then duplicated with a fresh set of chicken skins. Phages were counted on lawns of susceptible bacteria (6, 8).

When phages were applied at a MOI of 1, the mean *Salmonella* plate count fell from log₁₀ 3.91 CFU/cm² to 3.27 (22% of the zero-hour count) after 24 h of incubation (Table 1). After 48 h of incubation at 4°C, the *Salmonella* count had decreased further to 11% of the original count. In contrast, on the untreated controls, the *Salmonella* count fell from log₁₀ 3.92 to 3.71—the equivalent of 60% of the zero-hour count after 24 h of incubation and did not change further by 48 h postincubation. The differences between the counts from the treated and control samples at both times were analyzed with Student's *t* test and were found to be significant ($t = 5.0$, $P < 0.01$ at 24 h; $t = 5.74$, $P < 0.01$ at 48 h).

Phages inoculated onto skin that had been pretreated with *S. enterica* serovar Enteritidis increased threefold in numbers from a mean plaque count of log₁₀ 3.0 PFU/cm² at time zero to log₁₀ 3.49 PFU/cm² after 48 h, whereas those inoculated onto untreated chicken showed a reduction of log₁₀ 3.04 PFU/cm² at time zero to log₁₀ 2.11—changes which were also significantly different from each other ($t = 3.48$; $0.02 < P < 0.01$).

Similar experiments, with a slightly higher MOI used, were carried out using identical chicken skin pieces inoculated with approximately 10⁴ CFU of *C. jejuni* strain C222, which was obtained from the Oxford Brookes University collection, per cm². *C. jejuni* typing phage 12673 was obtained from the National Collection of Type Cultures, London, United Kingdom, cultured on the target organism by using standard procedures, and contained between log₁₀ 9.2 and 10.1 PFU/ml. This phage was used to treat half of the chicken skins at an approximate density of 10⁶ PFU/cm². Swab samples were taken in triplicate, just as was done for the *Salmonella* experiments, and numbers

TABLE 2. Effect of application of bacteriophage at high MOI on *S. enterica* serovar Enteritidis

Treatment	Viable counts of <i>S. enterica</i> serovar Enteritidis ^a	P value
Untreated control	4.66 ± 0.29	
Phage P22	2.83 ± 0.24 (1.3%)	<0.01
Phage 29C	1.92 ± 0.11 (0.08%)	<0.01

^aValues represent mean ± standard error. Percentages in parentheses signify ratio of original count remaining.

of surviving *Campylobacter* estimated after 24 h of incubation at 4°C by plate counts on modified CCDA (charcoal-cefazolin-deoxycholate agar) Preston medium (CM 739; Oxoid) containing CCDA cefoperazone and amphotericin B supplement (SR155; Oxoid) incubated under microaerobic conditions (12). Chicken portions were inoculated with log₁₀ 4.05 PFU of *C. jejuni* per ml. *Campylobacter* did not survive well on exposed chicken surfaces, and after 24 h at 4°C, the mean plate count from the non-phage-treated sample was 2.99 CFU/cm² (approximately 10% of the initial inoculum; Table 1). However, in the chicken portions treated with phage 12673, the count was significantly lower. Thus, treatment with phage at 10⁶ PFU/cm² appeared to reduce the recovered *Campylobacter* counts by approximately 95%, which was statistically significant ($t = 2.5$; $P = 0.04$).

Multiplication of a *Pseudomonas* phage has been observed on the skin of a burn patient infected with this opportunistic pathogen (J. Soothill, personal communication). Because the initial experiments indicated multiplication with a low MOI of phages, further experiments were carried out with higher MOI.

Application of phages at high MOI. For these experiments, virulent mutants derived from the transducing lambda-doid phage P22, HTint, and phage 29C, isolated on *S. enterica* serovar Enteritidis from sewage (8), were used in preference to phage 12 because of higher plaquing efficiency. The phage P22 preparation contained log₁₀ 9.14 PFU/ml and phage 29C contained log₁₀ 10.10 PFU/ml.

