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Competitive Exclusion of Heterologous *Campylobacter* spp. in Chicks

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Chicken and human isolates of *Campylobacter jejuni* were used to provide oral challenge of day-old broiler chicks. The isolation ratio of the competing challenge strains was monitored and varied, depending upon the isolates used. A PCR-restriction fragment length polymorphism assay of the flagellin gene (*flaA*) was used to discriminate between the chick-colonizing isolates. Our observations indicated that the selected *C. jejuni* colonizers dominated the niche provided by the chicken ceca. Chicken isolates from the *flaA* type 7 grouping generally had numerical superiority over the human isolates when they were administered in our 1-day-old chick model. Our results suggest that it is possible to use combinations of *C. jejuni* chicken isolates as a defined bacterial preparation for the competitive exclusion of human-pathogenic *C. jejuni* in poultry.

Campylobacter jejuni is one of the most common causes of bacterial gastroenteritis in the United States and worldwide (7, 20, 27), with an estimated annual incidence in excess of 2 million cases in the United States. Infection is usually foodborne, and poultry consumption has been identified as a significant risk factor (1, 6, 28), while other vehicles, including milk (8, 9), water (18, 29), and contact with pets and farm animals (3), have also been reported. Poultry can become colonized with *Campylobacter* at various stages of production by endogenous or exogenous sources (21, 23).

Preventing contamination of poultry by *C. jejuni* has proven to be a difficult task. *Campylobacter jejuni* occupies an intestinal niche distinguished from *Salmonella*, primarily swimming freely in the mucin layer covering the epithelia within the intestinal tract crypts (2). Typically, chicks do not become colonized before the age of 2 to 3 weeks (25), yet maternal antibodies are not protective against colonization (13), and young chicks can be colonized by low doses of the organism (4, 24). Competitive exclusion (CE) was first described by Nurmi and Rantala (16) and has potential to control pathogens in poultry. By prefeeding nonpathogenic microorganisms to the birds, the ecological niche may become occupied by antagonistic bacteria. Feeding undefined intestinal flora or defined *Escherichia coli* to chicks induces a 50% reduction of *Campylobacter* colonization (12, 19). Stern et al. (23, 24) reported that standard CE preparations, effective against *Salmonella*, are not consistently effective against chicken colonization by *C. jejuni*. An alternative approach using genetically modified *Campylobacter* mutants as a probiotic to control the colonization of *C. jejuni* in chickens has been proposed (30).

The objective of this study was to determine whether *C. jejuni* strains originating from chickens could outcompete human isolates in the colonization of 1-day-old chicks. To assess the presence of the various challenge strains, we characterized the flagellin gene of *C. jejuni* (14, 15, 17). A PCR-restriction fragment length polymorphism (RFLP) typing technique was used to monitor colonization progress of the *C. jejuni* strains, and we have provided further characterization of the colonizers by gene sequencing a portion of the flagellin gene (11).

MATERIALS AND METHODS

Bacterial strains and their cultivation. Human *Campylobacter* strains were obtained from V. Korolik (Royal Melbourne Institute of Technology, Melbourne, Australia) and from I. Nachamkin (University of Pennsylvania, Philadelphia) Chicken strains were isolated from cecal and fecal droppings collected from commercial flocks and from carcass rinses. All isolates were grown on Campy-Cefex agar at 42°C for 24 h in a microaerobic atmosphere (10% CO_2, 5% O_2, and 85% N_2) (10). The *C. jejuni* strains used were identified by typical colony formation on selective agar and microscopic examination for typical morphology and motility and were serologically confirmed [Meritec Campy (jcl) *Campylobacter* Culture Confirmation test kit; Meridian Diagnostics, Inc., Cincinnati, Ohio].

