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Correlation of *Campylobacter* Bacteriophage with Reduced Presence of Hosts in Broiler Chicken Ceca

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***Campylobacter jejuni* and *Campylobacter*-specific bacteriophage were enumerated from broiler chicken ceca selected from 90 United Kingdom flocks ($n = 205$). *C. jejuni* counts in the presence of bacteriophage (mean $\log_{10}$ 5.1 CFU/g) were associated with a significant ($P < 0.001$) reduction compared to samples with *Campylobacter* alone (mean $\log_{10}$ 6.9 CFU/g).**

Campylobacter jejuni is a major cause of acute bacterial enteritis worldwide. Domestic poultry are a reservoir for campylobacters and undercooked poultry meat, in particular chicken, is by implication an etiological source of human infections (11). One potential mechanism to control *Campylobacter* is the use of host-specific bacteriophage (3, 8). Previous studies have shown that there is a high prevalence of *Campylobacter* in broiler chicken flocks (5, 9, 13), which are able to survive abattoir processing and contaminate retail products (12). Similarly, we and others have shown that *Campylobacter* bacteriophages are present in the chicken intestinal tract (4, 7, 15, 16) and in all probability are the sources of bacteriophages demonstrated to survive processing (2). Although the chicken intestinal tract has been established as a source of *Campylobacter* and their phage, there has not been any attempt to enumerate naturally occurring populations of either in commercial broiler chickens. The aim of the present study was to investigate relationships between populations of *Campylobacter* and their bacteriophage found in the ceca of conventional barn-reared broiler chickens in the United Kingdom.

Commercial Ross broiler chickens ($n = 205$) from 90 flocks were obtained between August and September 2002 from 22 farms belonging to three national poultry producers based in the United Kingdom. The chickens were slaughtered between 22 and 46 days of age (mean 34 days), transported to the laboratory and stored at 4°C prior to dissection. All dissections were performed within 48 h of slaughter. The ceca of each chicken were removed individually, with thorough decontamination between dissections, and stored in sterile petri dishes at 4°C for up to 2 h prior to enumeration of bacteria and phage.

For the enumeration of bacterial populations, cecal contents (1 g) were diluted 1:10 in maximum recovery diluent (CODE CM733; Oxoid, Basingstoke, United Kingdom) and resuspended by pulse vortex mixing for ca. 2 min. This suspension was then serially diluted in maximum recovery diluent prior to spread plating 100-μl volumes of each dilution in duplicate on to the surface of modified charcoal-cefoperazone-deoxycholate agar (CCDA; Lab 112, Lab M, Bury, United Kingdom) with selective supplements (PL450; ProLab Diagnostics, Chester, United Kingdom). Plate count agar (0479-01-1; Difco, Oxford, United Kingdom) was used to enumerate the total aerobic, anaerobic, and microaerobic populations. CCDA plates were incubated under microaerobic conditions (5% H_2, 5% O_2, 10% CO_2, 80% N_2) at 42°C. Plate count agar plates were incubated at 37°C either aerobically, anaerobically, or microaerobically for the respective plates. Enumeration was performed after 24 to 48 h of incubation for all populations. Between four to eight presumptive *Campylobacter* colonies from each of 205 cecal samples were Gram stained and examined under a light microscope to confirm typical morphology prior to biochemical tests for oxidase, catalase, and hippurate hydrolysis. Macrorestriction profiles (SmaI and KpnI) of 124 *Campylobacter* chromosomal DNA were resolved by pulsed-field gel electrophoresis as described by Ribot et al. (14).

For the isolation of *Campylobacter* bacteriophage, cecal contents (1 g) were diluted 1:10 in SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 0.01% gelatin; Sigma, Gillingham, Dorset, United Kingdom) and resuspended by gentle inversion and pulse vortex mixing for up to 5 min. This suspension was then incubated aerobically at 4°C for 24 h on a gyratory platform shaker (60 rpm) to allow phage elution into the buffer. An aliquot (1 ml) of eluate was transferred to a sterile 1.5-ml microcentrifuge tube and subjected to centrifugation at 10, 000 × *g* for 3 min to remove bulk debris then passed through a 0.2-μm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany) to remove any remaining bacterial cells. This filtrate was then serially diluted in SM buffer prior to enumeration on lawns of bacterial cells by using the surface droplet technique (16). Each bacteriophage isolate was subjected to a minimum of three successive rounds of serial plaque purification and propagation prior to lytic spectrum profiling (6). These purified isolates were then screened for their ability to infect *Campylobacter* strains, possessing different phage types, obtained from the National Collection of Type Cultures. Phage isolates with different lytic

