

Evidence of cross-contamination by *Campylobacter* spp. of broiler carcasses using genetic characterization of isolates

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Abstract

Campylobacter is recognized as one of the leading cause of gastroenteritis worldwide, and is frequently isolated from the small intestines and ceca microflora of chickens. Twenty-one out of 81 *Campylobacter*-positive poultry flocks were selected to evaluate the genetic diversity of *Campylobacter* isolates and to study the distribution of genotypes among flocks. *Campylobacter* isolates recovered from chicken carcasses and ceca were analyzed by pulsed-field gel electrophoresis (PFGE). Little diversity was found among *Campylobacter* strains isolated from a given carcass, with a maximum of 2 different genotypes being present. However, at flock level, as many as 4 different profiles were observed. Typing of strains showed that most strains isolated from ceca were similar to those isolated from corresponding broiler carcasses. A total of 39 different macrorestriction profiles were observed, with evidence of *Campylobacter* cross-contamination among broiler flocks in Quebec slaughterhouses. Surprisingly, some flocks shared related genotypes both with and without sharing similar rearing practices. Existence of such cross-contamination must be considered to in developing strategies to control *Campylobacter* in chickens, and to avoid bacteria contamination of noncolonized flocks. Further typing studies of *Campylobacter* found in hatcheries, farm environment, and crates or trucks in Quebec might be helpful in elucidating the kinetics of broiler chicken *Campylobacter* contamination.

Résumé

Les *Campylobacter* sont reconnus mondialement comme étant parmi les plus importantes causes de gastro-entérite, et sont fréquemment isolés à partir de la flore du petit intestin et des ceca de poulets. Sur un total de 81 troupeaux positifs pour *Campylobacter*, 21 ont été choisis afin d'évaluer la diversité génétique des isolats de *Campylobacter* et étudier la distribution des génotypes parmi les troupeaux. Les isolats de *Campylobacter* obtenus des carcasses de poulets et des ceca ont été analysés par électrophorèse en champs pulsés (PFGE). Peu de diversité a été trouvée parmi les souches de *Campylobacter* isolées d'une carcasse donnée, avec un maximum de 2 génotypes différents présents. Toutefois, à l'échelle du troupeau on pouvait observer jusqu'à 4 profils différents. Le typage des souches a montré que la plupart des isolats obtenus des ceca étaient similaires à ceux isolés de la carcasse de poulet correspondante. Un total de 39 profils de macro-restriction différents a été observé, avec des évidences de contamination croisée par *Campylobacter* parmi les troupeaux de poulets à griller dans les abattoirs au Québec. Étonnamment, quelques troupeaux partageaient des génotypes apparentés tout en ayant ou non des pratiques de régie similaires. L'existence d'une telle contamination croisée doit être également considérée lors de l'élaboration de stratégies pour contrôler les *Campylobacter* chez le poulet, et empêcher la contamination bactérienne de troupeaux non-colonisés. Des études supplémentaires de typage des *Campylobacter* trouvés dans les couvoirs, l'environnement de la ferme, et les cages de transport et camions au Québec, seraient utiles afin d'élucider la cinétique de la contamination de la contamination par *Campylobacter* chez les poulets à griller.

(Traduit par Docteur Serge Messier)

Introduction

Campylobacter spp. is one of the leading cause of human bacterial foodborne infections, with an isolation rate of approximately 13 cases per 100 000 in the USA in 2004 (1). Some studies showed that poultry meat is a frequent source of human *Campylobacter* infections, although many other sources are suspected (2). Indeed, *Campylobacter* is frequently isolated from the small intestines and ceca microflora of chickens, and gut colonization is well-documented (3–6). Intestinal content is therefore suspected to be the main source of broiler carcass contamination at slaughter (7). Even if much effort has been made to decrease bacterial carcass contamination at slaughter with the implementation of HACCP programs, a significant proportion

of broiler carcasses is still contaminated with *Campylobacter* (8,9). Many studies in the USA and Europe have evaluated *Campylobacter* diversity in poultry and the significance of cross-contamination at the slaughterhouse level (10–12); however, to our knowledge, no such study has been performed in the province of Quebec. Occurrence and significance of cross-contamination is highly dependent of slaughter practices, and could potentially vary from country to country particularly if different slaughter procedures are used. Since slaughter procedures in Quebec are quite different from those in the US and Europe, a better understanding of *Campylobacter* contamination in Quebec slaughterhouses would help in reducing cross-contamination of *Campylobacter* during the slaughtering process.

