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Diversity of *Campylobacter* Isolates from Retail Poultry Carcasses and from Humans as Demonstrated by Pulsed-Field Gel Electrophoresis

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ABSTRACT

Campylobacter spp. are a major contaminant of poultry. Eating undercooked chicken and handling raw poultry have been identified as risk factors for campylobacteriosis in humans. Previous studies have found *Campylobacter* spp. on 90% of poultry carcasses. In the present study, pulsed-field gel electrophoresis (PFGE) was used to assess the genetic diversity of strains on retail poultry carcasses. PFGE patterns of isolates from campylobacteriosis cases were compared to those from the poultry isolates. Over a 1-year study period (March 2000 through February 2001), whole fresh young chickens ($n = 72$) were obtained from three retail outlets in an urban community in the south-central United States. *Campylobacter* spp. were isolated from 82% of these carcasses. Strains ($n = 70$) were defined on the basis of their PFGE pattern. Sixty-seven percent of the carcasses from which *Campylobacter* spp. were isolated were contaminated with more than one PFGE-distinguishable strain. During the 1-year study period, most of the PFGE patterns (59%) were limited to isolates obtained from a single carcass. Forty-one percent of the PFGE-distinguishable strains were recovered from more than one carcass. Ninety-seven percent of the carcasses contaminated with the same strain were purchased at the same time from the same store. To examine the degree of genetic stability, four strains were followed in vitro over an estimated 1,000 doublings. The PFGE pattern of one of these isolates underwent minor changes during in vitro growth. The data indicate extensive variability in the PFGE patterns of *Campylobacter* spp. isolated from humans and from poultry carcasses. In spite of difficulties caused by such diversity and the fact that some carcasses are contaminated with more than one strain, the pattern variation provides a useful method for linking a particular strain to its source.

There are an estimated 2.4 million cases of human campylobacteriosis each year in the United States *(14)*. The majority of cases are sporadic and peak during the summer months *(4)*. Eating undercooked poultry and handling raw poultry have been identified as risk factors for sporadic campylobacteriosis *(9, 10)*. Poultry are colonized with many of the same *Campylobacter* serotypes that cause disease in humans *(2, 16)*.

Intestinal colonization of healthy birds occurs by 4 weeks of age *(11)*. Surveys of live birds on farms demonstrate that birds can harbor large numbers of *Campylobacter* spp. without showing any signs of illness. *Campylobacter* counts on carcasses increase 10- to 100-fold during evisceration and subsequently decrease later in processing *(11)*. The Food Safety Inspection Service microbial baseline study estimated that 88.2% of broiler carcasses were contaminated with *Campylobacter* spp. *(20)*.

Subtyping of isolates is useful for epidemiologic investigation of *Campylobacter* spp. Serologic methods have been developed for subtyping *Campylobacter jejuni*; however, the methods are not adequately discriminatory, the availability of typing sera is limited, and the typing is largely carried out by reference laboratories *(1)*. Several different DNA-based subtyping schemes have recently been developed (see *(15)* for review). Among these is pulsed-field gel electrophoresis (PFGE), which has proven useful in epidemiologic investigations of salmonellosis and *Escherichia coli* O157:H7 infection *(19)*. When 39 epidemiologically unrelated and 18 outbreak-related strains of *C. jejuni* were compared by ribotyping, phage typing, and PFGE *(5)*, PFGE was found to be the most discriminating. The present investigation was undertaken to characterize the extent of diversity of *Campylobacter* spp. present on retail purchased poultry carcasses on the basis of PFGE polymorphisms. Specifically, the number of PFGE-distinguishable strains of *Campylobacter* spp. on individual retail poultry carcasses was determined. The diversity of PFGE strains as a function of time and site of purchase was analyzed to determine how common patterns were shared by isolates purchased throughout the year. Because the characterization of such diversity is useful in evaluating traceback studies of human infections, PFGE profiles of poultry and human isolates that were obtained during approximately the same time interval were compared.

