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Bacteriophage Therapy To Reduce *Campylobacter jejuni* Colonization of Broiler Chickens†

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Colonization of broiler chickens by the enteric pathogen *Campylobacter jejuni* is widespread and difficult to prevent. Bacteriophage therapy is one possible means by which this colonization could be controlled, thus limiting the entry of campylobacters into the human food chain. Prior to evaluating the efficacy of phage therapy, experimental models of *Campylobacter* colonization of broiler chickens were established by using low-passage *C. jejuni* isolates HPC5 and GIIC8 from United Kingdom broiler flocks. The screening of 53 lytic bacteriophage isolates against a panel of 50 *Campylobacter* isolates from broiler chickens and 80 strains isolated after human infection identified two phage candidates with broad host lysis. These phages, CP8 and CP34, were orally administered in antacid suspension, at different dosages, to 25-day-old broiler chickens experimentally colonized with the *C. jejuni* broiler isolates. Phage treatment of *C. jejuni*-colonized birds resulted in *Campylobacter* counts falling between 0.5 and 5 $\log_{10}$ CFU/g of cecal contents compared to untreated controls over a 5-day period postadministration. These reductions were dependent on the phage-*Campylobacter* combination, the dose of phage applied, and the time elapsed after administration. *Campylobacter*s resistant to bacteriophage infection were recovered from phage-treated chickens at a frequency of <4%. These resistant types were compromised in their ability to colonize experimental chickens and rapidly reverted to a phage-sensitive phenotype in vivo. The selection of appropriate phage and their dose optimization are key elements for the success of phage therapy to reduce campylobacters in broiler chickens.

Campylobacter jejuni and *C. coli* collectively account for the majority of acute bacterial enteritis cases reported in the United Kingdom and most of the developed world (12). More than 80% of birds in the United Kingdom harbor these organisms as a part of their normal intestinal flora (9, 26). Large numbers of these bacteria are disseminated upon abattoir processing and can be readily isolated from fresh chicken produce (20). The consumption of undercooked poultry meat and the cross-contamination of other foods in the preparation of poultry are significant risk factors in the transmission of campylobacters to human beings (20). A contemporary risk assessment has estimated that a $\log_{10}$ 2.0 reduction in *Campylobacter* numbers on retail chicken carcasses could reduce the frequency of human campylobacteriosis by up to 30-fold (32). To achieve this aim, intervention measures have focused on improving farm biosecurity and/or the decontamination of poultry carcasses postslaughter.

Ideally, decontamination techniques should not change the organoleptic or nutritional properties of the food. They should also be safe, inexpensive, and acceptable to the public. At present no single technique fits these criteria, although a number of different approaches to reduce *Campylobacter* carcass contamination have been suggested (10).

Intervention strategies focused at the start of the food chain (i.e., the broiler farm) are most likely to yield the greatest reductions of *Campylobacter* contaminating retail poultry meat. The upgrading of biosecurity has successfully produced *Campylobacter*-free chickens, but these measures are expensive and difficult to maintain. This is largely due to the susceptibility of chickens to infection by *Campylobacter* and its ubiquity in the environment (25). Moreover, unless increased biosecurity is applied globally, the benefits of reducing *Campylobacter* on some farms will be negated by cross-contamination from positive flocks at the abattoir (17). *Campylobacter*s are usually first detected in broiler house chickens when they are ca. 2 to 3 weeks old (25). The principle site of colonization is the gastrointestinal tract, in particular the ceca, small intestine, large intestine, and cloaca (6). Although it is generally accepted that campylobacters have evolved to rapidly and efficiently colonize the avian gut, some strains have been identified as poor colonizers (23, 31). Mutations in certain genes, for example, those with functions associated with motility, capsule formation, chemotaxis, and microaerobic respiration, result in a reduced ability to colonize chickens (16, 21). Models of colonization relevant to the hypothesis being tested are therefore of vital importance in studies aimed at the reduction of campylobacters in the food chain (26).

Another potential method of controlling campylobacters at the farm level is by competitive exclusion (CE). This technique has been successfully used to control *Salmonella*, but CE trials with *Campylobacter* have produced mixed results (26). Protection has been demonstrated with a strain of layer hen leading to the conclusion that CE effect was strongly dependent on

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bird variety to which it was applied (24). The success of vaccination in controlling *Salmonella* in United Kingdom layer flocks has prompted studies into a similar treatment for *Campylobacter*. Although this is a feasible strategy, toward which some progress has been recently reported (43), no such vaccine is currently available for commercial application (reviewed in reference 26).

Bacteriophages are naturally occurring predators of bacteria that are ubiquitous in the environment. Most bacterial species have their own specific bacteriophages, and campylobacters are no exception. *Campylobacter*-specific bacteriophages have been isolated from various sources including pig manure, abattoir effluents, sewage, and broiler chickens (2, 15, 22, 35). Some of these bacteriophages have been characterized to form the basis of the United Kingdom phage typing scheme (13, 34). Bacteriophages have been applied as a decontamination technique to reduce campylobacters on poultry meat under experimental conditions (3, 14). Since campylobacters are unable to multiply under refrigeration conditions, the phages may infect hosts on the surface of poultry meat but are unable to complete their replication cycle until environmental conditions are permissive for metabolic activity of their host. Whether the consumption of bacteriophage-infected campylobacters on contaminated meat would provide protection from enteric disease is unknown. The use of bacteriophages to reduce the numbers of campylobacters entering the food chain at farm level is a potentially useful intervention strategy (2), where reductions in numbers of campylobacters in chickens could lead to a measurable reduction in carcass contamination.

Bacteriophages are readily isolated from poultry excreta and, as a consequence, their potential therapeutic application to animals would not introduce any new biological entity into the food chain. A growing number of studies have successfully used phages to treat animal diseases (reviewed in references 5 and 29). These include applications to treat *Salmonella* (7, 37) and *Escherichia coli* (4, 18, 19) infections of young chickens. The use of phages to control *Campylobacter* in chickens diverges from these previous studies since campylobacters are considered natural commensals, not pathogens, of poultry species. Campylobacters colonize the chicken intestine to a high density and as such are a promising target for phage therapy. Concerns have been raised that campylobacters will simply become resistant to bacteriophages rendering this strategy ineffective in the long term (5). Bacteria rapidly mutate to become resistant to bacteriophages in vitro on laboratory media (1). However, unlike bacterial resistance to static chemotherapeutic agents such as antibiotics, bacteriophages constantly evolve to circumvent host barriers to infection. This leads to an evolutionary balance that allows both host and prey to proliferate.

Although Western countries have been slow in adopting bacteriophage therapy, primarily because of the antibiotics industry and a paucity of consistent evidence of phage efficacy, former Soviet Union countries have embraced the technology (41). The failures of Western scientists to achieve consistent results may have resulted from a general lack of understanding of phage biology and replication dynamics. Phage replication is critically dependent on the density of bacteria. There is predicted to be a distinct threshold above which phage numbers increase and below which they decrease, termed the phage proliferation threshold (29). The outcome of phage therapy also depends on the various life history parameters, including the inoculum size, and the inoculum timing (28, 42).