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The objectives of this study were to: 1) evaluate genetic diversity of *Campylobacter* isolates recovered from chicken carcasses and pooled ceca at slaughterhouse using pulsed-field gel electrophoresis (PFGE) genotyping; 2) study the distribution of genotypes among flocks; and 3) assess the significance of cross-contamination among them.

Materials and methods

Rearing, transportation, and slaughter conditions

A questionnaire was sent to producers and slaughterhouse foremen for each flock slaughtered. Questions were related to hatchery, rearing practices, feed mill origin, and transportation.

Sampling of broiler chicken flocks at the slaughterhouse

A flock was defined as a group of birds from the same hatchery raised in a broiler house during the same time period. Flocks used for this study were part of a larger study on *Campylobacter* prevalence and risk factors conducted on 81 broiler chicken flocks slaughtered in Quebec (13). Sampling in the present study was the same as that described and used by Arseneault et al (13). Briefly, a total of 30 carcasses were sampled by carcass rinsing for each chosen flock; a total of 2372 carcasses were sampled as previously described (14,15). Following evisceration and after a 20 ppm chlorinated shower, each carcass was placed in a sterile plastic bag containing 400 mL of Buffered Peptone Water [Beckton-Dickinson (BD), Franklin Lakes, New Jersey, USA] and vigorously shaken for 30 s. Carcass rinses were stored on ice in 1L bottles (Nalgene NUNC International, Rochester, New York, USA), brought to the laboratory within the next 3 to 8 h and analyzed for the presence of *Campylobacter*.

Both ceca from each sampled bird were also collected after evisceration, placed in a labelled sterile plastic bag, and stored on ice until further analysis. One of the 2 ceca was frozen at -80°C , and the other was used for *Campylobacter* detection.

Flock sub-selection

Of the 81 broiler chicken flocks initially sampled, 21 were chosen to use within the framework of this study. Each flock was selected because it contained a minimum of 5 *Campylobacter* positive carcasses. For logistical reasons, 5 carcasses were randomly selected among the 30 available from each flock to have a quick and representative view of *Campylobacter* diversity across every flock. Only *Campylobacter* isolated from carcasses and pooled ceca belonging to those 21 flocks were analyzed.

Campylobacter isolates recovered from carcasses

For carcass samples, 25 mL of each carcass rinse were mixed with the same volume of double-strength Bolton broth (Oxoid, Hampshire, UK.) supplemented with cefoperazone (20 mg/L), vancomycin (20 mg/L), trimethoprim (20 mg/L), and cycloheximide (50 mg/L) (supplement SR0183, Oxoid), and incubated for 24 h at 42°C . A portion of each sample (10 μL) was then inoculated onto mCCDA agar (Oxoid) supplemented with cefoperazone (32 mg/L) and amphotericin B (10 mg/L) (supplement SR0155; Oxoid) and incubated microaerobically (CampyEzPak, BD) for 48 h at 42°C in jars

(BD GasPak EZ Container Systems, BD), as described by Gun-Munro et al (16).

Campylobacter isolates recovered from bird ceca

At the laboratory, ceca sampled along with the carcasses were first separated into 3 groups of 10 ceca each; these "pools" were identified with the corresponding flock number. To obtain pools of fecal matter, the surface of each cecum was first sterilized by heat searing with a hot spatula, as described by Nadeau et al (8). Cecal content was collected from each cecum of each pool with a sterile swab, placed in a sterile stomacher bag (M-Tech Diagnostic, Warrington, Cheshire, England) containing 10 mL of phosphate buffered saline (PBS), and gently homogenized. A portion of the resulting mixture was put directly on a selective mCCDA medium in jars (BD) and incubated under microaerobic conditions for 48 h at 42°C , as previously described (16).