Flagellin gene typing. Flagellin gene typing was performed according to the method of Nachamkin et al. (14). The chromosomal DNA from the isolates was extracted and subjected to the PCR method described by Nachamkin et al. (14). A single pair of forward and reverse primers, FLA4F and FLA1728R, were used to generate a 1.7-kb PCR product. PCR was performed with a DNA Thermal Cycler (Perkin-Elmer Cetus, Branchburg, N.J.) under the following cycling conditions: 94°C for 1 min and then 94°C for 15 s, 55°C for 45 s, and 72°C for 1 min and 45 s for 35 cycles. After the last cycle, the sample was held at 72°C for 5 min. The PCR mixture was checked for the presence of the expected 1.7-kb amplicon representing the *Campylobacter* flagellin gene by electrophoresis on a 1% agarose gel.

RFLP analysis. After a pronounced band was obtained following electrophoresis, the PCR product was digested with 0.375 μl of the restriction enzyme *Dde*I (20,000 U/m; New England Biolabs, Inc., Beverly, Mass.) and analyzed by agarose gel electrophoresis with 4% Nusieve GTG agarose (FMC Bioproducts, Rockland, Maine). A 123-bp DNA ladder (Gibco BRL, Gaithersburg, Md.) was used as the molecular size standard. The gels were examined with a UV transilluminator and photographed with Polaroid type 55 high-speed 4-by-5 sheet film.

Image analysis. Computer image analysis was performed with Pro-RFLP Macintosh version 2 (DNA ProScan, Inc., Nashville, Tenn.). The digested DNA band images were electronically digitized and adjusted for contrast. The data were analyzed with the Pro-RFLP software program and processed according to the manufacturer's instructions. Each pattern was saved and compared with existing patterns (*Campylobacter* RFLP Database, version 1.0, 1994; Trustees of the University of Pennsylvania). Pattern comparisons were performed at the 5% stringency level, with one-to-one matching; i.e., the pattern being searched had to have the same number of bands, and each band had to be within 5% of the band size stored in the database.

Chick challenge test. Challenge isolates were harvested following growth on Campy-Cefex agar at 42°C for 18 to 24 h in the microaerobic atmosphere

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specified above. Cultures were suspended in phosphate-buffered saline (PBS), and suspensions were enumerated on Campy-Cefex agar. Each 1-day-old chick (10 per trial) was gavaged with 0.1 ml of the specified bacterial suspension. Chicks were sacrificed 7 days postchallenge, and ceca were aseptically removed before being diluted 1:3 (wt/vol) in PBS. Serial dilutions were plated onto Campy-Cefex agar and plated as previously described to determine the *C. jejuni* colonization level.

Colonization competition study. Chicken or human isolates which readily colonized chicks were mixed in a 1:1 ratio. Each 1-day-old chick (total of 6 birds for each cell mixture) was challenged orally with 0.2 ml of the cell mixture. Chicken ceca were dissected 7 days later, and the number of *C. jejuni* was counted as described above. Ten colonies on plates with a total number of colonies less than 100 (one plate from each cecum sample) were enumerated separately, and their flagellin gene types were identified by PCR-RFLP analysis (15), as described above. A positive control test was performed by challenging six 1-day-old broiler chicks with individual colonizing isolates of chicken or human origin. Chicken ceca were dissected 7 days after challenge, and the number of *C. jejuni* cells counted ensured that each isolate readily colonized the intestinal tracts of the chicks.

Protective efficacy study. Six 1-day-old broiler chicks were challenged with selected chicken isolates. The birds were then challenged with the selected human isolates 3 to 5 days later. Ten colonies per plate, containing less than 100 colonies, with one plate from each cecum sample, were enumerated separately, and their flagellin gene types were identified by PCR-RFLP analysis. Positive control tests were done by challenging six 1-day-old broiler chicks with chicken or human isolates only. Chicken ceca were dissected 3 to 5 days later, after the human isolates were challenged. The number of *C. jejuni* cells was enumerated as described above to ensure that chicks were capable of being colonized by each isolate.

