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Prevalence and numbers of coliphages and *Campylobacter jejuni* bacteriophages in New Zealand foods

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Abstract

Vegetable samples were tested for the presence of coliphages. None of the 55 samples contained these phages at concentrations greater than 10 g^{-1} (the limit of detection). Spiking and recovery experiments indicated that the method was efficient at detecting coliphage T4 added to the food, and so it was concluded that phage titres were not being falsely underestimated. In addition 51 samples of chicken skin from retail portions were tested for the presence and numbers of coliphages and for presence only of *Campylobacter jejuni* phages. Coliphages were isolated from 46 samples (90.2% positive), at up to 2570 PFU 10 g sample^{-1} but no *C. jejuni* phages were isolated. Several other methods were used to isolate *C. jejuni* phages from retail chicken but none was successful. However, when pooled whole chicken rinses from 39 flocks were tested for the presence of *C. jejuni* phages, 11 (28.2%) of the flocks were positive. It is possible that phages present on birds at the start of processing were either inactivated or simply diluted out during spin chilling. These data add to the body of information indicating that phages can readily be isolated from certain foods and indicate that consumers are exposed to them on a regular basis.

Keywords: Bacteriophage; Vegetables; Chicken; *Campylobacter*

1. Introduction

The potential use of bacteriophages (phages) to control bacteria of importance to food quality and safety has been the subject of recent reviews (Greer, 2005; Hudson et al., 2005) and a number of examples of such applications have been published (Atterbury et al., 2003a; Carlton et al., 2005; Greer and Dilts, 2002; Leverentz et al., 2003). Potential pitfalls in the use of phages to control bacteria on foods include limited host range (Greer and Dilts, 1990), a possible minimum host density requirement for phage replication (Ellis et al., 1973) or the inhibitory effect of non-target bacteria (Wilkinson, 2001). Consumers may also question their use on food as safe practice. Phage properties relevant to their safe use, such as lack of allergenicity and carriage of toxin genes have been well documented for at least one phage (Carlton et al., 2005) but further understanding of the distribution of naturally-occurring phages in foods may assist in better assessing risk. Coliphages have been detected in many foods including fresh chicken, pork, ground beef, mushrooms, lettuce, other raw vegetables, chicken pie and delicatessen foods (Allwood et al., 2004; Kennedy et al., 1984, 1986a), in some samples at numbers exceeding 10^4 10 g^{-1}. In other cases phages infecting particular host bacteria have been detected in foods likely to contain the host, for example *Campylobacter* phages in raw chicken (Atterbury et al., 2003b), phages infecting *Propionibacterium freudenreichii* in Swiss cheese (Gautier et al., 1995) and *Brocothrix thermosphacta* phages in beef (Greer, 1983).

No data regarding the presence of phages in foods are available for New Zealand, and so the work described here sought to obtain quantitative data on coliphages in vegetables and chicken skin, and prevalence data for *Campylobacter jejuni* phages in poultry.

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2. Materials and methods

2.1. Reference cultures and phage stocks

Phage T4 was kindly supplied by the University of Canterbury. *C. jejuni* host isolates NCTC 12662, NCTC 12664, and phage 12672 were purchased from the Health Protection Agency, Colindale, London. *Escherichia coli* strain C3000 was a gift from Dr. Lester Sinton (ESR Water Management Programme). Local isolates of *C. jejuni* from lamb's fry, chicken faeces and a clinical case were also used as hosts for the isolation of *Campylobacter* phages.

2.2. Isolation and recovery of coliphages from vegetables and chicken skin

Fifty-five organic, conventional and home-grown vegetable samples (asparagus, mung bean sprouts, spinach, watercress and silverbeet) were selected from 11 shops and three home gardens in the Christchurch area. In addition 51 fresh chicken samples were bought from sixteen supermarkets in Christchurch to investigate the distribution of coliphages. All samples were stored at 4 °C until processed.

The methods used for the detection of coliphages were essentially those of Kennedy et al. (1986b). For vegetables, 50 g of each sample was chopped and weighed aseptically into sterile barrier filter stomacher bags (Interscience, St. Nom, France), 50 ml chilled EC broth (Difco, Detroit, MI, USA) added and the sample stomached for 2 min. A 30 ml sub-sample of stomachate was centrifuged at 3000×*g* for 5 min, and a volume (25 ml) of the supernatant was filtered through a 0.22 μm filter (Millex, Millipore, County Cork, Ireland) if necessary. For each chicken skin sample, 10 g was stomached with 90 ml chilled EC broth for 2 min in a barrier bag, a 40 ml sub-sample of the stomachate centrifuged at 1615×*g* for 10 min and 20 ml of supernatant filter-sterilised prior to storage under refrigeration in the dark. All samples were tested for the presence of coliphages using the agar overlay technique (Carlson, 2005). Sample suspension (2.0 ml) was mixed with 0.2 ml log phase *E. coli* C3000 host culture and 4.0 ml molten agar. This was then poured onto five EC agar plates per sample and incubated at 37 °C for 16 to 20 h.

