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Application of a Group II *Campylobacter* Bacteriophage To Reduce Strains of *Campylobacter jejuni* and *Campylobacter coli* Colonizing Broiler Chickens

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ABSTRACT

Members of the genus *Campylobacter* are frequently responsible for human enteric disease worldwide. Persistent *Campylobacter* contamination of poultry meat is a common problem that represents a significant food safety risk through the consumption of undercooked poultry meat or through cross-contamination of other foods during the preparation of poultry. Bacteriophage therapy is one possible means by which this colonization of poultry could be controlled, thus limiting the entry of *Campylobacter* into the human food chain. Previously group III phages with genome sizes of approximately 140 kb had been administered to *Campylobacter jejuni*–colonized poultry. The application of a group II *Campylobacter* phage, CP220, with a genome size of 197 kb is described here. Phage CP220 was administered to both *C. jejuni*– and *C. coli*–colonized birds. A 2-log CFU/g decline in cecal *Campylobacter* counts was observed after 48 h in birds colonized with *C. jejuni* HPC5 and administered with a single 7-log PFU dose of CP220. The incidence of phage resistance developing in *Campylobacter*-colonized chickens upon exposure to virulent phages was determined to be 2%, and the resistant types remained a minor component of the population. To achieve a similar reduction in *Campylobacter* numbers in *C. coli* OR12–colonized birds, a 9-log PFU dose of CP220 was required. Using phage to reduce *Campylobacter* colonization in poultry offers the prospect of a sustainable intervention measure that may limit the entry of these pathogens into the human food chain.

Acute cases of bacterial gastroenteritis in the developed world are frequently caused by *Campylobacter jejuni* and *Campylobacter coli.* It is generally considered that the handling and consumption of poultry meat contribute greatly to this disease burden, given that *Campylobacter* can be isolated following slaughter from more than 80% of chickens raised for poultry meat in the United Kingdom *(22).* Carriage rates among live birds vary greatly between European countries, but prevalence rates of *Campylobacter*-positive birds are generally high (reviewed by Wagenaar et al. *(45)*). During transport and abattoir processing large numbers of *Campylobacter* organisms are disseminated. The numbers contaminating the surface of the meat are reported to be in the region of 5.9 log CFU per carcass following scalding and defeathering *(30),* and consequently *Campylobacter* can be readily isolated from fresh chicken produce (reviewed by Jacobs-Reitisma et al. *(21)*). Risk assessments have estimated that a 2.0-log reduction in *Campylobacter* numbers on retail chicken carcasses could reduce the incidence of human campylobacterosis up to 30-fold *(33).* To achieve this aim, intervention measures have focused mainly on improving farm biosecurity to prevent colonization from occurring and on reducing colonization during rearing and the decontamination of poultry carcasses postslaughter *(8, 11).* Even when it is possible to produce *Campylobacter*-free chickens, the benefits of this may be negated by cross-contamination from *Campylobacter*-positive flocks at the abattoir *(2, 30).*

Bacteriophages (often abbreviated to phages) are naturally occurring predators of bacteria that are ubiquitous in the environment. Their specificity against a particular bacterial species and their lack of impact upon other flora make them attractive antibacterial agents, and their use as such is usually referred to as phage therapy. While Western countries have been slow in adopting phage therapy, primarily because of the success of antibiotics in treating bacterial infections and a paucity of consistent evidence of phage efficacy, former Soviet Union countries have embraced the technology *(42).* 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),* although the nature of the threshold is subject to controversy *(23).* The outcome of phage therapy also depends on the various life history parameters including the inoculum dose and the inoculum timing *(28, 46).*

There is a wide range of potential applications of phag-

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es to treat human and animal bacterial infections *(27, 39)* but also to prevent food spoilage and reduce pathogen levels in food (reviewed by Connerton and Connerton *(9),* Greer *(18),* Hudson et al. *(20),* Sulakvelidze et al. *(42),* and Summers *(43)*). The use of phages to control *Campylobacter* in chickens diverges from most other studies because campylobacters are not overtly pathogens of poultry species. *Campylobacter* colonizes the chicken intestine to a high density and as such is a promising target for phage therapy *(8).* Concerns have been raised that *Campylobacter* will simply become resistant to phages, rendering this strategy ineffective in the long term *(6).* A variety of spontaneous phage-resistant bacterial mutants arise readily in in vitro populations *(1).* However, resistance to phages has been correlated with reduced virulence in vivo *(10, 24, 40).* Unlike bacterial resistance to bacteriostatic chemotherapeutic agents, such as certain antibiotics, phages constantly evolve to circumvent host barriers to infection. This leads to an evolutionary balance that allows both host and prey to proliferate. For phage therapy to be effective, artificially shifting the equilibrium in favor of phage results in a temporary window of opportunity to reduce bacterial numbers. For the application described here the return to equilibrium is broken by the slaughter of the birds while the *Campylobacter* numbers are lowered.