Statistical analysis. The isolation ratio of chicken isolates used in colonization competition study and in the protective efficacy study was analyzed by analysis of variance ($P < 0.05$), by using the Statistical Analysis System (SAS) (22).

SVR sequencing. Twelve PCR products generated as described by Nachamkin et al. (14) were used as DNA templates. A single pair of forward and reverse primers, FLA242FU and FLA625RU, were used to generate a short variable region (SVR) from the *flaA* sequence (11). Sequencing of the DNA templates was performed with sequence data gathered with an ABI 373a automated DNA sequencer (ABI-Perkin Elmer, Foster City, Calif.), and the sequences were compiled into contiguous fragments with the AssemblyLIGN program (Intelligenetics Division, Oxford Molecular Group, Campbell, Calif.). Sequences were aligned by using the Pileup program in the GCG (Genetics Computer Group) suite (5), and Plotsim was used to generate similarity scores. Aligned sequences were compared, and dendrograms were generated by phylogenetic analysis with parsimony (PAUP), version 3.1 (26).

RESULTS

In our study, a pair of universal primers was used to amplify a 1.7-kb amplicon of the *C. jejuni* flagellin gene (*flaA*). The PCR products were then digested with *Dde*I enzyme to generate various *flaA* RFLP profiles for the discrimination of *C. jejuni* strains from different sources. A total of 57 *C. jejuni* strains (21 from chickens and 36 from humans) were analyzed for their respective *flaA* types.

Chick challenge test. The ability of *C. jejuni* strains to colonize chicks varied among the strains possessing the same *flaA* type. Tables 1 and 2 show the colonization efficiency of *C. jejuni* strains isolated from humans and chickens, respectively, in 1-day-old broiler chicks. For the human isolates tested, the chick-colonizing strains had a limited relationship to *flaA* type. Of particular interest were strains within the *flaA*-21 type, which was the fifth-most-frequently-isolated *flaA* type by Nachamkin et al. (15). This *flaA* type from humans did not colonize 1-day-old broiler chicks. Several superior colonizing human isolates, AU423 (*flaA*-1), UP4 (*flaA*-1), AU886 (*flaA*-15), and UP1 (*flaA*-27), were chosen for further chicken challenge studies.

The most important factor influencing chicken isolate colonization may have been the source of the bacterial strains. Strains isolated from ceca or cecal droppings, e.g., W92.4ENR (*flaA*-7) and CE05 (*flaA*-59), were good colonizers; whereas those isolated from broiler wash, e.g., P108 (*flaA*-1) and P74 (*flaA*-13), were poor colonizers. Only those strains with good colonizing ability, e.g., CE05, CE09, W92.4ENR, and P62, were used for further competitive exclusion testing.

TABLE 1. Chick colonization by *C. jejuni* isolates of human origin[a]

FlaA type	Bacterial strain	Challenge dose (CFU)	No. of chicks colonized/ 10 challenged
1	AU331	3.2×10^6	10
	AU351	3.1×10^6	0
	AU423[b]	5.0×10^5	10
	AU933	1.5×10^6	10
	UP4[b]	1.2×10^5	9
	84-194	5.3×10^6	0
2	UP9	3.1×10^6	9
7	AU108[b]	7.2×10^5	9
	AU860	7.5×10^5	5
	UP13	1.4×10^6	9
	UP17	6.5×10^5	10
	UP18	1.0×10^5	3
10	AU8001	3.2×10^6	6
	AU904	4.9×10^5	1
15	AU007	9.7×10^5	8
	AU311	8.0×10^5	5
	AU413	5.0×10^5	1
	AU886[b]	3.1×10^5	10
	AU913	3.0×10^5	0
	UP15	2.7×10^5	9
20	UP19	1.3×10^5	0
21	D3083	4.0×10^6	0
	D3141	2.1×10^6	0
	3145	6.2×10^6	0
	D3468	5.3×10^6	0
23	UP5	1.4×10^5	10
27	UP1[b]	1.5×10^6	10
	UP8	2.2×10^5	2
33	AU464	9.0×10^5	4
40	AU315	5.8×10^5	0
40	UP7	1.7×10^6	8
	UP25	4.6×10^5	8
	UP27	5.0×10^7	10
48	UP11	1.8×10^6	10
	UP26	1.7×10^6	10
49	UP16	2.1×10^6	0