Here we describe the use of *Campylobacter* bacteriophages to reduce numbers of campylobacters in experimentally infected chickens and the optimization of the phage therapy regime. Targeted intervention measures to reduce colonization require that a suitable colonization model be established to ensure that differences in the colonization potential of *Campylobacter* strains do not affect the outcome (26). We also describe in vitro experiments of bacteriophage and host proliferation as a basis for the use of the phages and host in model chicken studies. Finally, we describe studies on the acquisition of phage resistance by the test campylobacters exposed to phage in the chicken intestine and the implications of this for the use of phage therapy to control campylobacters.

MATERIALS AND METHODS

Bacterial strains and growth media. Campylobacters were isolated and identified from broiler chicken excreta, free-range chicken excreta and from the surface of poultry meat by direct plating on cefoperazone charcoal deoxycholate agar (mCCDA) selective medium (LAB 112 [Lab M, Bury, United Kingdom]; with cefoperazone and amphotericin B supplement from Pro Lab Diagnostics, Neston, United Kingdom) using standard techniques. The isolates and reference strains were cultured on horse blood agar (blood agar base no. 2 CM0271 [Oxoid, Basingstoke, United Kingdom] with 5% defibrinated horse of blood [TCS, Buckingham, United Kingdom] added) under microaerobic conditions (5% O_2, 5% H_2, 10% CO_2, and 80% N_2 produced by the evacuation and replacement technique) at 42°C for 24 h. Strains of *Campylobacter* were stored at −80°C in Microbank storage beads (Pro Lab Diagnostics).

Experimental birds. Commercial *Campylobacter*-free male Ross broiler chickens were obtained as hatchlings (PD Hook, Oxfordshire, United Kingdom). Birds were housed in a controlled environment in individual pens under strict conditions of biosecurity. To ensure that the experimental birds remained free of naturally occurring infection, feces and cloacal swabs were taken each day and tested for *Campylobacter* by direct plating on mCCDA agar and for *Salmonella* by enrichment in Rappaport-Vassiliadis soya peptone broth (CM 866; Oxoid) then plating on xylose-lysine desoxycholoate agar (CM 469; Oxoid).

The chickens were killed and the ceca and upper (proximal small intestine) and lower intestines of the birds were first separated by ligature and then removed by sterile dissection; the lumenal contents were collected for *Campylobacter* and bacteriophage isolation. Essential organs (liver, pancreas, heart, and kidney) were also dissected and homogenized to examine whether the bacteria or bacteriophages could be found in other tissues.

Chicken colonization model. Broiler chicken *C. jejuni* isolates HPC5 and GIIC8 were suspended in phosphate-buffered saline (PBS; BR14a; Oxoid) and diluted to produce a range of colonization doses from $\log_{10}$ 2.7 to 7.8 CFU (n = four birds per dose tested). Each chicken was dosed with 1 ml of bacterial suspension in PBS or PBS alone for the controls by oral gavage at 20 to 22 days of age. Chickens were killed after 48 h, and the campylobacters were enumerated from the contents of the ceca and upper and lower intestines. Colonization levels were recorded as the $\log_{10}$ CFU per gram of intestinal contents for each dose of the *C. jejuni* isolate administered. Persistence over a longer period was examined by using an established dose of $\log_{10}$ 8 CFU in 1 ml of PBS to colonize chickens; thereafter, groups of birds ($n \geq 5$) were killed at 24-h intervals and the campylobacters present in their intestinal contents were enumerated as stated above.

Enumeration of campylobacters. Serial dilutions of cecal contents and upper and lower intestine contents were made in maximum recovery diluent (CM0733; Oxoid) and enumerated using the Miles and Misera technique on mCCDA agar with additional agar (L13; Oxoid) to a total of 2% added to reduce swarming. Plates were incubated under microaerobic conditions at 42°C for 48 h before typical *Campylobacter* colonies were counted.

PFGE of SmaI digests of genomic DNA. In order to ensure that no contaminating campylobacters had infected the birds in the containment facility, pulsed-field gel electrophoresis (PFGE) was carried out (8) on SmaI-digested DNA and compared to the known profiles of the test strains.

MLST of *Campylobacter* isolates. Selected isolates were also analyzed by using a multilocus sequence typing (MLST) scheme (11) to ensure single strain colo-

nization. Analysis of the sequence alleles was performed with reference to the *C. jejuni* MLST database (http://mlst.zoo.ox.ac.uk).

Bacteriophage selection, growth, and enumeration. Bacteriophages were isolated from ceca and upper and lower intestines as previously described (2, 8). Briefly, a 10% suspension of intestinal contents was prepared in SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, and 0.01% gelatin; Sigma Aldrich, Gillingham, United Kingdom) and centrifuged at 13,000 × *g* for 5 min to remove bacteria. The resulting supernatant was filtered through a 0.2-μm-pore-size disposable filter (Minisart; Sartorius, Gottingen, Germany) to remove any remaining bacteria. The filtrate was then applied to lawns of the appropriate host *C. jejuni* prepared using a modification (2) of the method described by Sambrook et al. (36). Primary isolates were selected on *Campylobacter* host derived from the same sample or the *C. jejuni* reference stain NCTC 12662 (PT14). The plates were then incubated for 24 h at 42°C under microaerobic conditions. Plaques were extracted from the overlay using a pipette and suspended in 100 μl of SM medium. Single plaques were propagated in this way a total of three times to ensure that the isolates represented a single clone. Bacteriophages were amplified by using the plate lysis method (13). Stability of phage titers at 4°C was determined over a 12-month period. Tolerance to different pH values from pH 2.2 to pH 9 was also explored by examining phage titers after exposure and neutralization through the use of appropriate buffers.

Lytic profiles. The lytic activities of selected bacteriophages were determined with 50 broiler chicken *Campylobacter* isolates and 80 reference strains of human origin, whose phage type had been previously defined on the basis of the 16 bacteriophages currently in use in the United Kingdom phage typing scheme (13). Lytic activity was assessed by pipetting 10 μl containing $\log_{10}$ 6 to 7 PFU onto prepared bacterial lawns and allowing the phages to absorb into the overlay agar. The plates were then incubated for 24 h at 42°C under microaerobic conditions. If ≤20 plaques were produced from $\log_{10}$ 6 to 7, the bacteria being tested were regarded as being insensitive to the phages.

Enumeration of bacteriophages. Enumeration of bacteriophages were carried out by making serial dilutions of the filtered suspensions of cecal and upper or lower intestinal contents, prepared as described above. Each dilution of phage suspension was applied as a 10-μl spot in triplicate to the prepared bacterial lawn and allowed to absorb into the overlay agar. Plates were incubated in the normal way, and the number of plaques was counted on the appropriate dilutions giving between 10 and 100 plaques to obtain the phage titer per gram for each section of the chicken intestine sampled.