The targeted use of phages to reduce the numbers of *Campylobacter* entering the food chain at the farm level is a potentially useful intervention strategy *(3, 7, 8),* where reductions in numbers of *Campylobacter* in chickens could lead to a measurable reduction in carcass contamination. Phages specific for *Campylobacter* have been isolated from numerous sources including pig manure, sewage, broiler chickens, and poultry carcasses *(4, 13, 17, 19, 25, 35).* Some have been used to develop phage typing schemes *(15, 17).* The 16 phages used in the United Kingdom typing scheme *(15)* have been characterized *(34).* All the phages had icosahedral heads and long contractile tails that were classified as members of the *Myoviridae,* but they could be separated into three different groups as proposed by Sails et al. *(34),* based on genome size and head diameter. Two phages with head diameters of 140.6 and 143.8 nm with large genome sizes of 320 kb were classified into group I. Five phages, classified into group II, had average head diameters of 99 nm and average genome sizes of 184 kb. Group III contained nine phages with average head sizes of 100 nm and average genome sizes of 138 kb. It is also notable that the three phage groups ascribed by Sails et al. *(34)* may target alternative hosts among *Campylobacter* populations based on the observation that the three most common *Campylobacter* phage types show susceptibility to only one of the three phage groups assigned on the basis of genome size and morphology *(34).* Further characterization of these phages using spontaneous and transposon mutants indicated that capsular polysaccharide and flagella are important components of the infection process *(12).*

Phages of *Campylobacter* have been used successfully to reduce *Campylobacter* numbers present on poultry meat *(5, 16)* and to reduce the carriage of *Campylobacter* in the ceca of chickens *(24, 38, 44).* Selection of the most appropriate phages and their dose optimization are key elements for the success of phage therapy to reduce *Campylobacter* in broiler chickens *(8, 24).* The efficacy of a phage in vitro does not necessarily translate to efficacy in vivo *(24),* so it is important to generate experimental data from the administration of phages to chickens. The phages that were chosen for the poultry treatment experiments were broiler phage isolates CP8 and CP34 *(24)* and CP30 *(37)* and typing phages NCTC 12669 and NCTC 12671 *(44)* from the United Kingdom phage typing scheme *(15).* All these phages belong to group III and represent those most frequently isolated from poultry sources *(25).*

This study examines the therapeutic value of *Campylobacter* phage CP220 to reduce *Campylobacter* numbers in the ceca and upper and lower intestines of experimentally infected chickens. In contrast to previous studies, this phage belongs to the group II *Campylobacter* phages *(34).* Phage CP220 displays a wide host range, which extends to *C. coli* strains that include a dominant *C. coli* that has been shown to displace established *C. jejuni* from colonized chickens *(14).* Various studies from around the world have shown that the numbers of *C. coli* isolated from poultry may range from 6 to 50% *(11),* with the highest incidence associated with free-range birds and the lowest from birds reared in conventional barns *(13, 32).* Development of therapeutic agents that target many different strains and include both of the main *Campylobacter* species found in the chicken intestine is clearly advantageous. Selecting a phage from a different group that had previously been used for phage therapy was therefore prudent. We also describe studies on the acquisition of phage resistance by the test *Campylobacter* exposed to phage in the chicken intestine and discuss the implications of this for the use of phage therapy to control *Campylobacter* in commercial poultry production.

MATERIALS AND METHODS

Bacterial strains. *C. jejuni* HPC5 was isolated from a United Kingdom broiler chicken *(24),* and *C. coli* OR12 was isolated from a United Kingdom organic chicken farm *(13).* Broiler strains for lytic profiling were from laboratory stocks. The isolates were routinely cultured on horse blood agar (blood agar base No 2 CM0271 [Oxoid, Basingstoke, UK] with 5% [vol/vol] defibrinated horse blood [TCS, Buckingham, UK] 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. Isolates of *Campylobacter* were stored at −80°C on Microbank storage beads (Pro Lab Diagnostics, Cheshire, UK).