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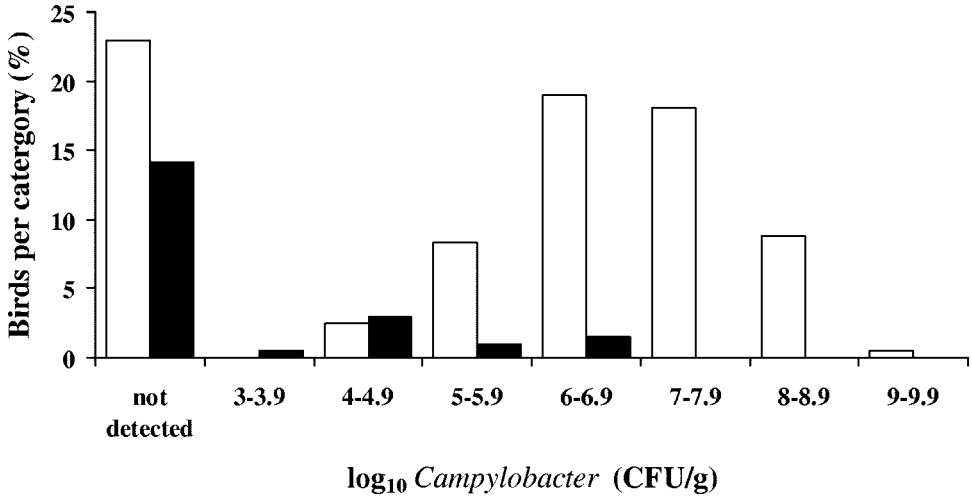

FIG. 1. Distribution of *C. jejuni* enumerated from chicken ceca with (■) and without (□) phage expressed as a percentage of birds ($n = 205$) per category (intervals of 1 $\log_{10}$ CFU/g).

spectra were screened against a further 80 *Campylobacter* strains isolated from human sources and are distinguishable by their sensitivity to the phages used to form the basis of the United Kingdom phage typing scheme (6).

C. jejuni was isolated from 63% (129 of 205) of the ceca of birds tested and lytic bacteriophage of *C. jejuni* in 20% (41 of 205). No *C. coli* isolates were recovered in the present study. The enumeration of campylobacters from chicken ceca in the presence of bacteriophage (mean $\log_{10}$ 5.1 CFU/g) was associated with a significant ($P < 0.001$) reduction in numbers compared to samples with *Campylobacter* alone (mean $\log_{10}$ 6.9 CFU/g). The detection limit was determined as $\log_{10}$ 2.0 *Campylobacter* CFU/g of cecal contents and similarly for bacteriophage $\log_{10}$ 2.0 PFU/g of cecal contents. The calculation of mean colonization values excluded samples where *Campylobacter* was not detected. The *Campylobacter*-positive ceca were ranked according to their colonization titer recorded as the CFU/gram of cecal contents (Fig. 1). In the absence of phage, the colonization levels could be fitted to a normal distribution with a median of $\log_{10}$ 6.9 CFU/g of cecal contents. Whereas when bacteriophages were present in conjunction with campylobacters, a reduction in titer of ca. 2 logs was observed. It was notable that viable *Campylobacter* cells could not be recovered from 29 of 41 (71%) of the phage-positive chicken ceca by using conventional selective plating techniques. This may be compared to 47 of 164 (29%) of the phage-negative ceca. The difference between these incidences may also be due to the influence of the phage populations acting to reduce the cecal host colonization levels below the limit of detection and possibly during experimental recovery. To investigate the possibility of other bacterial populations affecting the colonization of *Campylobacter* or the presence of bacteriophage, the total populations of aerobic, anaerobic, and microaerobic bacteria in the broiler chicken cecal contents were determined. No discernible differences evident in the total aerobic counts which remained constant (mean count of $\log_{10}$ 7.4 ± 0.6 CFU/g of cecal contents). The microaerobic and anaerobic counts determined were more variable with mean counts of $\log_{10}$ 7.5 ± 2.4 and $\log_{10}$ 8.9 ± 2.1 CFU/g of cecal contents, respectively, but no significant differences were distinguishable between any of the subgroups.