Examination of *C. jejuni* bacteriophages morphology by electron microscopy. Bacteriophages CP8 and CP34 were examined by electron microscopy as previously described (2). Briefly, glutaraldehyde-fixed phage suspensions on Pioloform grids were negatively stained with 0.5% uranyl acetate. The specimens were observed with a JEOL 100CX transmission electron microscope at an acceleration voltage of 80 kV.

Phage genome size determination using PFGE. Genomic DNAs from bacteriophages CP8 and CP34 were prepared and run by using a CHEF DRII (Bio-Rad, United Kingdom) as previously described (2). A 2-mm slice of each agarose plug was inserted into the wells of a 1% agarose gel. The gel was run by using a Bio-Rad CHEF DRII system in 0.5× Tris-borate-EDTA for 18 h at 6 V/cm with a switch time of 30 to 60 s. Lambda concatemers (Sigma Aldrich, United Kingdom) were used as markers.

Bacteriophage DNAs. Genomic DNAs were prepared from each bacteriophage by using a standard protocol described for Lambda phage (36) using proteinase K digestion followed by phenol-chloroform extraction and precipitation. DNAs were digested with restriction enzymes (EcoRI, EcoRV, HhaI, HindIII, PstI, and SspI; Promega, Ltd., Southampton, United Kingdom), and the enzyme digests were run on a standard 0.6% Tris-acetate (0.04 M)-EDTA (0.001 M) buffer (pH 8.0) (36) agarose gel and stained with ethidium bromide after electrophoresis. Random DNA fragments were produced by DNase I digestion and repaired by using Klenow fragment and T4 DNA polymerases before molecular cloning as blunt-end fragments into the SmaI site of the plasmid vector pUC8 (36). The pUC8 clones were sequenced to produce phage DNA sequences from which prospective primer pairs were selected for PCR amplification in order to produce DNA amplicons that would discriminate phage and *Campylobacter* host DNAs (see additional information in the supplemental material). DNA hybridization was performed by using the digoxigenin labeling system as recommended by the supplier (Roche Diagnostics, Ltd., Lewes, United Kingdom).

In vitro experiments of phage efficacy. *C. jejuni* strain HPC5 was grown to mid-exponential-growth phase in nutrient broth no. 2 (CM 67; Oxoid) under microaerobic conditions in conical flasks on an orbital shaker. The cultures were diluted in fresh medium to give approximately $\log_{10}$ 5 CFU/ml, and bacteriophages (CP34 or CP8) were added to give multiplicities of infection (MOIs) of <0.1, ~1, and >10. Accurate determinations of *Campylobacter* and bacteriophage counts were performed at the same time by serial dilution to give the actual MOI of each experiment. Samples were taken over a period of 24 h.

Phage efficacy in chickens. Birds were infected with *C. jejuni* (HPC5 or GIIC8) at 18 to 20 days old depending on the experimental protocol. Each bird received $\log_{10}$ 3 to 8 CFU *Campylobacter* in 1 ml of PBS by oral gavage. A *Campylobacter*-free control group of bird were maintained for each experiment to ensure no environmental infection was occurring. Bacteriophages CP34 or CP8 were administered at 25 days old, once *Campylobacter* infection was established in control birds. The bacteriophage doses of $\log_{10}$ 5, 7, and 9 PFU were administered in 1 ml of 30% (wt/vol) $CaCO_3$ by oral gavage. Control groups of birds (with or without *Campylobacter*) were administered with 30% (wt/vol) $CaCO_3$ (1 ml) without bacteriophages. Birds were sacrificed at 24-h intervals, and the numbers of both *Campylobacter* and phages in the cecal contents and the upper and lower intestinal contents were enumerated. Colonization values are recorded as the mean $\log_{10}$ *Campylobacter* count per gram of intestinal contents ± the standard deviation. The liver, pancreas, heart, and kidney were also dissected to determine whether campylobacters and/or bacteriophages could be recovered from homogenates of these tissues.

Statistical treatment of data. Interbird variance in control colonization levels were examined by using $\log_{10}$-transformed *Campylobacter* counts using Bartlett's test (40). Statistical differences between paired control and treatment groups (using $\log_{10}$-transformed *Campylobacter* counts) were assessed by using the Student *t* test. Differences between experimental groups were analyzed by analysis of variance.

Acquisition of phage resistance. In order to establish the frequency of resistance to bacteriophages postintervention, 10 colonies from the isolation plates from each phage-treated bird were selected and tested for resistance first to the test phage they had been exposed to and subsequently a wider panel of bacteriophages used in the United Kingdom typing scheme (13). A bacterial lawn was prepared from each colony pick, and the test bacteriophage was applied at a range of dilutions from 0 to $\log_{10}$ 6 PFU to establish whether resistance had been acquired and to what degree. The in vitro frequency of phage resistance was also determined in the same way from single colony *Campylobacter* isolates picked from plates representing the 24-h time points of the growth experiments.

Stability of the resistant phenotype in vivo and in vitro. Resistant single-colony isolates from both the in vitro and in vivo experiments were subcultured on horse blood agar more than five times (representing more than 100 generations) and retested for resistance phenotype. In an experiment to determine the stability of resistant phenotypes in vivo, resistant variants of HPC5 from the CP34 treatment trial were selected (R14 and R20). Their colonization potential was assessed by inoculation of a new batch of birds with these isolates. After 6 days the numbers of colonizing campylobacters in the cecal contents and upper and lower intestines were determined as before. Single-colony isolates from these birds were tested for their resistance phenotypes.

RESULTS

Selection of bacteriophages. Bacteriophages were isolated from a range of sources, including broiler chicken excreta, free-range layer-chicken excreta, and processed chicken meat as described previously (2, 8). Bacteriophage isolates ($n = 53$) were selected for phage therapy trials on the basis of their ease of propagation, maintenance of phage titers over time (stable for ≥12 months at 4°C), and their stability at pH values between 4.0 and 9.0. All *Campylobacter* bacteriophage isolates were inactivated at pH 2.2, a pH that they could experience upon ingestion and transit of the proventriculus and gizzards of birds. Two phage candidates were selected for further investigation (CP8 and CP34) that exhibited the ability to lyse a range of different campylobacters, including two characterized *C. jejuni* hosts (HPC5 and GIIC8) that were subsequently utilized in chicken colonization trials. Bacteriophages CP8 and CP34, respectively, lysed 84 and 63 of 130 campylobacters tested (these included 50 broiler chicken isolates collected from farms at different geographic sites within the United Kingdom and the 80 different phage types represented in the United Kingdom bacteriophage typing scheme). The phages CP8 and CP34