Propagation and characterization of phage CP220. Propagation of high-titer stocks of phage CP220 was carried out as previously described *(4)* using the plate lysis method *(15)* with *C. jejuni* NCTC 12662 as host. Genomic DNA from phage CP220 was prepared and analyzed by pulsed-field gel electrophoresis (PFGE) as previously described *(4).* The size of the genome was determined by comparison to standard concatenated lambda DNA markers (Sigma Aldrich, Gillingham, UK). The morphology of CP220 was examined by electron microscopy as previously described *(4).* The effects of different pH values on the viability of phage CP220 were determined by incubation of a 7-log PFU/ml suspension in a range of buffered SM (50 mM Tris-HCl, 0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$ and 0.01% gelatin; Sigma Aldrich)

from pH 2 to pH 11. Viability was determined after 24 h and again after 4 days of incubation at room temperature. The lytic activity of CP220 was determined using 50 broiler chicken *Campylobacter* isolates. Lytic activity was assessed by pipetting 10 μl containing 4 to 5 log 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. The concentration of phage applied was selected to mirror the routine test dilution for phage typing *Campylobacter* isolates *(15).* The routine test dilution represents the phage titer required to produce subconfluent lysis of the control strain and was selected to allow for sufficient phage particles to be available to overcome host cell restriction and produce visible plaques. This number is frequently considerably less than would be expected on the control strain to which the phage is already adapted. A strain is considered to be sensitive to a phage (according to Frost et al. *(15)*) if more than 20 plaques are produced from the 10^4 to 10^5 phage particles that are applied to the lawn. If a small number of plaques (<20) are produced, the strain is considered resistant to the phage either due to the host cell restriction being too great to be overcome or because the host has a small population of sensitive clones that permit a low level of phage replication. In either case phage therapy with this strain-phage combination is unlikely to be successful, and more appropriate phages should be sought.

Experimental birds. Commercial *Campylobacter*-free male Ross broiler chickens were obtained as hatchlings (P. D. Hook, Oxfordshire, UK). 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 modified cefoperazone charcoal desoxycholate agar (mCCDA; CM0739, Oxoid) agar supplemented with cefoperazone supplement (Pro-Lab Diagnostics) and for *Salmonella* by enrichment in Rappaport-Vassiliadis soya peptone broth (CM 866, Oxoid) and then plating on xylose-lysine desoxycholate agar (CM 469, Oxoid). In order to ensure that no contaminating *Campylobacter* had infected the birds in the containment facility, PFGE of *Sma*I digests of genomic DNAs was carried out *(31)* from random phage-sensitive isolates recovered from the chickens and compared to the known profiles of the test strains.

Phage efficacy in chickens. Birds were colonized with *C. jejuni* HPC5 or *C. coli* OR12 at 20 days of age. Each bird received 8 log CFU C*ampylobacter* in 1 ml of phosphate-buffered saline (Oxoid) by oral gavage. A *Campylobacter*-free control group of birds was maintained for each experiment to ensure that no environmental infection was occurring. Phage CP220 were administered when the birds were 25 days old, once *Campylobacter* infection was established in control birds. The phage doses of 5, 7, and 9 log PFU were administered in 1 ml of 30% (wt/vol) $CaCO_3$ (Sigma Aldrich) by oral gavage. Control groups of birds (with and without *Campylobacter*) were administered 30% (wt/vol) $CaCO_3$ (1 ml) without phage. Three birds were euthanized from each dose group and from the control group at 24-h intervals, and the numbers of both *Campylobacter* and phages in the ceca and upper and lower intestinal contents were determined. Colonization values are recorded as the mean log *Campylobacter* count per gram of cecal contents, upper intestinal contents, or lower intestinal contents ± standard deviation. Interbird variance in control colonization levels was examined by using log-transformed *Campylobacter* counts, using Bartlett's test *(41).* Statistical differences between control and treatment groups (using log-transformed *Campylobacter* counts) on a daily basis were assessed by analysis of variance (testing the hypothesis that means from two or more samples are equal) from the Excel Data Analysis package (Microsoft Corporation, Redmond, WA).