Identification of strains to the species level

For each carcass and pool of ceca, presumptive *Campylobacter* colonies were analyzed by Gram stain morphology and mobility under phase-contrast microscopy. Typical colonies were then inoculated on 5% sheep blood agar (Quelab Laboratories, Montreal, Quebec) and incubated for 48 h at 42°C under microaerobic conditions. In order to achieve identification at the species level, selected biochemical tests were done on colonies as previously described (17). Oxidase and catalase reactions were done (Remel; Lenexa, Kansas, USA) to assess the *Campylobacter* genus, and Indoxyl acetate and hippurate hydrolysis (BD) tests were conducted to confirm the species level. Isolates that gave a positive result to both hippurate hydrolysis and indoxyl acetate tests were considered to be *Campylobacter jejuni*. Those that were only positive for the indoxyl acetate test were considered to be *Campylobacter coli*. *Campylobacter* isolates were then frozen at -80°C in Brucella broth (BD) containing 15% glycerol (BD) until further analysis.

Campylobacter diversity analysis

To provide a first evaluation of *Campylobacter* genotypic diversity on each positive carcass, 5 *Campylobacter* colonies were first analyzed using the PFGE technique for the 5 first flocks of the study. At this point, since there was only little diversity observed among analyzed colonies, and for logistical reasons, it was decided that only 3 colonies per positive carcass would be typed with the PFGE technique for the subsequent carcasses at the slaughter level. However, 5 colonies were analyzed for each pool of ceca that was positive.

Indeed, 4 different sub-analyses of *Campylobacter* genotypes have been made: within bird, between birds within a flock, between flocks, and between slaughterhouses.

Pulsed-field gel electrophoresis (PFGE)

Deoxyribonucleic acid (DNA) preparation was done according to the protocol established by Michaud et al (18), with some modifications. Briefly, *Campylobacter* were grown on 5% sheep blood agar (Quelab Laboratories) under a microaerobic atmosphere for 48 h at 42°C . Colonies were harvested and homogenized in 1000 μL of cold cell suspension buffer [100 mM Tris, 100 mM EDTA, (pH 8.0)], and optical density (OD) was adjusted to a value of 2.0 at

numbers 2 and 20 of each gel were filled with a *Campylobacter jejuni* (LSPQ 3234) digested with *KpnI* as a reproducibility control.

Choice of restriction enzyme

In a recent study, it was found that the discriminatory power of *KpnI* was greater than that of *SmaI* (21). Moreover, in a preliminary study conducted over 10 carcasses from 2 different broiler flocks, *KpnI* gave consistently a higher number of different genotypes than *SmaI*, which confirmed the good discriminatory power of *KpnI* (data not shown). Given that information and the significant number of isolates we had to analyze, only *KpnI* was used to digest DNA samples.

Gel analyses

Macrorestriction profiles were analyzed using BioNumerics Software (Applied Maths; Sint-Martens-Latem, Belgium). Restriction fragments were identified visually, and normalized by interpolation to the nearest reference lane. An optimization of 1% and a position tolerance of 2% were applied. Dice Coefficients were established on the basis of pairwise comparisons of the PFGE patterns of isolates. Coefficients matrix was used to generate dendograms based on the unweighted pair group method using arithmetic averages (UPGMA).

Results

Campylobacter isolation rates from broiler chicken carcasses and pooled ceca

For the 81 flocks sampled between April 2003 and February 2004 (13), *C. jejuni* were isolated from 89% of the carcasses and 100% of the *Campylobacter* positive pooled ceca. *Campylobacter coli* were isolated from 11% of *Campylobacter* positive carcasses and from 0% of positive pooled ceca. However, the 21 flocks included in the current study were only colonized with *C. jejuni*.