To assess the method's efficiency of recovery, several samples were prepared in duplicate and spiked with approximately 10^3 plaque-forming units (PFU) of phage T4 diluted in Suspension Medium (SM) buffer (Carey-Smith et al., 2006) prior to enumeration as described above.

2.3. Isolation of C. jejuni phages

For whole birds, five exsanguinated chickens from a single flock each week were processed by the following method for 39 weeks. A string was tied around the neck and the cloaca plugged with a tampon (Libra, Springvale, Australia) to prevent the leakage of internal contents. A volume (600 ml) of Buffered Peptone Water (BPW, Oxoid, Basingstoke, UK) was added to the chicken in a bag and the contents massaged by hand through the bag, followed by continuous agitation for 5 min. The rinse was drained into a stomacher bag and stomached for 1 min. A volume (100 ml) of rinse was transferred to a sterile bottle and centrifuged at 5000×*g* for 8 min at 4 °C. The supernatants from 5 carcasses were pooled, 1 ml filter sterilised and stored at −20 °C if not tested immediately. A volume (100 μl) of supernatant and 400 μl host culture (NCTC 12664 log phase) were added to 4 ml molten Nutrient Broth no. 2 (Oxoid) overlay (with 0.01 M $MgSO_4$ and 0.001 M $CaCl_2$ added). This mixture was stored molten for 20 min prior to pouring onto a base plate prior to incubation at 42 °C. All *Campylobacter* plates were incubated in a 10% CO_2 incubator (Fraser et al., 1992).

To isolate phages from fresh chicken skin, the skin was aseptically removed and stomached in EC broth for 2 min. The stomachate was centrifuged at 1615×*g* for 10 min, and the supernatant was stored in the dark at 4 °C. A 2 ml volume of supernatant was used to inoculate overlays as described above.

2.4. Additional methods used to detect C. jejuni phages in chicken meat and offal

Five different approaches were used for the recovery of *C. jejuni* phages from 33 samples of chicken meat and offal. In most cases two or three locally isolated hosts were used for phage detection. Firstly, a direct isolation using a previously described method was attempted (Grajewski et al., 1985) on 29 samples. Briefly, a 1 g sample was mixed in 9 ml Brucella Broth (Difco) plus $MgSO_4$ and $CaCl_2$, the sample mixed, incubated at 4 °C for 45 min., filtered through a 0.45 μm filter and centrifuged at 2000 ×*g* for 25 min. The filtrate from a second filtration step (0.22 μm pore-size) was applied in 10 μl volumes to agar overlays which were incubated at 42 °C for 18–24 h prior to examination for plaques.

In a second approach 28 samples were tested by enrichment with no inoculum. Sample (1 g) was mixed with 9 ml of modified Exeter Broth plus $MgSO_4$ and $CaCl_2$, and incubated at 37 °C for 24 h. The enrichment was then centrifuged at 2000×*g* for 5 min and filtered (0.22 μm pore-size). A volume (1 ml) of the filtrate was mixed with 1 ml of host culture and left at 37 °C for phage to adsorb for 15 min. One millilitre of this was added to 3 ml of soft-overlay and poured onto the base plate. After incubation the plates were examined for plaque formation.

The last three approaches applied to a total of 42 samples involved enriching with either hosts isolated from the sample, local *C. jejuni* isolates or host NCTC 12662. Enrichments were prepared as described above with 100 μl of exponential phase host inoculated into the sample before incubation at 37 °C for 24 h. It was then either treated by adding 100 μl of chloroform, mixing briefly, incubating at room temperature for 20 min and then centrifuging at 2000×*g* for 5 min or centrifuging at 2000×*g* for 5 min and then filtering (0.22 μm pore-size). Testing for plaques was by spotting onto a lawn or agar overlay as described above.

2.5. Recovery of spiked C. jejuni phages from poultry products

To determine the limit of detection in fresh chicken products, 10 g portions of liver, stir fry, and minced meat were spiked with

100 µl of *C. jejuni* phage NCTC 12672 diluted in SM. The two inoculum levels used were 5.5×10^2 pfu g^{-1} and 5.5 pfu g^{-1}. The spiked samples were left at room temperature for 30 min before extraction with 10 ml SM and direct isolation. The filtrate was assayed using the overlay method.