Enumeration of *Campylobacter.* Serial dilutions of cecal contents from chickens were made in maximum recovery diluent (CM0733, Oxoid), and *Campylobacter* organisms were enumerated from plate counts on mCCDA agar supplemented with cefoperazone 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 *Campylobacter* colonies were counted. Colonies on mCCDA that were shiny, grey, and of typical size and shape were presumed to be *Campylobacter.* Gram stains of randomly selected isolates were examined to confirm typical *Campylobacter* morphology. In addition, *Sma*I digests of genomic DNAs were prepared *(31)* from 10 isolated colonies from the final sample day and subjected to PFGE to confirm that the isolates were identical to the *Sma*I digest profile of the strain administered.

Enumeration of phages. Phages were isolated from ceca and upper and lower intestines as previously described *(24).* Briefly, a 10% suspension of intestinal contents was prepared in SM buffer and centrifuged at 13,000 × *g* for 5 min to remove solid debris and bacteria. The resulting supernatant was filtered through a 0.2-μm-pore-size disposable filter (Minisart, Sartorius, Goettingen, Germany) to remove any remaining bacteria. Enumeration of phages was carried out by making serial dilutions of the filtered suspensions. The dilutions were then applied to lawns, prepared using a modification *(4)* of the standard method *(36),* of either *C. jejuni* HPC5 or *C. coli* OR12, depending on which strain had been used to colonize the birds. The plates were then incubated for 24 h at 42°C under microaerobic conditions.

Testing of recovered isolates for resistance to CP220. The development of resistance in *C. jejuni* HPC5 following treatment with CP220 was determined on isolated colonies recovered from the mCCDA plates from the day 5 birds treated with 9 log PFU of CP220. Bacterial lawns were prepared for each isolate as above, and a series of dilutions of phage CP220 were applied in 10-μl volumes to the surface of the lawn. The plates were then incubated for 24 h at 42°C under microaerobic conditions. If no plaques were formed, the isolate was considered to be resistant to CP220.

RESULTS

Characterization of phage CP220. Phage CP220 was identified as a group II phage *(34)* following examination of genomic DNA by PFGE. The genome size by this method was calculated to be 197 kb (Fig. 1A), which was slightly larger than the average size (184 kb) for members of this group of phages. The morphology of phage CP220 was examined by transmission electron microscopy (Fig. 1B). The results revealed that CP220 was typical of the *Myoviridae* family of phages with an icosahedral head and a contractile tail. The size of the CP220 head was approximately 96 nm in diameter, which was typical of a group II phage, and the tail was approximately 110 nm in length by 16 nm in width. Lytic profiling revealed that this phage had a fairly broad host range with 15 of 50 *C. jejuni* isolates from broiler chickens being sensitive to this phage, and three of three *C. coli* strains tested were also found to be sensitive.

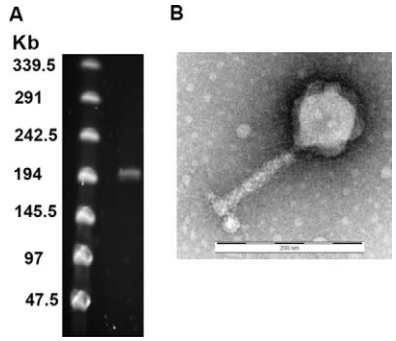

FIGURE 1. *(A) PFGE (pulsed-field gel electrophoresis) of genomic DNA from* Campylobacter *phage CP220 (right) with lambda concatemer marker DNAs (left). (B) Electron micrograph of CP220 phage.*

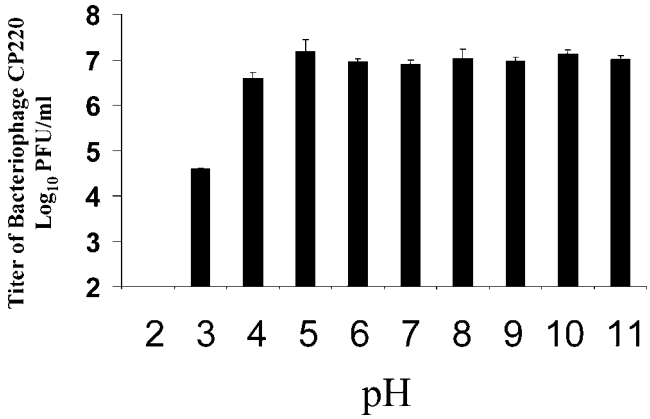

FIGURE 2. *pH tolerance of CP220 following room temperature incubation of 7 log PFU/ml phage at different pHs for 24 h.*

pH stability of CP220. The ability of CP220 to survive in SM buffer adjusted to a variety of pH values between pH 2 and pH 11 was investigated to ensure that CP220 was able to withstand passage through the poultry digestive system. The phage was found to be stable for at least 4 days at all pH values tested between pH 5 and pH 11 (results not shown). At pH 3, however, a decline of 2 log PFU in titer was recorded after 1 day, while at pH 2 no phage was recoverable within 24 h of exposure (Fig. 2). For this reason the phage was suspended in 30% $CaCO_3$ when administered to chickens to ensure protection from the low pH encountered in the proventriculus and gizzard, which is normally between pH 1 and pH 4 *(26)* as previously described *(24).*