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MATERIALS AND METHODS

Experimental design. During the 2-year period, 1 July 1999 through 30 June 2001, 419 cases of *Campylobacter* spp. infection were reported to the Arkansas Department of Health. Isolates from 61 cases were submitted to this agency. Isolates from 54 cases (one isolate from each case) were analyzed by PFGE. Isolates from seven cases were not typeable.

Packages labeled whole fresh young chickens were purchased from three randomly selected supermarkets (A, B, and C) in Little Rock, Ark. The three supermarkets are part of two grocery store chains, one having 12 (A and B) and the other 9 (C) retail outlets in the city. All of the other grocers in the city were single retail outlets. Two chickens were purchased each month from each supermarket, so that a total of six chickens were analyzed every month for 1 year (March 2000 through February 2001). Overall, 72 carcasses were evaluated. Supermarkets A and B sold chickens from one producer; supermarket C sold chickens from a different producer. The two chickens purchased from supermarkets A and B were purchased at the same time whenever possible. Each month, all purchases were made during a 1-week interval.

Isolation of *Campylobacter* spp. To isolate *Campylobacter* spp., each carcass was placed in a sterile Ziplock bag with 50 ml of a 0.9% NaCl–0.1% peptone rinse and placed on a platform shaker at room temperature (Innova 2000 Shaker, New Brunswick Scientific, Edison, N.J.) (40 rpm, 5 min). The carcass was aseptically removed from the bag, and the rinse was filtered through sterile gauze. The filtrate was centrifuged (relative centrifugal force 315, 5 min) in 50-ml tubes, and the supernatant was filtered through a 0.2-μm sterile Nalgene filter to concentrate organisms. After approximately 10 min, the resultant 20- to 25-ml prefiltrate was used to wash the membrane and was transferred to sterile 1.5-ml tubes and centrifuged (relative centrifugal force 12,800, 5 min) in an Eppendorf microfuge. The supernatant was aspirated, and pellets were suspended in 0.9% NaCl–0.1% peptone; contents of each tube combined for a total volume of 500 μl. The 500-μl suspension was plated directly on a CVA (20 μg/ml cefoperazone, 10 μg/ml vancomycin, and 20 μg/ml amphotericine B) plate (Catalog no. 01270; Remel, Lenexa, Kans.) and incubated (48 h, 42°C) under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) in a CO_2 incubator. After 48 h, all colonies suspected to be *Campylobacter* spp. were subcultured for 24 h on fresh CVA plates. Because of the technical limitations of performing PFGE analysis, not more than 10 colonies from each carcass were subcultured. These isolates were then subjected to a polymerase chain reaction assay to confirm that they were genus *Campylobacter* spp. *(3).*

PFGE. The *Campylobacter* isolates from the 72 poultry carcasses ($n = 448$) and from 54 human cases were grown overnight on CVA plates, and washed cells were embedded in 1.0% agarose plugs (BioWhittaker Molecular Applications, Rockland, Maine) *(17).* The plugs were treated with achromopeptidase (500 μg/ml, 0.01 M Tris, pH 8.0, 37°C, 2 h) and proteinase K (1 μg/ml, 56°C, 48 h) to lyse the bacteria. After lysis, the bacterial DNA was restricted with *Sma*I (50 U, 16 h, 25°C). The plugs containing the restricted DNA were placed in an agarose slab gel and electrophoresed under pulsed-field conditions (24 h, 5.8 V/cm^2, 5 to 35 s, 120° angle). Molecular-weight standards (48.5-kb Lambda Ladder, Bio-Rad, Hercules, Calif.) were included in each gel. The gel was stained with ethidium bromide, and the chromosomal fragments were visualized by UV transillumination. Isolates that displayed indistinguishable *Sma*I PFGE patterns and that were isolated from different carcasses were subjected to a secondary PFGE after restriction with *Kpn*I (100 U, 16 h, 37°C) to confirm their identity. Secondary PFGE with *Kpn*I was also used to confirm matches between clinical isolates and poultry isolates.