Additionally, three groups of triplicate samples of chicken liver were inoculated with 3.6×10^2 PFU g^{-1}, 44 PFU g^{-1}, and 6.5 PFU g^{-1} of the same phage and left at room temperature for 1 h before resuspension in 10 ml SM. All samples were also tested for phages that may already have been present.

Stomached chicken skin samples were tested in the same way as for the recovery of coliphages. Four samples were inoculated with 3.2×10^3 PFU of the phage, stomached in EC broth and centrifuged as described above. A volume (2 ml) of the supernatant and 400 µl of exponential host culture (NCTC 12664) were added to 4 ml Nutrient No. 2 agar overlay containing 0.01 M $MgSO_4$ and 0.001 M $CaCl_2$, phage was allowed to adsorb for 20 min, and five overlay plates were then poured per sample and incubated.

3. Results and discussion

3.1. Prevalence in, and recovery of, coliphages from vegetables

Vegetable samples were only tested for the presence of coliphages as previous work demonstrated the presence of *E. coli* in 14% of commercial hydroponically grown leafy vegetables and 12.8% of seed sprout samples tested in New Zealand (Graham and Dawson, 2002), while *C. jejuni* was not isolated. Other studies have also reported that *E. coli* can be isolated from a variety of vegetables at prevalences up to, for example, 16.8% in field-grown produce (Mukherjee et al., 2005) and 20% in fresh lettuce (Kennedy et al., 1986a). It was therefore reasonable to assume that a significant proportion of the vegetables would have been contaminated by this organism.

We were unable to detect coliphages in any of the samples tested (0%, 95% CI 0–6.5%). However, in the five samples spiked with 10^3 PFU T4, the mean recovery was 79% (asparagus 104%, mung bean sprouts 83%, spinach 21%, watercress 74% and silverbeet 116%), with values greater than 100% reflecting the experimental effects of sampling and enumeration. The results for the unspiked samples are therefore unlikely to be due to methodological problems since phage T4 added to vegetable samples was recovered efficiently by the method. These data contrast with those for retail vegetables tested in the USA, where F-specific RNA coliphages could be isolated from various vegetables even in the absence of *E. coli* (Allwood et al., 2004). Host C3000 should detect both somatic and F+ phages (Harwood et al., 2005). In another American study lettuce was shown to be one of the raw foods which contained phages infecting *E. coli* C3000 the least frequently (Kennedy et al., 1986a). In ground beef and chicken F-specific RNA phages were generally detected less frequently and at lower numbers than somatic coliphages tested on *E. coli* C (Hsu et al., 2002). Another possible reason for differences between these studies is the method used to elute phages from the foods, but the method followed here was essentially the same as that used by Kennedy et al. (1986a).

3.2. Prevalence and recovery of coliphages in retail chicken skin

Of the 51 samples of chicken skin tested, 46 (90.2%, 95% CI 78.6–96.7%) contained coliphages. The distribution of counts obtained from the samples is shown in Fig. 1. Those samples recorded as <10 PFU 10 g^{-1} may have still contained phages at levels beneath the limit of detection. Most counts fell in the range of 10–99 PFU 10 g^{-1} sample. Previous results have also shown chicken skin to contain coliphages at a high prevalence (Kennedy et al., 1986a). The highest count obtained by us was 2570 PFU 10 g^{-1} sample, which is around an order of magnitude lower than the maximum reported by Kennedy et al. (1986a), and somewhat higher than that in another study using *E. coli* C as the host (Hsu et al., 2002).

The recovery rate of phage T4 added to five fresh chicken samples was 136% (range 87% to 157%), possibly as the result of some replication occurring during processing of the samples or variability in the counting methodology.

3.3. Prevalence of C. jejuni phages in chicken skin and whole chickens

Unlike a similar UK survey (Atterbury et al., 2003b) no sample of retail chicken skin in the initial survey was found to contain *C. jejuni* phages capable of infecting host NCTC 12664 (0% positive, 95% CI 0–7.1%). In contrast, and again using host NCTC 12664, 11 of 39 (28.2%, 95% CI 15.0–44.9%) pooled whole bird rinses tested yielded *C. jejuni* phages. Given that the same host strain was used for the isolation of phages in both chicken samples, it is of interest that phages were isolated only from whole birds and not from samples of chicken skin purchased at retail level (although it is to be noted that the isolation methods were not identical). It seems likely that broiler processing may either inactivate or reduce the numbers of *C. jejuni* phages to the point where they are no longer detectable at a high prevalence (the sample size used, $n=51$, gives a 95% probability of detecting *C. jejuni* phages on chicken skin if 6% of chicken skin samples contain phages).