Activity of CP220 against *C. jejuni* HPC5 in chickens. The efficacy of CP220 against *C. jejuni* HPC5 was tested by administering 5, 7, or 9 log PFU of CP220 to *C. jejuni* HPC5–colonized chickens ($n = 3$ birds for each dose or control per day) and determining *Campylobacter* counts, each day, over the following 5 days, in three parts of the intestine. Colonization levels of the three parts of the intestine with the HPC5 strain were similar to those previously observed *(24).* Figure 3A through 3C shows the effects of administering the three different doses on the *Campylobacter* count from individual birds, in the three parts of the intestine. Treatment with 7 log PFU of phage CP220 (Table 1) resulted in a significant reduction in mean cecal *Campylobacter* counts, by 2.1 log CFU/g ($P = 0.01$), 24 h following administration, compared to controls. Similar reductions (of between 1.9 and 2.3 log CFU/g) were observed in the upper and lower intestines ($P = 0.004$ and $P = 0.01$, respectively) at 24 h. A significant reduction in *Campylobacter* count compared with controls continued for a further 24 h ($P = 0.001$ for the upper intestine, $P = 0.02$ for the ceca, and $P = 0.02$ for the lower intestine). From 3 days onwards, however, the treatment became less effective, with considerable variation between birds, but the effects of phage treatment were still clearly evident when comparing the phage-treated birds to controls (Fig. 3B). Treatment with a higher dose (9 log PFU) (Fig. 3A) produced similar reductions to those obtained with the 7-log dose 24 h after administration in the upper intestine, ceca, and lower intestine (Table 1). At 48 h the effect was diminished compared with the 7-log dose in all three parts of the intestine. For the lower dose (5 log PFU) (Table 1), days 1 and 5 produced the best reductions in *Campylobacter* count, in the upper intestine, ceca, and lower intestine, but the 5-log PFU dose was less effective than the 7-log PFU dose.

Development of resistance to CP220. An investigation into the development of resistance to CP220 revealed that only 2 (2.1%) of 98 *C. jejuni* HPC5 isolates from the day 5 birds treated with 9 log PFU of CP220 displayed resistance to the phage.

Activity of CP220 against *C. coli* OR12 in chickens. The efficacy of CP220 against *C. coli* OR12 was tested by administering 5, 7, or 9 log PFU of CP220 to *C. coli* OR12–colonized chickens ($n = 3$ birds for each dose or control per day) and determining *Campylobacter* counts, each day, over the following 5 days, in three parts of the intestine (Fig. 3D through 3F). It was immediately apparent that colonization of the upper intestine by *C. coli* OR12 was poor (mean of 3.0 ± 1.6 log CFU/g), with many of the individual bird counts being below the limit of detection (2.0 log CFU/g). This was in contrast to *C. jejuni* HPC5, which efficiently colonized the upper intestine (mean of 6.7 ± 0.5 log CFU/g). It was also apparent from the control birds that the normal mean colonization level of the ceca (at 8.6 ± 0.7 CFU/g) was much higher for this strain than for *C. jejuni* HPC5 (7.4 ± 0.3 CFU/g), although it was similar to previous levels of colonization observed for this strain (7.8 ± 0.7 log CFU/g *(14)*). Treatment with 9 log PFU of phage CP220 (Table 1) resulted in a significant reduction in mean cecal *Campylobacter* counts, by 1.9 log CFU/g ($P = 0.02$), and in a 3.7-log CFU/g reduction in the lower intestine ($P = 0.02$), 48 h following administration, compared to controls. The 7- and 5-log PFU doses (Fig. 3E and 3F) resulted in small or insignificant declines. It was difficult to draw any conclusion with regard to the effects of phage on the upper intestinal counts of *C. coli* OR12 because of the poor colonization of the control birds in this part of the intestine.