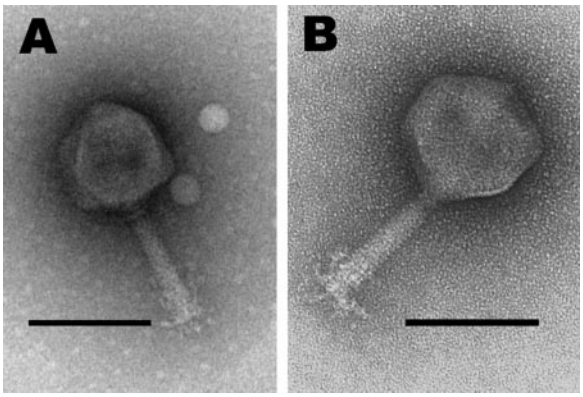

FIG. 1. Electron micrographs of bacteriophages CP8 (A) and CP34 (B). Bars, 0.1 μm.

were examined by electron microscopy (Fig. 1) and found to be similar in structure, each possessing an icosahedral head (diameters of 96 and 92 nm, respectively) and 100-nm contractile tails with tail fibers at their distal end. These morphologies are typical of the *Myoviridae* family of bacteriophages that exhibit lytic lifestyles and are exemplified by T4 phage of *E. coli*. Consistent with this, the genomic DNA sizes of each of these phages were approximately 140 kb, as estimated by PFGE. CP8 and CP34 phage DNAs were refractory to the restriction enzymes EcoRI, EcoRV, HindIII, PstI, and SspI. However, the restriction enzyme HhaI, which has previously been reported to discriminate other *Campylobacter* bacteriophage DNAs, (2, 34), could cut CP8 but not CP34 genomic DNAs. Random DNA fragments were generated from each of the phage DNAs by using DNase I and cloned into the plasmid vector pUC8. Despite the inherent difficulties in cloning phage DNAs, a number of clones were obtained, and their DNA sequences were determined. These DNA sequences were used to select primer pairs that were designed to generate PCR amplicons that could discriminate CP8 and CP34 bacteriophages. These amplicons then formed the basis of a PCR-based method to discriminate phage recovered from phage-treated chickens.

Chicken colonization model. We sought to create a model of *Campylobacter* colonization of broiler chickens that could be used specifically to evaluate the efficacy of phage therapy. Low-passage *C. jejuni* isolates (subcultured less than four times after isolation) from broiler chickens were initially screened against a panel of lytic bacteriophages. Two isolates (*C. jejuni* HPC5 and GIIC8) that were sensitive to infection by a broad range of phages, including CP8 and CP34, were selected for the colonization trials. The rate and level of colonization was determined in 20- to 22-day-old birds. The age of the birds was selected to parallel the first observation of colonization often observed in commercial broiler chickens (25). The effect of dose on the level of colonization of HPC5 ($\log_{10}$ 2.7, 3.8, 5.8, and 7.9 CFU) at 48 h is presented in Fig. 2A. The lowest doses resulted in the greatest variance in *Campylobacter* colonization of the small intestine, large intestine, and ceca. However, the $\log_{10}$ 7.9 doses resulted in reproducible colonization of all intestinal sites examined at 48 h. To establish the persistence and variation of the *C. jejuni* colonization levels over the experimental time frame, 20-day-old chickens were given a single 8-$\log_{10}$ CFU dose of *C. jejuni*, and the campylobacters enumerated from the intestinal contents of groups of these birds killed at 24-h intervals. Figure 2B shows the colonization levels of chickens following inoculation with *C. jejuni* HPC5 over a 7-day period. *C. jejuni* HPC5 could be detected in all parts of the intestine within 24 h. Maximum colonization was achieved within 48 h, and these colonization levels could be shown to be maintained over 9 days (chickens at 22 to 30 days of age). From 48 h onward the $\log_{10}$ counts of *C. jejuni* per gram of cecal contents showed equality of variance between all experimental groups sampled at daily intervals (Bartlett's test, $P > 0.05$). Mean viable counts and standard deviations of *C. jejuni* HPC5 in the upper intestine, lower intestine, and ceca were $\log_{10}$ 6.3 ± 0.8, 6.7 ± 0.8, and 7.4 ± 0.7 CFU/g of lumenal contents, respectively. Similarly, time and dose responses were obtained by using *C. jejuni* GIIC8, where the mean viable counts in the upper intestine, lower intestine and ceca were $\log_{10}$ 6.4 ± 0.5, 8.0 ± 0.4, and 7.8 ± 0.4 CFU/g of lumenal contents, respectively. Chickens colonized by these *C. jejuni* isolates did not show invasion of any of the non-gastrointestinal-tract tissues examined (liver, pancreas, heart, and kidney) throughout the course of the experiment. These results indicated that HPC5 and GIIC8 were both able to colonize experimental birds within the ranges of those reported for naturally infected birds (33) and maintain colonization levels over a suitable time period. The data collated from the colonization trials were subsequently used to design the phage treatment experiments.

In vitro studies of phage efficacy. In vitro growth experiments were carried out with phages CP8 and CP34 mixed with *C. jejuni* host in liquid culture under microaerobic conditions to give approximate MOIs of <0.1, 1, and >10. Bacteria and phage were enumerated over a 24-h period. The growth of *C. jejuni* host HPC5 and the replication of CP8 and CP34 are presented in Fig. 3. Viable counts of the initial *Campylobacter* inoculum and bacteriophage titer were performed to give the actual values for the initial MOI, which for CP8 were 0.09, 6, and 900 and for CP34 were 0.03, 3, and 300. Figure 3A to C shows infection by bacteriophage CP8 of *C. jejuni* HPC5 at the three different MOIs. Although the lowest MOI of 0.09 (Fig. 3A) resulted in the greatest reduction in *Campylobacter* counts ($\log_{10}$ >2) at the experimental end point of 24 h, the final counts were within $\log_{10}$ 0.5 for all three initial MOIs. At the lowest MOI (0.09) the bacteriophage titers remained similar for the first 12 h but increased rapidly over the second 12 h. Bacteriophage applied at the highest MOI of 900 (Fig. 3C) declined in numbers during the first 8 h but recovered to their initial titer by the end of the experiment.