It is possible that the processing of chickens differs between the UK and New Zealand, as in the UK chicken for the fresh meat market is "generally" air-dried (Hartnett, 2001), whereas in New Zealand spin chilling is used for all chicken. The extra dilution effect of the spin chiller may remove most *C. jejuni* phages from retail chicken. A general reduction in numbers of

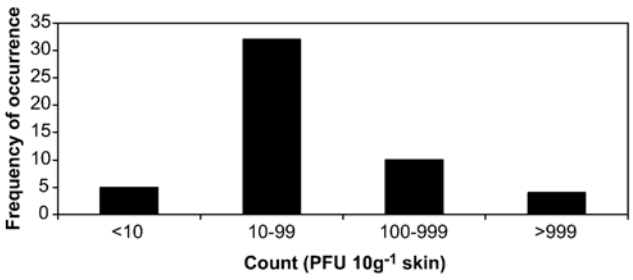

Fig. 1. Distribution of counts of phages infecting *E. coli* C3000 on chicken skin sampled at retail.

Table 1
Recovery of *C. jejuni* phages added to chicken products

Food type	Number added	mean % recovery (standard deviation)
Minced chicken [a]	5.5×10^2	12.7 (1.8)
	5.5	Detected*
Diced chicken [a]	5.5×10^2	5.5 (3.6)
	5.5	Not detected
Chicken liver [b]	3.6×10^2	44.4 (5.3)
	44	12.6 (13.0)
	6.5	Detected*
Chicken skin [c]	3.2×10^3	105.6 (18.9)

*The small number of phages added and recovered preclude calculation of statistics.
[a] Single samples tested in triplicate.
[b] Mean of two samples enumerated in triplicate.
[c] Mean of four replicates.

F-specific RNA and somatic coliphages has been shown at various points along chicken processing in the USA (Hsu et al., 2002).

3.4. Recovery of spiked C. jejuni phages from chicken products

The recovery of phages inoculated onto fresh chicken products was calculated from spiking the products with phage at different concentrations (Table 1). These extraction methods could reliably detect less than 3.0×10^2 PFU g^{-1} and phages could still be detected on most occasions when the inoculum was even lower than this. Given the small number of experiments it is not possible to comment on differences in recoveries between foods or at different levels of inoculum.

For chicken skin, the phages were recoverable from the skin at numbers indicating a slightly greater than 100% recovery. *C. jejuni* phage replication was unlikely during processing as the minimum growth temperature for the organism is 30 °C and so the slight increase in numbers is most likely to be due to uncertainties surrounding counting.

3.5. Additional experiments to isolate C. jejuni phages from retail chicken

As discussed above, a possible reason for the difference in the ability to detect *C. jejuni* phages in retail chicken may have been due to the methods used. The methods applied to whole birds and retail chicken were different because they were considered to be the best for the sample being tested, mainly on the grounds of the size of the sample. To examine the possibility that the differences observed reflected methodological differences, 33 chicken meat and offal samples were tested by up to five methods so that a total of 89 sample/method combinations were examined. Despite the fact that enrichments were used with different hosts to amplify any phages present, it was still not possible to isolate *C. jejuni* phages from the samples. Given this information and that the recovery of *C. jejuni* phages from foods was reasonably efficient, it was concluded that the result was unlikely to be a methodological artefact.

3.6. Conclusions

Chicken meat samples were free of *C. jejuni* phages, which was a surprising result since the host bacterium can readily be isolated from chicken both in New Zealand (Hudson et al., 1999) and overseas (e.g. Shih, 2000). While there may be good reasons for these observations, the data suggest that using phages as an indicator of the presence of the host bacterium would not, in this case, be useful. It could be argued that phages do not adhere to foods in the same way as the host does, and so may be differently removed during processing. This is supported by the slightly greater than 100% recovery of phages added to chicken skin samples as it indicates that a very small proportion, if any, of the added phages irreversibly adhered to the chicken skin.

We have shown that coliphages are commonly present on chicken skin, while they could not be detected on vegetables. This difference may be due to the number of host present; Kennedy et al. (1986a) measured a mean *E. coli* concentration of 3.32 $\log_{10}$ in 100 g samples, while for lettuce the mean was 0.70 $\log_{10}$. The fact that phages could be readily isolated from some foods in New Zealand is consistent with the overseas data, and this information should be considered when assessing safety aspects of the application of phages to foods.

When attempting to isolate phages from foods, consideration needs to be given to the likely prevalence and concentration of the host bacterium as well as any processing steps that may remove any phages present.

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