Phage counts following administration to *Campylobacter*-colonized chickens. Phage counts determined for

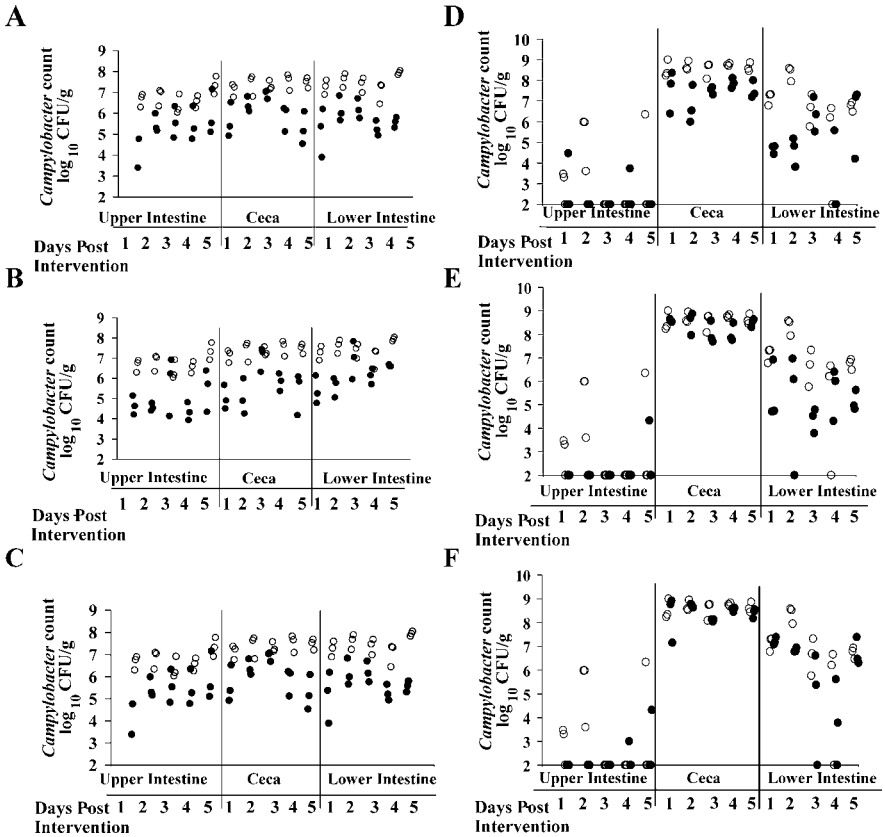

FIGURE 3. *Comparison of the efficacy of CP220 against* C. jejuni *HPC5 and* C. coli *OR12 in three parts of the intestine. Chickens colonized by* C. jejuni *HPC5 or* C. coli *OR12 were treated with either a 9-, 7-, or 5-log PFU dose of CP220.* ○, *control* Campylobacter *counts of intestinal contents;* ●, *phage-treated* Campylobacter *counts (log CFU per gram) for individual birds (*n = *3 for each dose or control per day). (A)* C. jejuni *HPC5 with 9 log PFU CP220; (B)* C. jejuni *HPC5 with 7 log PFU CP220; (C)* C. jejuni *HPC5 with 5 log PFU CP220; (D)* C. coli *OR12 with 9 log PFU CP220; (E)* C. coli *OR12 with 7 log PFU CP220; (F)* C. coli *OR12 with 5 log PFU CP220.*

each section of the intestine for each of the host strains and those for the 7-log PFU dose are shown in Figure 4. The phages were clearly able to colonize and replicate using either of the *Campylobacter* strains tested as host. Irrespective of the initial phage dose administered (the 7-log PFU doses are shown in Figure 4), the phage titers recovered on day 1 were similar, ranging between 4 and 4.8 log PFU/g for the upper intestine, between 4.3 and 5.9 log PFU/g in the ceca, and between 4 and 5.2 log PFU/g for the lower intestine.

DISCUSSION

Phage CP220 was identified as a *Campylobacter* group II phage *(34)* on the basis of its genome size and head diameter and as a member of the family *Myoviridae* based on the morphological characteristics observed by electron microscopy. Although the genome size was slightly larger than the average size of 184 kb for this group, it bore no resemblance to the group I phages *(34),* as their genome size was reported to be 320 kb and can be as large as 425 kb (unpublished observation).