[a] Each day-old chick was gavaged with 0.1 ml of the specified strains and challenge dose. Chicks were sacrificed 7 days postchallenge, and ceca were plated onto Campy-Cefex agar to determine *C. jejuni* colonization.
[b] Strains used in colonization competition and protective efficacy studies.

Colonization competition study. The isolation ratios of the human and chicken isolates are represented in Table 3. The chicken isolate with the *flaA*-7 type (W92.4ENR) was most successful at competing with human isolates with *flaA*-1 types

TABLE 2. Chick colonization of *C. jejuni* isolates of poultry origin[a]

FlaA type	Bacterial strain	Challenge dose (CFU)	No. of chicks colonized/ 10 challenged
1	mf23899	3.2×10^6	1
	P108	2.8×10^5	1
4	2932	1.8×10^5	2
5	25216	2.1×10^4	0
6	P54	7.0×10^4	0
7	W92.4ENR[b,c]	3.2×10^4	10
13	P74	1.3×10^6	0
15	P94	2.2×10^6	1
23	1645	1.1×10^5	0
39	P50	7.7×10^8	3
	P51	2.3×10^8	5
49	P110	7.7×10^7	2
	PRC15	1.5×10^8	9
	PCR63	5.0×10^6	10
59	P57	1.7×10^8	6
59	CE-0-5[b,c]	6.1×10^7	10
	CE-0-9[b,c]	1.6×10^8	10
72	P88	1.8×10^5	8
80	P62[b]	2.3×10^8	10
	P86	1.7×10^9	6
	P104	1.6×10^8	10

[a] Each day-old chick was gavaged with 0.1 ml of the specified strains and challenge dose. Chicks were sacrificed 7 days postchallenge, and ceca were plated onto Campy-Cefex agar to determine *C. jejuni* colonization.
[b] Strains used in colonization competition study.
[c] Strains used in protective efficacy study.

in the chicken ceca, but had a more limited effect on *flaA*-15 and *flaA*-27 isolates; producing reductions of only 27 and 25%, respectively.

Protective efficacy study. Even when *C. jejuni* colonized the chicken ceca for a period of time, colonization by other *Campylobacter* strains was not consistently reduced. Human isolates were gavaged into chicks 3 or 5 days after the chicken isolates were administered. Chicken isolates with the *flaA*-59 type (CE05 and CE09) had no protective effect against subsequent challenge with human isolates (*flaA*-15 and *flaA*-27 types). These chicken isolates did not prevent colonization by human isolates even after they had occupied the ceca for 5 days. On the other hand, after colonizing the ceca for 5 days, a chicken isolate with the *flaA*-7 type (W92.4ENR) had the capability of preventing colonization of the human isolate with the *flaA*-15 type (AU886), although there was little effect on the *flaA*-27 type (UP1). These results suggest that the superior colonizing strains, of either chicken or human origin, will be dominant in the chicken ceca, regardless of when the competing challenge event occurs.

SVR sequencing. The sequences of many isolates with the same *flaA* type were classified into the same clades, with as little as a 1-bp difference (data not shown). Strains from different *flaA* types were grouped into different clades, and their base pair differences were also calculated. UP1 (*flaA*-27), a unique isolate, had more than 50 bp different from other isolates. Further molecular investigation is required to determine why this isolate had the strongest colonization efficacy and could not be excluded by other strains used in this study.