PFGE typing reproducibility and establishment of a cut-off for analysis

By PFGE typing of the *Campylobacter jejuni* LSPQ 3234 reference strain used as a reproducibility control, a 94% similarity level has been obtained, mainly due to variation in gel migration. It is important to note that this similarity level was based on raw data contained in the similarity matrix generated with the BioNumerics analysis. Therefore, all strains showing a similarity level $\geq 94\%$ following BioNumerics analysis were considered to be identical. However, all the isolate patterns presenting that similarity level were controlled visually to ensure that genotypes were really similar. Each distinct genotype was identified with a capital letter (A to Z) or a double capital letter (AA to MM). Figure 1 provides a representative portion of the dendogram obtained.

Diversity of PFGE fingerprinting patterns within birds

Despite several attempts, 6 isolates were nontypable using the PFGE technique (Table I: NT). A low diversity level has been observed among colonies isolated from carcasses and pooled ceca. Following diversity analysis, only 6 of the 97 carcasses showed at least 2 different colony profiles among those analyzed within a

carcass [Table I: flock 128 (R); flock 129 (B); flock 160 (AA); flock 175, (II); flock 191, (CC); flock 198, (LL)]. For pooled ceca, the same genotype was obtained for all 5 colonies in 15 out of 16 pools. The other pool of ceca showed 2 out of 5 colonies associated with a different genotype [Table 1: Flock 160, pool 178, (AA)].

Diversity of PFGE genotypes between birds within a flock

Thirty-nine different genotypes were obtained from 21 chicken flocks (Table I; A to MM), regardless of sample source (carcasses or pooled ceca). Within a flock, it was possible to identify up to 4 different profiles on chicken carcasses. (Table I, flock 197). Generally, when there was more than 1 genotype within a flock, one would prevail over the others (Table I). It was also possible to observe that different genotypes could be equally distributed within a flock (Table 1: flocks 88, 190, and 197).

In most of the "ceca positive flocks," macrorestriction profiles found in the ceca were the same as those found on carcasses. Exceptions were flocks 82, 128, and 187, which appear to be colonized by different genotypes for their respective carcasses and ceca [Table I; flock 82 (profiles G and O vs. profile K); flock 128 (profile W vs. profile R); flock 187 (profile EE vs. profile T) and Figure 1, flock 128, profiles W and R].

Diversity of PFGE genotypes between flocks

For a given slaughter day, and when flocks were slaughtered consecutively, carcasses genotypes were most of the time shared by the 2 sampled flocks [Table I: flocks 109 and 112 (S), 128 and 129 (W), 175 and 176 (GG), 187 and 188 (EE), 190 and 191 (JJ)]. However, in some consecutively slaughtered flocks, the *Campylobacter* genotypes found on carcasses could also be different between the 2 sampled flocks [Table 1: flocks 128 (R) and 129 (B and V); 175 (II) and 176 (MM); 190 (V, Z) and 191 (CC)], but this occurred less frequently.

Flocks slaughtered on a same day, but not consecutively, generally harbored different genotypes on their carcasses (Table I: flocks 160 and 164, 196 and 197). Nevertheless, a genotype recovered from a pool of ceca on a given day was also found on carcasses of the flock slaughtered and sampled several hours later [Table 1: flocks 109 and 112 (S)].

Diversity of PFGE genotypes between slaughterhouses

In this study, it was also possible to observe that some flocks slaughtered on different days and slaughterhouses can be colonized by identical *Campylobacter* genotypes. Even if this occurred only twice during the study (flocks 109, 112, and 149; and flocks 164, 175, and 176), it is interesting to note that birds from flocks 109 and 149 came from the same hatchery (A), and were fed with feed purchased from the same feed mill (C) (Table I). However, no such other relationships could be established between other flocks that shared similar profiles.