An important difference between CP220 and the group III phages previously used in phage therapy trials of *Campylobacter*-colonized chickens was its ability to lyse *C. coli* strains including *C. coli* OR12 and 30% of typical broiler chicken strains (15 of 50), including some that were not lysed by group III phages. A phage for inclusion in a cocktail for phage therapy would ideally have a broader host range than 30% of isolates, but the fact that it lysed these different *Campylobacter* strains warranted further investigation of the potential of CP220 because it could potentially complement a broad-host-range group III phage to give maximum coverage of the *Campylobacter* population.

Failure to neutralize gastric pH is purported to be one of the major reasons for the failure of early phage therapy trials. Therefore, the stability of CP220 at different pHs was examined by incubation of the phage in SM buffer adjusted between pH 2 and pH 11 and found to be similar to that of other *Campylobacter* phages *(24).* It was determined that CP220 was stable if the pH was above pH 4; so provided it was administered in a form that would protect it from low pH in the proventriculus and gizzard, in this case by suspension in $CaCO_3$, it would be likely to survive long enough to reach target intestinal *Campylobacter* populations.

The ability of phage CP220 to replicate on the *C. jejuni* host HPC5 in chickens resulted in significant reductions in the cecal *Campylobacter* counts with the 7-log PFU dose being most effective. In general phage CP220 was less effective in reducing *C. coli* OR12 populations colonizing chickens but was still able to decrease the *Campylobacter* count by almost 2 log CFU/g in the ceca when an increased dose of 9 log PFU was administered. By being subjected to an increased phage dose, the *C. coli* organisms are likely to be lysed without replication (passive inundation). It is not clear why there should be a difference between the doses required to reduce the *Campylobacter* populations for each species, and possibly between the outcomes of phage administration in terms of active replication or passive inundation. These outcomes may reflect the availability of the host bacteria to the phage that may be different between the species. Evidence for this can be drawn from the poor

TABLE 1. *Decline in average* Campylobacter *counts*[a]

Dose (PFU)	Days after phage treatment	Decline in avg *Campylobacter* counts (log CFU/g) compared with controls (*P* value)		
		Upper intestine	Ceca	Lower intestine
A. *C. jejuni* HPC5 counts in birds phage treated with CP220 compared with HPC5 control				
9 log	1	2.6 (0.02)	1.5 (0.04)	2.1 (0.04)
	2	1.3 (0.02)	1.0 (0.05)	1.2 (0.02)
	3	NSD	NSD	1.2 (0.03)
	4	NSD	1.7 (0.02)	1.8 (0.01)
	5	NSD	2.2 (0.01)	2.4 (0.001)
7 log	1	2.0 (0.004)	2.1 (0.01)	1.9 (0.01)
	2	2.3 (0.001)	2.4 (0.02)	2.0 (0.02)
	3	NSD	NSD	NSD
	4	2.2 (0.0002)	1.7 (0.01)	NSD
	5	1.9 (0.05)	2.1 (0.03)	1.3 (0.0005)
5 log	1	1.7 (0.04)	1.7 (0.005)	1.1 (0.05)
	2	NSD	NSD	NSD
	3	1.3 (0.01)	NSD	1.0 (0.03)
	4	1.9 (0.01)	NSD	NSD
	5	2.0 (0.03)	0.9 (0.03)	1.2 (0.002)
B. *C. coli* OR12 counts in birds phage treated with CP220 compared with OR12 control				
9 log	1	NC	NSD	2.5 (0.0003)
	2	NC	1.9 (0.02)	3.7 (0.02)
	3	NC	1.0 (0.02)	NSD
	4	NC	0.9 (0.003)	NSD
	5	NC	1.1 (0.02)	NSD
7 log	1	NC	NSD	NSD
	2	NC	NSD	NSD
	3	NC	NSD	2.2 (0.01)
	4	NC	0.7 (0.04)	NSD
	5	NC	NSD	1.6 (0.005)
5 log	1	NC	NSD	NSD
	2	NC	NSD	1.5 (0.002)
	3	NC	NSD	NSD
	4	NC	0.2 (0.02)	NSD
	5	NC	NSD	NSD

[a] NSD, no significant difference ($P > 0.05$); NC, not sufficiently colonized to do analysis.

colonization of the upper intestine by the *C. coli* strain, which is likely to be the first encounter of an appropriate metabolically active host *Campylobacter* for the phage administered, via the oral route. Initial rounds of phage replication at this intestinal site could influence the subsequent course of the treatment if the progeny phages have been acclimatized to their host. *C. coli* and *C. jejuni* are rarely enumerated in the upper intestine, making comparison of their colonization levels in this part of the intestine with those of other strains difficult. It is therefore not possible to determine if the poor colonization of the upper intestine by *C. coli* OR12 is widespread within the species or a strain-specific phenomenon.