Base pair similarity may not have any bearing upon colonization or protective efficacy. Strain W92.4ENR, which had the best colonization and protective efficacy, differed by 8 bp from AU886. The P62, CE05, and CE09 isolates had 2-, 24-, and 23-bp differences from AU886, respectively. The base pair differences between UP1 and P62, W92.4ENR, CE05, and CE09 were 55, 56, 58, and 57, respectively. Isolates with similar SVR sequences did not have the same colonization or protective efficacy, also suggesting that SVR sequence analysis may not relate to the colonization of chick ceca by *C. jejuni*.

The SVR sequencing method had better discriminatory power than PCR-RFLP for differentiating between various sources of *C. jejuni*, because base pair differences could still be found in those strains within the same *flaA* type. This result correlates with those described by Meinersmann et al. (11), who compared two variable regions in the *flaA* gene for the discrimination of 22 *C. jejuni* strains from an outbreak.

DISCUSSION

CE preparations, whether defined or undefined, showed limited success in preventing colonization by *Campylobacter* spp. in chicken ceca (12, 19, 22, 24). In our study, we evaluated the feasibility of using selected chicken *C. jejuni* strains not previously having an *flaA* type associated with human disease. These isolates were used as a defined CE preparation to prevent the colonization of pathogenic human isolates in the chicken ceca.

The colonizing abilities of different *C. jejuni* strains varied even among strains with the same *flaA* type. Since strains originally isolated from humans having higher colonizing abilities had the *flaA* types most frequently isolated, e.g., *flaA*-1 and *flaA*-7, it seemed that the RFLP typing scheme might correlate with the colonization ability of *C. jejuni* in the chicken ceca. On the other hand, four human isolates with the *flaA*-21 type, having an association with Guillain-Barré syndrome (15), could not colonize 1-day-old chicks. This result infers that poultry may be a limited source to transmit *C. jejuni* strains that cause Guillain-Barré syndrome in humans. Further research is needed to support this hypothesis.

In the present study, strains with superior colonizing ability always dominated the *Campylobacter* population in chicken ceca, regardless of when the chicken was exposed to those strains. UP1 was always the best colonizer among the chicken isolates tested, whereas AU886 was excluded only by W92.4ENR after it had occupied the ceca for 5 days. This phenomenon may be explained because *C. jejuni* occupied the intestinal niche, but was not attached directly to the surface of epithelial cells, and so was readily displaced. Bacteriocin activity among the isolates may also provide a further explanation. This result also suggested that once nonpathogenic *C. jejuni* strains with strong colonizing ability have been identified, there is a potential to exclude pathogenic *C. jejuni* from chicken intestines.

TABLE 3. Isolation ratio of chicken isolates of *C. jejuni* in the colonization efficacy study

Human isolate (*flaA* type)	Isolation ratio of chicken isolate ($P_{avg} \pm$ SE)[a]			
	P62	CE05	CE09	W92.4ENR
AU423 (1)	0	0.47 ± 0.13	0.98 ± 0.22	0.93 ± 0.17
UP4 (1)	0.02 ± 0.12	0.83 ± 0.20	1.00 ± 0.04	1.00 ± 0.03
AU886 (15)	0	0	0	0.27 ± 0.15
UP1 (27)	0	0	0	0.25 ± 0.15

[a] P_{avg}, average probability; SE, standard error.

A PCR-RFLP method was used to differentiate *C. jejuni* strains isolated from chicken ceca. A total of 57 *C. jejuni* isolates (21 from chickens and 36 from humans) were classified into categories of *flaA* type, depending on their electrophoretic fragment profiles produced after digestion with *Dde*I enzyme. The total time needed for completing PCR-RFLP analysis was about 48 h, and this method was highly accurate for tracking *C. jejuni* strains originating from different sources.

The results of SVR sequencing correlated with those of PCR-RFLP analysis, which classified strains with the same *flaA* type into the same clades. Also, the method has the capacity to identify base pair differences in strains with the same *flaA* type. UP1 had the strongest colonizing ability among the strains tested in our study, and it had the most unique SVR sequence among the strains.

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