The replication of bacteriophage CP34 in *C. jejuni* HPC5 was markedly different to that of CP8. At the highest MOI (i.e., 300) bacteriophage CP34 caused the numbers of campylobacters to decline for 8 h (Fig. 3F), but after this time bacterial counts increased until they were $\log_{10}$ 1 CFU below that of the controls by 24 h. In contrast, the lowest MOI of 0.03 had little effect on the growth of the bacteria until 8 h, but thereafter a reduction of $\log_{10}$ 2 in *Campylobacter* count compared to the control was observed. An MOI of 3 (Fig. 3E) was the least effective dose at the 24-h sample point. Bacteriophage enumeration of CP34 (Fig. 3D to F) revealed the highest MOI of 300 to result in a decline in phage titer by more than $\log_{10}$ 1 PFU/ml during the first 4 h, corresponding with a decline in

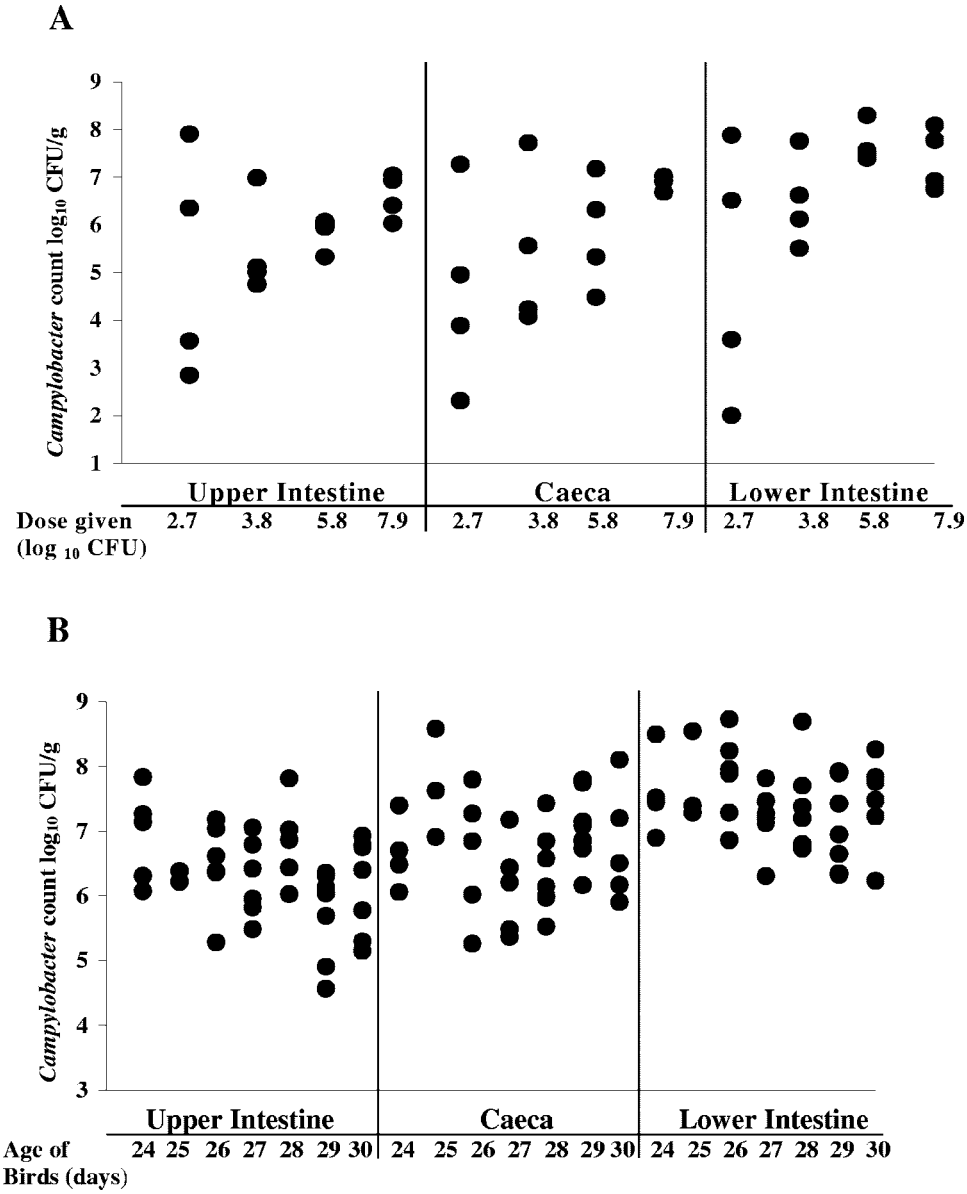

FIG. 2. Colonization of Ross broiler chickens with *C. jejuni* HPC5. (A) Dose-dependent colonization of 21-day-old male Ross broiler chickens with *C. jejuni* HPC5 (doses administered were $\log_{10}$ 2.7, 3.8, 5.8, and 7.9 CFU). Colonization was assessed 48 h postinfection for each intestinal site. (B) Colonization of Ross broiler chickens with *C. jejuni* HPC5 over a 7-day period. Chickens were administered at 20 days of age with $\log_{10}$ 8 CFU.

numbers of host cells (Fig. 3F). Using the lowest MOI (0.03; Fig. 3D) the phage titer increased over the first 4 h, while an MOI of 3 (Fig. 3E) resulted in phage numbers remaining relatively constant over the same period but increasing after 8 h. By 12 h, CP34 phages were approximately $\log_{10}$ 6 PFU/ml under all three experimental conditions and increased to $\log_{10}$ 7 to 8 by 24 h regardless of the original MOI. The frequencies of phage resistance after phage treatment were 8 and 11% for CP8 and CP34, respectively. Subculturing these isolates five times did not result in reversion to the sensitive phenotype.

Bacteriophage treatment of experimental chickens. Preliminary experiments were performed to assess whether the use of $CaCO_3$ as an antacid was appropriate to protect the phages from exposure to low pH during passage through the proventriculus and gizzard. Phages could be efficiently recovered from the $CaCO_3$ mixture and therefore was administered to control chickens colonized with *C. jejuni* HPC5 or GIIC8. The control birds remained colonized within the predetermined range for each isolate, in all experiments. Host *C jejuni* HPC5 was used to colonize chickens at 20 days of age and three different doses of bacteriophage CP8 ($\log_{10}$ 5, 7, and 9 PFU) administered separately in antacid to birds 5 days after colonization. Figure 4A shows the effect of administering a $\log_{10}$ 7 dose of CP8 on the colonization levels of individual birds. The numbers of campylobacters and bacteriophages in the intestinal contents of the ceca and upper and lower intestine were determined and