Phages, like most other predators, seldom eliminate their prey in nature; instead, population numbers rise and fall in a cyclic manner. It is likely that the increase in numbers of *Campylobacter* observed after 3 days is due to such a fluctuation and also contributes to the variability of counts

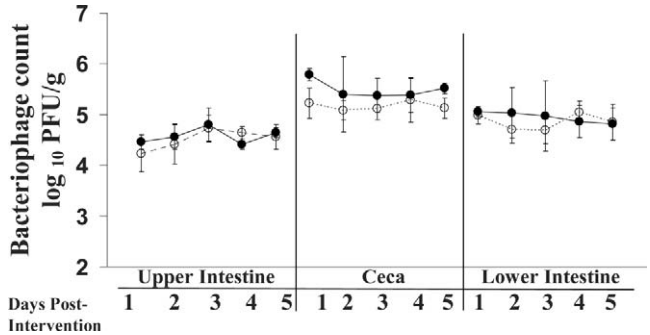

FIGURE 4. *Mean phage counts in chickens colonized with either* C. jejuni *HPC5 or* C. coli *OR12 and treated with 7 log PFU CP220, in three parts of the intestine.* ○, C. jejuni *HPC5 colonized birds;* ●, C. coli *OR12 colonized birds.*

in the individual birds. Administering the phage as a single dose appears to result in a synchronous decline in *Campylobacter* numbers for approximately 48 h.

The 9-log dose of CP220 has the potential to constitute an effective dose for both strains within 24 h as a result of passive inundation of host without time for phage replication (lysis from without). However, the 7-log dose produced similar reductions after 24 h, and in fact the greatest reductions for *C. jejuni* HPC5 were observed with this dose after a 48-h period. This implied that the active replication of phage in the intestine was required to reduce colonization. Empirical determination using different doses and times is therefore, at present, the only way to evaluate the effectiveness of phage treatment, as there is a general lack of understanding of the dynamics of phage replication in vivo as opposed to in vitro, coupled with the number of variables associated with animal experiments. We therefore undertook to extend these empirical observations through investigation of an alternative type of phage (group II as opposed to group III) with a host range that extended to *C. coli* in addition to *C. jejuni.* In practical terms, the fact that replication is clearly required for phage treatment to be effective has advantages and disadvantages. The advantages are that there is no need to match the dose administered to the level of *Campylobacter* colonization in the bird because the phages will replicate according to the numbers of host present and that it is cheaper to administer fewer phage to the birds. A possible disadvantage is that increasing the rounds of replication could result in the development of host resistance. However, our empirical determination did not indicate that this would be a significant problem even after 5 days. We envisage that slaughter of birds 2 days following administration of phages would be the most effective treatment allowing replication while reducing the risk of developing host resistance.

The differentiation of phages into groups based on significantly different physical characteristics such as shape and size indicated the possibility of fundamental differences in their biology and mode of action. The experimental results described here suggest an ability similar to that of group III phages, i.e., to reduce colonization by *Campylobacter,* and this could not have been determined otherwise. The work described therefore increases the number of po-

tential candidates for phage therapy (as previously only group III phages had been evaluated) and the breadth of their coverage in terms of host range. The fact that the results of the experiments presented here closely mirror those of previous experiments *(24, 37)* in terms of the optimum timing, dose required, and the reductions in *Campylobacter* carriage achieved would indicate that there is no advantage or disadvantage to using a group II phage over a group III phage other than its differing host range, which includes *C. coli.* The most likely successful treatments for chickens will probably include a cocktail of different phages that cover a broad range of strains, and it is likely that it would include both groups of phages to achieve maximum strain coverage. Thus, extending the efficacy of phage therapy to group II phages is a useful step forward in the development of phage treatment to reduce *Campylobacter* in chickens.

The selection of resistant bacteria has always been perceived as a potential drawback to phage therapy and has been reported following phage treatment *(40)* but can also be correlated with a reduced ability to compete in the intestinal environment *(24, 37).* It has also been shown that *Campylobacter* may use genomic instability as a means to temporarily avoid phage predation *(38).* Here, as in previous experiments, 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. The data presented here provide further fundamental information necessary if the potential offered by phage therapy as a sustainable biological control measure is to be harnessed for the reduction of *Campylobacter* emanating from farmed poultry sources.

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