Analysis of PFGE patterns. An image of the gel was recorded in the Eagle Eye II gel documentation system (Stratagene, Cedar Creek, Tex.). Images of the individual lanes were analyzed with Molecular Analyst Fingerprinting software (Bio-Rad). PFGE profiles of each isolate were stored in a database and compared with those of previously fingerprinted isolates. Patterns were compared by grouping analysis on the basis of their similarity index (percentage of isolates having fragments of identical size). When an indistinguishable pattern was observed from two or more chickens, the isolates were compared directly by electrophoresis on the same gel. At least one isolate of each pattern was analyzed by a polymerase chain reaction assay that differentiates between *C. jejuni, Campylobacter coli,* and *Campylobacter lari (3).*

Pattern stability analysis. Four isolates were selected to determine genetic stability during multiple in vitro passages. Each isolate was cultured on a CVA plate from frozen stock. A single colony of each was inoculated into 5 ml of Brucella broth (Difco Laboratories, Detroit, Mich.). Approximately 2.0×10^5 to 10^7 CFU of each isolate were transferred to 10 ml of fresh broth every 24 h for 50 days. After every 10 passages, approximately 50 to 100 CFU were transferred to CVA plates and incubated (48 h, 42°C) under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2). Ten colony-forming units per isolate were selected, subcultured on CVA plates, and then embedded in agarose plugs. Restriction with *Sma*I and PFGE analysis of the subisolates was carried out according to the procedure described above.

RESULTS

Isolates from poultry. A total of 72 carcasses were purchased during the 1-year study period. *Campylobacter* spp. were isolated from 59 of 72 (82%) of the carcasses, i.e., 19 of 24 (79%) from supermarket A, 22 of 24 (92%) from supermarket B, and 18 of 24 (75%) from supermarket C. There was no significant difference in the recovery of *Campylobacter* spp. from the three supermarkets surveyed (chi-square test: $P = 0.402$).

C. jejuni was isolated from all but 3 of the 59 *Campylobacter*-contaminated carcasses. Of the 72 carcasses, *C. jejuni* alone was isolated from 39, *C. coli* alone was isolated from 1, both *C. jejuni* and *C. coli* were isolated from 15, *Campylobacter* spp. were isolated from 2, and both *C. jejuni* and *Campylobacter* spp. were isolated from 2; however, from 13, no *Campylobacter* spp. were isolated. A mean of 6.2 isolates were obtained from each carcass, with a maximum of 10 isolates obtained from any one carcass. The number of isolates per infected carcass ranged from 1 to 10. Of the 448 isolates obtained, 359 (80%) were *C. jejuni,* 63 (14%) were *C. coli,* and 26 (6%) were unidentified *Campylobacter* spp.

The 448 isolates from the 59 contaminated carcasses displayed 70 distinct PFGE patterns when restricted with *Sma*I. Isolates on the same carcass that shared the same *Sma*I PFGE pattern were considered identical. Some of the patterns were displayed by only one isolate; others were shared by up to 27 isolates. The number of distinct PFGE patterns present on an individual carcass ranged from 1 to 6 (Fig. 1), with an average of 2.3 patterns/carcass. Among

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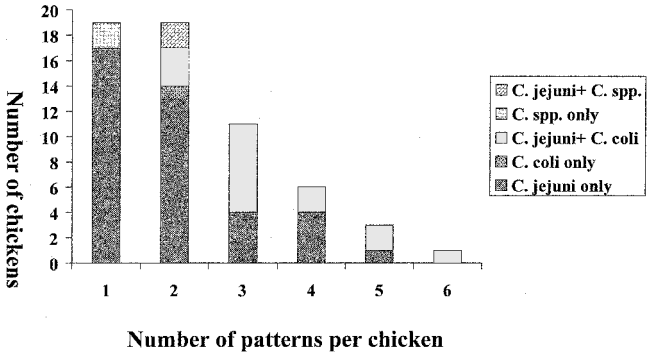

FIGURE 1. *Distribution of PFGE profiles of* Campylobacter *spp. recovered from chicken carcasses.*

the carcasses from which more than one strain was isolated, 17 of 40 (42.5%) were contaminated with more than one species of *Campylobacter.* Of the 70 patterns, 51 (73%) were from *C. jejuni,* 17 (24%) were from *C. coli,* and 2 (3%) were from unidentifiable *Campylobacter* spp.