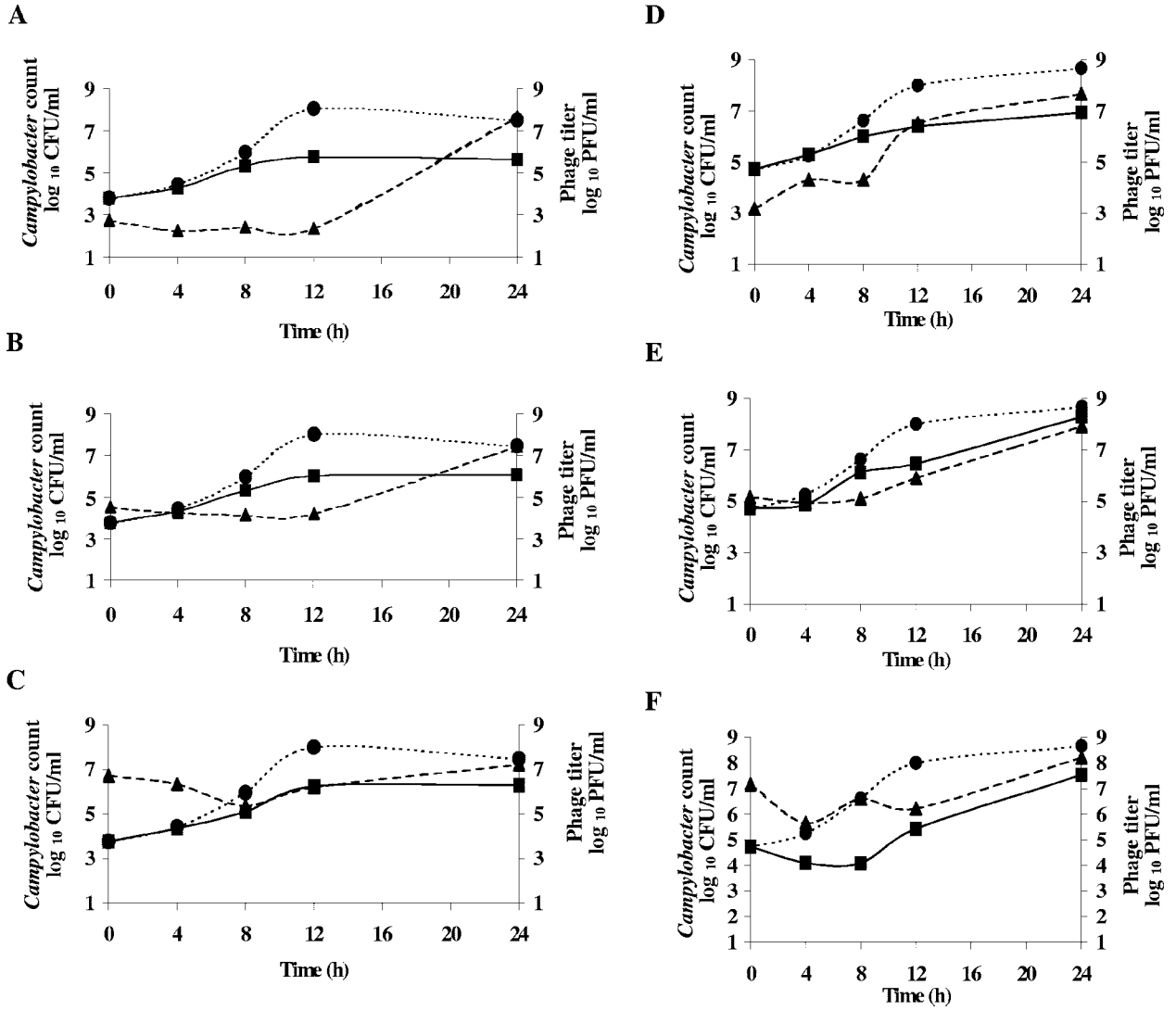

FIG. 3. In vitro replication of phages CP8 and CP34 with host *C. jejuni* HPC5 using different phage inoculation doses. (A to C) Replication of CP8 with initial MOIs of 0.09 (A), 6 (B), and 900 (C). ●, Control *Campylobacter* count $\log_{10}$ CFU/ml; ■, *Campylobacter* count $\log_{10}$ CFU/ml with phage; ▲, phage titer $\log_{10}$ PFU/ml. (D to F) Replication of CP34 with initial MOIs of 0.03 (D), 3 (E), and 300 (F) annotated as described for panel A.

compared to the colonization levels in the control group receiving $CaCO_3$ alone, and these were referenced to the values established in the colonization model.

In common with all of the experimental phage treatments, bacteriophage CP8 persisted over the course of the 5 days of the trial with HPC5 *C. jejuni*-colonized birds. These observations indicated that the phages could be maintained with their host within the chicken intestine. This may be compared to phages administered to the control groups of non-*C. jejuni*-colonized birds, within which phages were variably found in the range of $\log_{10}$ 3.5 to 4.1 PFU/g of intestinal contents after 24 h and not at all thereafter. The phage titers recovered from intestinal contents of HPC5 *C. jejuni* colonized chickens were in the range of $\log_{10}$ 3.2 to 6.5 PFU/g of cecal contents, which are consistent with the distribution of phage titers detected in the cecal contents of commercial broiler chickens naturally colonized with phages (ranging between $\log_{10}$ 3 and 6.9 PFU/g of cecal contents with the mode at $\log_{10}$ 4.2 PFU/g). The phage titers recorded did not correlate with simple dilution of the input dose, implying viral replication was occurring within 24 h of administration. The non-gastrointestinal-tract tissues of liver, pancreas, heart, and kidney were examined for the presence phages in these experiments, but none were recovered from any bird.

Treatment of *C. jejuni* HPC5 colonized chickens with phage CP34 at doses of 5, 7, and 9 $\log_{10}$ PFU resulted in significant falls in *Campylobacter* counts at one or more of the three intestinal sample sites during the course of the experiment. Figure 4B shows the effect of administering a 7-$\log_{10}$ dose of CP34 on the colonization levels of individual birds. Significant falls in the cecal *Campylobacter* colonization levels were recorded for the 5- and 7-$\log_{10}$ PFU doses at 24 h after phage administration. The highest phage dose of 9 $\log_{10}$ PFU did not result in significant decreases in cecal *Campylobacter* coloniza-