Discussion

Poultry colonization with *Campylobacter* is a well recognized phenomenon (5,9,22–24). Even if *Campylobacter* has often been

Table I. Genotypes of *Campylobacter* isolates recovered from broiler carcasses and pooled ceca for different broiler flocks and slaughter days in Quebec, using *KpnI* digestion and PFGE

Flock number	Sampling dates ^a	Slaughterhouse	Carcasses genotypes ^{b,c}	Pooled ceca genotypes ^{b,c,d}	Hatchery ^e	Floor-milling ^e
82	21/08/2003	D	G (5/25), O (20/25)	K (15/15) — pools 95-96-97	H	C
88	28/08/2003	C	X (5/10), DD (5/10)	Negative	H	mixmill
93	04/09/2003	B	Q (25/25)	Negative	K	F
109^f	25/09/2003	D	S (25/25)	S (10/10)	A	C
112	25/09/2003	D	S (25/25)	Negative	J	J
128	16/10/2003	A	R (11/30), W (19/30)	R (15/15) — pools 140-141-142	K	V
129	16/10/2003	A	B (1/14), V (3/14), W (9/14) NT ^g (1/14)	Negative	C	I
149	12/11/2003	C	S (12/15), NT (3/15)	Negative	A	C
150	18/11/2003	A	HH (15/15)	Negative	A	Z
160	03/12/2003	D	AA (1/15), BB (14/15)	BB (8/10) — pools 177-178, AA (2/10) pool 178	H	W
164	03/12/2003	D	GG (15/15)	GG (15/15) — pools 179-180-181	H	C
175	07/01/2004	B	GG (14/15), II (1/15)	Negative	A	I
176	07/01/2004	B	MM (3/15), GG (11/15), NT (1/15)	Negative	C	D
183	14/01/2004	C	D (3/15), N (12/15)	Negative	F	R
187	21/01/2004	D	EE (14/15), NT (1/15)	T (5/5) — pool 201	H	W
188	21/01/2004	D	EE (15/15)	Negative	A	C
190	26/01/2004	A	U (6/15), Z (3/15), JJ (6/15)	Z (5/5) — pool 206	A	N
191	26/01/2004	A	CC (1/15), JJ (14/15)	Negative	J	J
196	03/02/2004	D	L (3/15), Y (6/15), M (3/15), H (3/15)	M (5/5) — pool 218	H	G
197	03/02/2004	D	A (3/15), F (6/15), I (3/15), J (3/15)	Negative	H	P
198	05/02/2004	D	E (3/9), KK (5/9), LL (1/9)	Negative	J	J

^a Sampling date (dd/mm/year).

^b Fractions between brackets represent the number of isolates associated with the given genotype.

^c Each letter or group of letters represents a different genotype.

^d The numbers between brackets represent the number of pools of ceca analyzed.

^e Each letter under hatchery and floor-milling represents different hatchery and floor-milling. Hatchery and floor-milling are not related.

^f For the same slaughter dates, bolded flocks numbers were slaughtered consecutively.

^g NT — nontypable.

isolated from chicken feces at both farm and slaughterhouse level (23,25,26), only limited data are available regarding the level of chicken carcass contamination with *Campylobacter* and its carriage at slaughterhouses (10–12,25). Some authors have suggested the existence of cross-contamination between slaughtered flocks (8); however, it has seldom been demonstrated

(10–12,25). Those studies have reported that *Campylobacter* isolates found in feces of chicken were the same as those found on bird's carcasses at slaughterhouse in Japan, the United Kingdom, and France. To our knowledge, no such study has been performed in Quebec; this highlights the importance of the current study.

As shown in Table I, the number of different PFGE profiles recovered from carcasses and pools of ceca was high ($n = 39$). There was a low *Campylobacter* genetic diversity on carcasses and in pooled ceca from individual broilers at slaughter. The maximum number of different isolates observed on a given carcass or pooled ceca was 2; similar to another study (11). Even if we had detected as many as 4 different genotypes in a given flock, one predominated. These findings could help in understanding the previous discrepancies between the studies of Nadeau et al and Hiatt et al (8,25). Nadeau et al (8) observed only 1 single dominant genotype in feces of positive flocks in Quebec, whereas Hiatt et al (25) reported as many as 6 distinct clones within a flock. According to the actual results, a predominant genotype was present with several minor ones most of the time, which could indicate that both studies were right. However, it reinforces the importance of analyzing more than 1 colony for each positive sample. Although the number of carcasses analyzed in each flock was relatively limited in this study, since carcasses were randomly chosen within every flock, we believe that colonies recovered from each flock provided a good indication of the *Campylobacter jejuni* populations in the various flocks.