Macrorestriction profiling of six individual *Campylobacter* colonies from each of 12 phage-positive and 12 negative ceca was carried out. This analysis produced 19 unique profiles from 144 single-colony isolates. None of the profiles showed any significant association with either the phage-positive or -negative groups. However, it was notable that the ceca of the 5 of 12 phage-positive birds contained two or more *Campylobacter* types compared to 2 of 12 in which the phage were absent. The presence of phage in chicken ceca is likely to impose a considerable selective pressure on their hosts. The demise of *Campylobacter* strains sensitive to infection creates a niche for either phage-resistant subpopulations or new strains from the environment. This may ultimately result in a greater diversity of campylobacters in chickens which harbor phage. Recent studies have demonstrated the succession of phage-insensitive *Campylobacter* types within broiler flocks that naturally harbor phage (4).

A wide range of bacteriophage titers ($\log_{10}$ 1.5 to $\log_{10}$ 6.9 PFU/g of cecal contents) were recovered from chicken ceca. The range in titers probably reflects the availability of hosts due to the dynamic nature of *Campylobacter* populations in the chicken gut. Previous studies with *Salmonella enterica* serovar Typhi and *Escherichia coli* have shown that phage seldom eliminate their prey (1). Often in these cases, the numbers of predator and prey increase and decline with characteristic out-of-phase population oscillations (10, 18). Providing such interactions occur between phage and *Campylobacter* in the chicken gut, the numbers of phage recovered could vary according to the stage of the oscillatory cycle at which the sample was collected. The lytic spectra of the broiler phage isolates could be organized into nine lytic classes by using the phage typing scheme adopted in the United Kingdom (6). The assignment of phage to each lytic class was based on whether they are able to infect each of 80 *Campylobacter* type strains recovered from patients suffering from campylobacteriosis (6). The number of *Campylobacter* type strains infected by each phage lytic class varied considerably from 26 of 80 to 52 of 80.

Of the 41 birds from which bacteriophage were isolated, only 12 had recoverable campylobacters, and of these only four birds yielded sensitive *Campylobacter* hosts capable of propagating phage from the same source. The apparent absence of the host could have arisen due to the isolation procedures adopted, for example, if they were biased toward the recovery of certain phage and/or campylobacters, or be due to changes in host susceptibility that arose upon subculture. However, the oscillations in the predator and prey could also explain the observation if not all of the campylobacters present were sensitive to the phage. Depending on the amplitude of the predator and prey oscillation, the phage-sensitive hosts may become a minor component of the total *Campylobacter* population and present a lower stochastic chance of recovery from birds that are cocolonized. Given this advantage, an insensitive *Campylobacter* could displace the original host, resulting in the succession of a new population or of a subpopulation of the original in which phage resistance had developed. All other fitness characteristics being equal, the phage-resistant population could colonize the chicken gut with relative impunity. However, previous studies have shown phage-resistant mutants of *E. coli* to be less-virulent and less-efficient colonizers than the wild type (17). This may in part explain why fewer campylobacters were isolated from ceca also containing phage. For those birds harboring *Campylobacter*-specific phage but from which *Campylobacter* could not be recovered, explanations based on simple predator-prey relationships cannot easily be applied. This behavior could be a consequence of alternative bacterial flora competing for the same niche without the encumbrance of phage infection. If this was the case, it was not evident in the gross populations since the total aerobic, anaerobic, or microaerobic counts determined from *Campylobacter*-negative, phage-positive birds showed no significant differences compared to those determined without phage. However, it is possible the relative proportions of the bacterial species could change without altering the total counts.

Understanding the dynamics of the interaction between bacteriophage and their hosts in vivo is necessary if bacteriophages are to be used to reduce the incidence of pathogens in animal reservoirs. The results of the present study suggest that phage may affect the numbers of *C. jejuni* in commercial broiler chickens to various degrees, in that we demonstrate a correlation between the presence of natural environmental phage and a reduction in the numbers of *Campylobacter* colonizing broiler chicken ceca. These observational data are encouraging in that they provide evidence that phage-mediated control of *Campylobacter* in poultry could be utilized under commercial operating conditions. However, further studies are required to understand the interaction of *Campylobacter*-phage and their hosts in chicken poultry production if practical intervention measures are to be established.

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