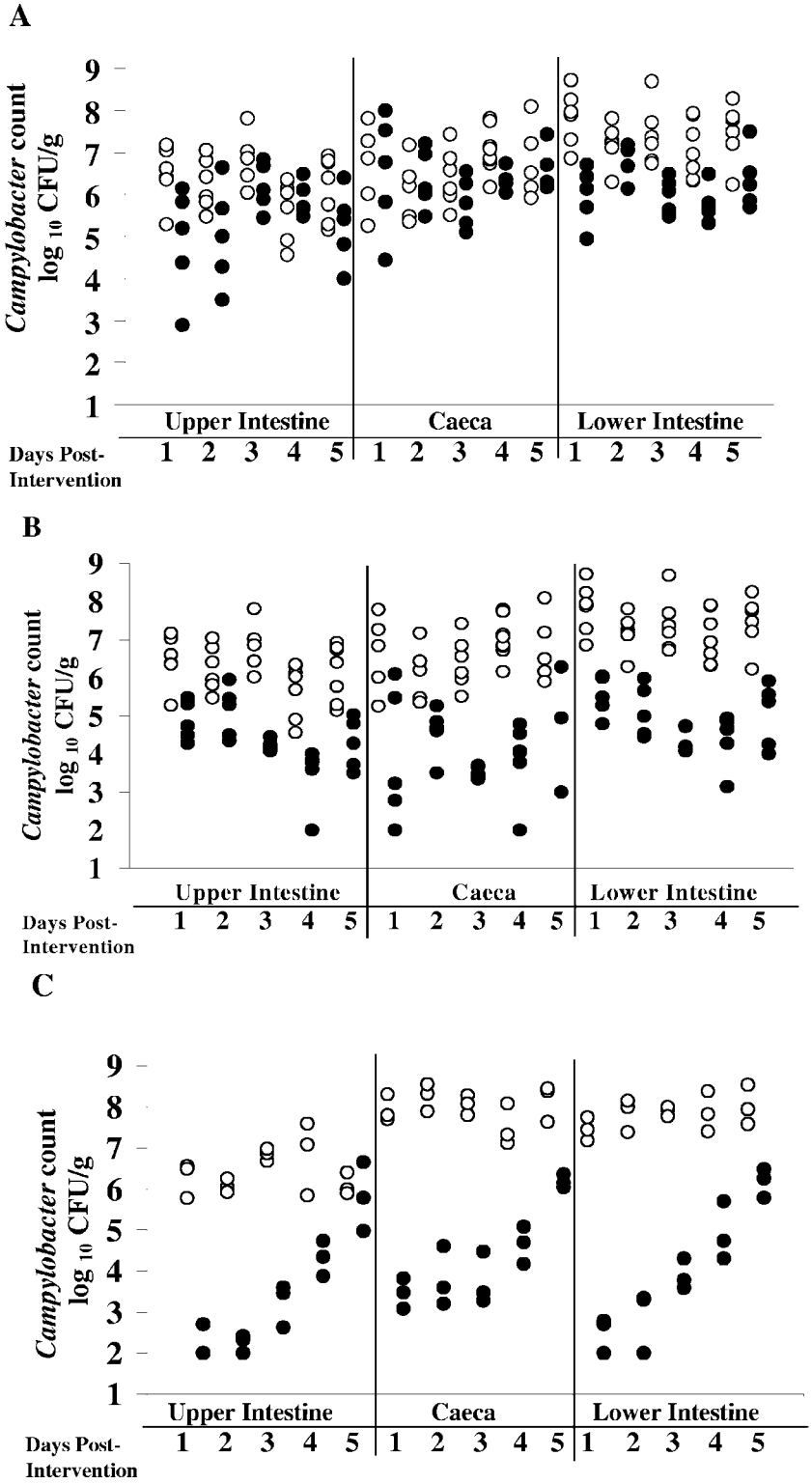

FIG. 4. Comparison of the efficacy of CP8 and CP34 phages against *C. jejuni* HPC5 and GIIC8. Chickens colonized by *C. jejuni* HPC5 or GIIC8 were treated with a $\log_{10}$ 7 PFU dose of either CP8 or CP34. ○, Control *Campylobacter* counts of intestinal contents; ●, bacteriophage-treated *Campylobacter* counts ($\log_{10}$ CFU/g). (A) Phage CP8-treated chickens colonized with *C. jejuni* HPC5; (B) phage CP34-treated chickens colonized with *C. jejuni* HPC5; (C) phage CP8-treated chickens colonized with *C. jejuni* GIIC8.

tion levels in the early days of the experiment, but a significant decrease of 1.4 $\log_{10}$ CFU/g ($P = 0.0007$) was recorded on day 4 (results not shown). The most effective dose was $\log_{10}$ 7 PFU with respect to the decline in mean cecal *Campylobacter* counts, with a drop to $\log_{10}$ 3.9 CFU/g over the first 24 h post-phage administration ($P = 0.012$).

Overall, phage CP34 was more effective in the reduction of *C. jejuni* at all intestinal sites compared to CP8. After the first day of CP8 treatment, the *Campylobacter* counts fell in the upper and lower intestines but the cecal counts were not significantly different from the control birds.

A single $\log_{10}$ 7 PFU dose of bacteriophage CP8 was administered to birds infected with *C. jejuni* GIIC8 (Fig. 4C). A substantial decline in the mean numbers of campylobacters in cecal contents of $\log_{10}$ 5.6 CFU/g within 24 h of phage administration was observed with this phage-host combination ($P = 0.00005$). However, it is evident that the *C. jejuni* counts start to recover from 72 h after phage administration. At 5 days after phage treatment, the cecal and lower intestinal colonization values still exhibit significant differences of $\log_{10}$ 2.1 and 1.8 CFU/g, respectively, but the upper intestinal count had recovered and to show no significant difference with the colonization values recorded for the control group. It is clear that the bacteriophage CP8 is much more effective in its ability to kill the *C. jejuni* isolate GIIC8 than the HPC5 isolate in the chicken intestine.

Acquisition of phage resistance in vitro and in vivo. Examination of single colony isolates of *C. jejuni* HPC5 that survived CP34 phage infection in vitro revealed that 11% of these were resistant to further CP34 infection. In contrast, 4% of the single colony isolates recovered from broiler chickens post-treatment with CP34 were resistant to the bacteriophage. Spontaneous bacteriophage resistance was not detected in *C. jejuni* reisolated from control chickens that were not exposed to phages, where 10 independent colonies were selected and tested from each control bird. Resistant *C. jejuni* strains from phage-treated birds were confirmed to be congruous to the original strain by MLST (sequence type 356). This was necessary to ensure that cross-contamination with different strains of *Campylobacter* had not occurred. Two independent phage resistant isolates of HPC5 (R14 and R20) were selected from two independent phage-treated birds and examined for the presence of CP34 phage DNA in their genomic DNAs. DNA hybridization of R14 and R20 genomic DNAs against digoxigenin-labeled phage DNA did not produce a hybridization signal and PCR amplification with phage-specific primers using R14 and R20 genomic DNAs as a template did not produce a positive amplicon. The phage-resistant isolates R14 and R20 were further tested in the colonization model and compared to the original strain. The average cecal contents *Campylobacter* counts were $\log_{10}$ 6.2 $\pm$ 0.98 CFU/g for R14 and 5.9 $\pm$ 0.68 CFU/g for R20. This was compared to the model colonization level of $\log_{10}$ 6.8 $\pm$ 0.3 CFU/g, which was significantly higher than R20 ($P < 0.02$) but not R14 ($P = 0.13$). Ninety isolates from birds initially colonized with either R20 or R14 were retested for a phage-resistant phenotype; of these, 97% were found to have reverted back to a phage-sensitive phenotype. These isolates were again confirmed as being genotypically derived from the original isolate by MLST.