Hiatt et al (25) also reported that while 1 predominant clone was observed, the diversity found in the final products seemed low compared with the high number of clones found on the farm. Since our protocol involved only slaughterhouse sampling, the hypothesis of a higher number of genotypes at farm level could also be considered. However, a larger and more varied sampling would be required in order to properly assess this hypothesis.

Rivoal et al, Newell et al, and Hiatt et al (10,11,25) have previously shown that carcasses at slaughter were mainly contaminated by *Campylobacter* originating from chicken ceca. Our results concur with those observations. Table I shows that genotypes in pooled ceca were found in 6 out of 8 flocks, and also found on chicken carcasses of the respective flocks. This could suggest that a significant proportion of carcass contamination has occurred at the slaughterhouse.

Another finding of this study is the recovery of common genotypes on carcasses of consecutively slaughtered flocks. As shown in Table I, this phenomenon was observed whenever flocks were slaughtered consecutively (flocks 109,112; 128,129; 175,176; 187,188; 190 and 191). This carriage of strains from one flock to another has been shown in previous studies (10,11,25), but this is the first demonstration of cross-contamination of *Campylobacter* between slaughtered flocks in the province of Quebec. However, part of this contamination could have also originated from bird feces that may have contaminated skin during transportation from the farm to slaughter or during the slaughter process. It could also have resulted from *Campylobacter* contamination on crates that could have contaminated birds during transportation.

The possibility of such cross-contamination needs to be considered to develop strategies for controlling *Campylobacter* contamination in chickens and to avoid bacterial contamination of noncolonized flocks. Some slaughter practices should be revised accordingly to reduce the spread of *Campylobacter*. A possible solution would be to slaughter negatively affected flocks prior to those that are positive for *Campylobacter*.

Another interesting finding is that some genotypes were found on carcasses of flocks not slaughtered on the same day, and at different slaughterhouses [Table I, flocks 109, 112 and 149 (S) and flocks 164, 175 and 176 (GG)]. After further analysis of rearing conditions of these flocks, it appears that flocks 109 and 149 only shared the hatchery and the feed mill. However, as flocks 109 and 112 were slaughtered consecutively, and that the genotype of flock 109 was also "S," it is possible that strain carriage occurred from flock 109 to flock 112. This hypothesis implies that flocks 109 and 149 were contaminated with *Campylobacter* that had originated from a common source; either hatchery, feed mill, trucks, or slaughterhouse staff. Flocks 164, 175, and 176 did not share any common hatchery or feed mill. It was, therefore, not possible to establish any relationship between possible contamination sources of those flocks. There were also flocks in our study that had common hatcheries and feed mills, but did not share similar genotypes. Nevertheless, this suggests that some flocks can be colonized with similar strains, without showing clear evidence of common rearing origins (flocks 164, 175 and 176; 196 and 198). Some authors (8,10,11,25–27) have also suspected the existence of external sources of contamination, such as hatchery, breeder hens, or transport. However, there is no specific evidence to support these hypotheses.

We have examined the possibility that the enrichment media used in this study could have influenced the genotype diversity found on broiler carcasses. However, since enrichment was used only for carcass rinses, and similar genotypes were found both on carcasses and in pooled ceca, this variable does not seem to have had an important impact on strain selection and diversity.

This study has found that a large genetic diversity of *Campylobacter* strains exist among broiler flocks. It suggests that there may be various reasons for broiler carcass contamination at the slaughterhouse, the major one being fecal contamination of carcasses. As samples were collected after evisceration, it is difficult to assess if contamination occurred before, during, or after evisceration. However, pooled ceca profiles were similar to those of carcasses, indicating that contamination effectively happened at the slaughterhouse. Further typing studies of *Campylobacter* found in hatcheries, the farm environment, and in crates or trucks might be helpful in elucidating the kinetics of broiler chicken *Campylobacter* contamination in Quebec.

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