Fifteen smaller skin pieces were sprayed with a 10⁴ dilution of the *S. enterica* serovar Enteritidis phage type 4 (log₁₀ 5 CFU/ml) to produce a density of ca. log₁₀ 3.7/cm². The skin pieces were inoculated with a hand-operated plant spray, delivering approximately 0.5 ml per s. The skin samples were inoculated using a short 0.5-s burst to the exterior surface. This was allowed to dry under gentle airflow for 30 min. Dilutions of phage preparations were then applied by the same method with a short 0.5-s spray to both sides of the skin. Five skin pieces were left untreated and five were each treated with a 10² dilution of phage P22 (log₁₀ 7.1 PFU/ml) or 29C (log₁₀ 8.1 PFU/ml) to produce a MOI of between 100 and 1,000. The skin samples were then processed within 30 min by adding 2 ml of phosphate-buffered saline to each piece in a sterile bottle followed by gentle partial homogenization with a mortar and pestle to remove bacteria from the skin without totally disintegrating the tissue. The number of viable bacteria per skin piece was determined immediately on Brilliant Green agar containing sodium nalidixate and novobiocin as mentioned above.

The mean log₁₀ viable bacterial count obtained from the control pieces was 4.66 per tissue piece, and from the pieces

treated with either P22 or 29C, the counts were 2.83 and 1.92, respectively (Table 2). This corresponded to reductions of 98.7 and 99.2%, respectively, both of which were highly significant ($P < 0.01$). Phage 29C may have been more efficient, with a reduction of more than nearly 1 log₁₀ unit, although this may have been solely the result of a higher phage density of phage 29C in the preparation used. Phage 29C was chosen for further studies.

Nine skin pieces were sprayed with a 10⁶ dilution of the *S. enterica* serovar Enteritidis strain (10³ CFU/ml), thus giving a density of between 10 and 10² CFU per cm² of skin. Three were left as untreated controls, three were sprayed with LB broth as phage-free controls, and the remaining three were sprayed with a 10² CFU per cm² preparation of phage 29C. This produced a MOI of ca. 10⁵. The log₁₀ viable numbers of the inoculated *Salmonella* on the three control skin pieces were 1.93, 2.00, and 2.16 (mean 2.03); those on the LB-treated pieces were 1.65, 1.73, and 1.74 (mean 1.71); and the nontransformed counts on the phage 29C-treated pieces were all zero.

Ten further pieces were treated with the same *Salmonella* dose, and five were left as untreated controls and five were treated with the same dose of phage 29C. The mean count in the controls pieces was (log₁₀) 1.09 ± 0.31 and the individual counts from the phage 29C-treated pieces were all zero.

Berchieri et al. (8) demonstrated reductions in mortality in newly hatched chickens caused by *S. enterica* serovar Typhimurium strains by administration of lytic phages in high numbers soon after oral infection with the salmonella (a MOI of 10³) but not when the phages were given some hours later or when lower numbers of phages were given (MOI of 1), suggesting lysis without phage multiplication. Given the relatively low numbers of *Salmonella* that normally contaminate the surface of chicken carcasses, (usually 1 to 30 CFU/100 g of skin, cited by Bryan and Doyle [9]), the present study has shown that this risk may be virtually eliminated by thorough application of a suspension of lytic phages. In one study (10), carcass *Campylobacter* counts were considerably higher, on the order of 10⁵ to 10⁶ CFU per carcass. However, this translates into a much lower number per cm², which might nevertheless be amenable to this approach.

Lysis of additional *Salmonella* strains. The effect of using phage 29C on two additional *Salmonella* strains, which were susceptible to attachment of this phage but were resistant to multiplication because of restriction (Barrow and Lovell, data not shown), were tested. The two smooth (O-12⁺) strains were *S. enterica* serovar Heidelberg 17705 and a PT6 *S. enterica* serovar Enteritidis. The two were each used to inoculate six chicken skin pieces, three of each of which were then treated with undiluted phage 29C. This was equivalent to a very high MOI of 10⁷. The log₁₀ viable numbers of *S. enterica* serovar Heidelberg from the three control pieces were 1.25, 1.92, and 2.03 (mean, 2.07) and were zero from the three phage-treated pieces. The corresponding control counts for *S. enterica* serovar Enteritidis PT6 were 2.13, 2.19, and 2.38 (mean, 2.23), and those for the phage-treated pieces were 0, 0, and 0.3 (mean, 2 CFU; log₁₀ 0.3).

Phages such as P22 or 29C, which attach to a common lipopolysaccharide antigen such as O-12, might therefore be used effectively to lyse most *Salmonella* strains within Kauffman-White groups A, B, and D. Groups B and D contain the

TABLE 3. The effect of EGTA in culture media on phage activity

Organism counted	Counts ^a when:		
	Not treated with phages	Treated with phages	
		Cultured with EGTA	Cultured without EGTA
Bacteria	5.01 ± 0.55	2.95 ± 0.32	3.35 ± 0.46
Phage		2.05 ± 0.52	6.78 ± 0.67

^a Values represent means of three independent experiments ± standard error.

serotypes that are currently of major significance. Other phages that would be lytic for additional serogroups could be found equally easily.