DISCUSSION

Before adopting bacteriophage therapy as a means of controlling campylobacters in broiler chickens, it is important to understand the dynamics of bacteriophage infection in experimental birds that are typical of commercial breed stock and colonized by appropriate host *C. jejuni* strains (25). We therefore considered it important to demonstrate that the *C. jejuni* selected for the present study could establish and maintain colonization, such that any changes in *Campylobacter* colonization could be ascribed to the introduction of phage. Our data confirm that the *C. jejuni* isolates were maintained in the experimental chickens over a 9-day period (21- to 30-day-old birds) and that the levels of *Campylobacter* are consistent with those reported for naturally colonized chickens, which are in the range of $\log_{10}$ 4.0 to $\log_{10}$ 8.0 CFU/g of cecal contents (33). Experimental bacteriophage treatments of *C. jejuni*-colonized birds resulted in phage titers persisting over the course of the trial implying that the phages administered were delivered to the intestinal sites colonized by *C. jejuni* and were able to replicate within the environment of the chicken intestinal tract. Chickens possess a pair of ceca at the junction of the ileum and rectum. The ceca represent a reservoir of microbes off the main gastrointestinal tract, where microflora actively enter the ceca by retrograde peristalsis of digesta that are not discharged.

The kinetics of bacteriophage replication and host growth were examined in vitro in order to gauge the course of infection and provide a basis on which to select the most suitable bacteriophages for subsequent in vivo experiments. The highest dose applied (MOI > 10) was intended to examine whether campylobacters could be reduced by passive inundation (28). Passive inundation refers to where bacterial numbers are depleted by the attachment of overwhelming numbers of phage but without productive replication of the bacteriophage. The lower phage doses (MOI < 1) were expected to initiate active proliferation of the phage and bacteria, with the phage eventually overwhelming their host. The former was initially considered as an appropriate strategy for the phage treatment of chickens from the point of view of reducing the possibility of acquired host resistance to phages over time. The low dose with active replication of phage is the scenario that is probably most similar to the primary entry of phages into the intestinal tract of *Campylobacter* colonized chicken flock. However, once an individual bird within the flock has been colonized by phage, all subsequent birds are likely to be exposed to higher doses as the phage are shed.

It was clear from our in vitro experiments that CP8 and CP34 bacteriophages exhibited some fundamental differences in their ability to predate the host HPC5. Comparison of these two phages in vitro with the same host over a comparable range of MOI revealed CP8 to be the most effective phage with respect to the reduction in the *Campylobacter* viable count at the experimental end point (24 h). Although phage-resistant variants could be isolated at this point the majority of the *Campylobacter* population remained sensitive to phage. Bacteria encountering high phage densities may be "lysed from without" leading to large quantities of bacterial cell debris to which phages may adhere in competition with live bacterial cells (30). This may lead to an initial drop in titer as many of the phages

are unable to replicate. Under conditions where high initial MOIs of CP8 and CP34 phages were applied to *C. jejuni* HPC5, the phage titer was observed to fall, possibly due to nonproductive binding as described above. However, in contrast to CP8, phage CP34 had recovered some of the initial fall in titer by 8 h due to its tendency to replicate earlier.

After the in vitro experiments, the same phage and host combinations were tested in chickens. The ability of bacteriophage CP34 to replicate on the *C. jejuni* host HPC5 in chickens resulted in significant reductions in the cecal *Campylobacter* counts with doses of $\log_{10}$ 5 and $\log_{10}$ 7 PFU, but $\log_{10}$ 9 PFU was less effective. Phages were evident in the ceca of the $\log_{10}$ 9 PFU-treated birds, so why they were ineffective in bringing about a reduction in the *Campylobacter* population is not clear. The first encounter of the phages administered with metabolically active host *C. jejuni* is likely to occur in the upper reaches of the intestinal tract and could influence the subsequent course of the treatment. The adherence of the phages to host bacterial cells leading to cell death without phage replication will effectively diminish the dose carried to later sections of the gut. Higher phage densities are also prone to phage aggregation and nonspecific association with digesta or non-host bacteria (30).

In general, bacteriophage CP 8 was less effective than CP34 against *C. jejuni* HPC5 colonizing broiler chickens. The preceding in vitro experiments and the recovery of CP8 from phage-treated chickens over 5 days clearly indicates that CP8 is able to replicate on *C. jejuni* HPC5 but not appreciably reduce *C. jejuni* HPC5 populating the chicken intestinal tract. The superior ability of bacteriophage CP34 over CP8 to kill *C. jejuni* HPC5 in chickens is contrary to the observations in vitro, which would clearly favor the ability of CP8. However, despite the inability of CP8 to affect *C. jejuni* HPC5 in chickens, the treatment of chickens with the same bacteriophage but colonized with an alternative host, *C. jejuni* GIIC8, yielded a $>\log_{10}$ 5 reduction in cecal *Campylobacter* counts. The greatest reductions in cecal *Campylobacter* counts were observed at 24 and 48 h after phage treatment, after which the *Campylobacter* counts recovered to a level of $\log_{10}$ 2 less than the control chickens over the next 3 days. Phage CP8 clearly has the capacity to function as a therapeutic agent in chickens, where it survived ingestion, discriminated nonspecific materials, and encountered enteric defense mechanisms, but was still able to replicate in the intestinal tract. Therefore, the lack of success of this phage with *C. jejuni* HPC5 is intriguing; at least a proportion of the host must remain permissive and accessible, since *C. jejuni* HPC5 can support the propagation of phage CP8 over the 5 days of the experiment. It is possible that a subpopulation of bacteria may become resistant to the phage CP8, but currently there is no evidence for this in the bacteria recovered after phage treatment. However, if the resistance was transient, say, for a population of bacteria that were physiologically downshifted and capable of regrowth, then the bacteria would escape the phage but remain fundamentally phage sensitive. Alternatively, a proportion of the *C. jejuni* HPC5 population may colonize intestinal sites that are either inaccessible for or a hindrance to CP8 attachment. For example, close proximity to the intestinal mucosa.

The avian intestinal tract is a complex environment where various physical (for example, the constant flow of digesta and mixing with non-host bacteria) and physiological (for example, oxygen levels) host defenses and biochemical factors (such as proteolytic enzymes and adverse pH) all influence the populations of colonizing bacteria. In addition, the kinetics of phage adsorption in the intestine may be quite different from laboratory media due to the viscosity of the mucus layer (42). It is evident that the direct translation information gained from homogeneous bacteria and phages in a well-mixed and controlled laboratory environment to the situation in vivo is not going to be a reliable measure of the efficacy of the phage to kill host bacteria.

The selection of resistant bacteria has always been perceived as a potential drawback to phage therapy and has been reported following phage treatment (37–39). However, resistance can be correlated with reduced virulence (27, 39). Here the incidence of phage-resistant phenotypes after phage challenge did not become the dominant population in phage-treated chickens despite the continued presence and replication of these phages. Chickens subject to environmental challenge will encounter diverse *Campylobacter* genotypes, it is therefore more likely that succession by phage insensitive genotypes will occur rather than through the selection of phage resistant strains (8). In the present study we have elected to use a single phage in each experiment to provide comparative data between phages. However, in commercial production it might be necessary to administer a cocktail of phages.

These data represent the first steps toward an understanding of the kinetic processes and the characteristics of *Campylobacter* bacteriophages that are necessary if the potential offered by phage therapy as a sustainable biological control measure is to be harnessed for the reduction of campylobacters emanating from farmed poultry sources.

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