Demonstration of lysis on chicken skin. To ensure that phage activity was occurring on the chicken skins and not simply on the surface of the agar plate during counting, additional experiments were carried out with phage P1, attachment of which is known to be sensitive to 10 mM EGTA. When a preparation of P1 was counted on a lawn of a prototrophic *E. coli* K12 strain (a gift from H. Williams Smith, Houghton Poultry Research Station, Cambridgeshire, United Kingdom) on LB agar, the count obtained was log₁₀ 10.3. On a lawn of K12 growing on this agar containing 10 mM EGTA, the count was log₁₀ 3.6. Twelve pieces of chicken skin were seeded with a 10⁴ dilution of the K12 strain as indicated in the above experiments, three being left untreated and six treated with the undiluted phage preparation, of which three were plated on LB agar containing EGTA and three on LB agar without EGTA after they were all homogenized. The remaining three pieces were sprayed with phage but not with the bacteria. The mean of the *E. coli* count obtained from the untreated skin decreased whether or not counting was carried out with or without EGTA (Table 3). The count from the skins treated with phage only, made on a lawn of the K12 strain on LB agar with and without EGTA, showed a considerable reduction in phage numbers. It seemed likely, therefore, that reduction in bacterial numbers as a result of phage activity was taking place on the skin and not on the surface of the agar plate.

The application of bacteriophages to chicken skin contaminated with *S. enterica* serovar Enteritidis or *C. jejuni* will thus significantly reduce the numbers of these bacteria. Confirmatory experiments with an EDTA-sensitive phage, P1, indicated that the phages were attaching to the bacteria while on the skin samples rather than on the agar surface.

The phages persisted without a reduction in particle numbers on the skin for at least 48 h. This suggests that phage application may also protect carcasses against cross-contamination with pathogens from other carcasses or surfaces. The value of nonspecific lysis in such a procedure is that it might also be used for strains of *Salmonella* and *Campylobacter* that possess the attachment antigen but which, in a normal test of sensitivity under laboratory conditions, can be regarded as resistant because of DNA restriction.

The extent to which *C. jejuni* is able to multiply on chicken carcasses is a point of debate. Although it is generally accepted that *C. jejuni* will not multiply below 31°C (14), recent findings by Lee et al. (18) indicated that there could be multiplication on chicken carcasses at 4°C. If there is little or no growth on

chicken carcasses at low temperatures, nonspecific lysis may be more appropriate, since vegetative phage replication requires bacterial growth. However, attachment of a low number of phage particles to a susceptible bacterium will eventually result in lysis once the bacteria have begun to multiply within the intestine of an infected animal or humans.

A good coverage of the outer and inner surface of the carcass would be necessary for this model to function effectively. This could be done with appropriate spray equipment. A high MOI was used for some experiments, but this could be easily reached in practice.

One of the objections raised to the use of phages in disease therapy and prophylaxis is the potential for selection of resistant clones, which are either altered in their antigenicity by loss of the attachment antigen or by other changes—for example, in the bacterial restriction-modification system. This may conceivably occur with high levels of bacterial contamination on chicken carcasses, albeit at a low rate. It is unlikely that any resistant mutants would be transferred back to their origins in poultry flocks or pig herds in numbers sufficient to initiate extensive infections. The phages used would be likely to contaminate abattoir staff and also food handlers as well as some consumers in the same way that phages—which arise from the normal microbial flora and are normally present on carcasses—also infect people. However, unless such people were directly associated with poultry rearing or were themselves colonized by the target bacterium, the phages would be highly unlikely to enter a cycle of infection in poultry. Unless the target bacterium is present in numbers in the gut of above ca. 10⁶ CFU per gram of contents, phages appear to be unable to multiply (8, 26, 27). In addition, it has been demonstrated recently that rough mutants of *Salmonella*, which appear to be the phage-resistant mutants selected most frequently, are much less able to colonize the alimentary tract of poultry (11, 33). For the same reasons, such mutants would be unlikely to multiply in all but a very small number of humans.

We have demonstrated that phages can be effective at reducing bacterial contamination of the carcass surfaces. A more detailed analysis would appear to be warranted.

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