The patterns present on a single carcass showed extensive diversity (Fig. 2). Lanes 2 through 11 demonstrate the PFGE patterns of 10 isolates obtained from a single carcass that displayed six distinct PFGE patterns, three of which are *C. jejuni* and three of which are *C. coli.* Lanes 12 through 16 demonstrate PFGE patterns displayed by five *C. jejuni* isolates from another carcass.

Dendrograms of the isolates obtained from each carcass were compared to determine the diversity of the PFGE patterns on each individual carcass. Of the 40 carcasses from which *Campylobacter* spp. displaying more than one PFGE pattern were isolated, 24 (60%) demonstrated isolates with PFGE patterns that were less than 20% homologous to other isolates from the same carcass. That is, the *Campylobacter* spp. isolated from each of these carcasses showed less than 20% homology to other isolates from the same carcass. The remaining 16 carcasses harbored multiple *Campylobacter* isolates on the basis of PFGE patterns that ranged from 1 to 85% homology (average homology/carcass = 43%).

Most (59%) of the PFGE patterns appeared on only one carcass, but 29 (41%) of the patterns appeared on two or more carcasses. The identity of isolates obtained from different carcasses was confirmed by electrophoresing representatives of each pattern from each carcass on the same gel. Of the 29 shared patterns, 14 were present on two carcasses, 5 were present on three carcasses, 5 were present on four carcasses, 3 were present on five carcasses, 1 was present on seven carcasses, and 1 was present on nine carcasses. These shared patterns were further analyzed according to the date and location of carcass purchase (Fig. 3). Attempts were made to purchase chickens from supermarkets A and B on the same date, and this was accomplished for 9 of the 12 months. Supermarkets A and B sold chickens from the same producer, whereas a different producer supplied supermarket C. All except one of the 29 shared patterns (97%) were found on chickens purchased on the same date at the same supermarket. Eleven of the patterns (38%) were shared by carcasses purchased on various dates

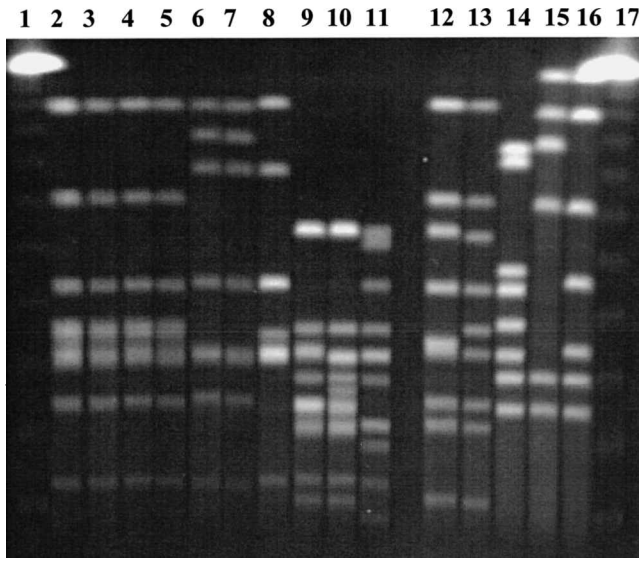

FIGURE 2. *PFGE patterns of* Campylobacter *isolated from chicken carcasses. Lanes 1 and 17, λ phage DNA size standard. Lanes 2 through 11, DNA from 10 isolates from a single carcass (no. 58) (lanes 2 through 8,* C. jejuni; *lanes 9 through 11,* C. coli*). Lanes 12 through 16,* C. jejuni *isolates from a single carcass (no. 35) demonstrating five different profiles.*

throughout the year at the same supermarket. Six patterns (21%) were shared by carcasses purchased on the same date but at different supermarkets (A and B) (same producer). Carcasses purchased on various dates from either supermarket A or B shared eight patterns (28%). Two patterns (7%) were shared by carcasses purchased on various dates from supermarkets selling chickens from different producers. Carcasses obtained from supermarkets A and C shared one of these patterns; carcasses obtained from supermarkets B and C shared the other pattern.

Because several of the profiles appeared at various times throughout the year, the duration of the patterns was analyzed (Fig. 4). Pattern duration was investigated for pat-

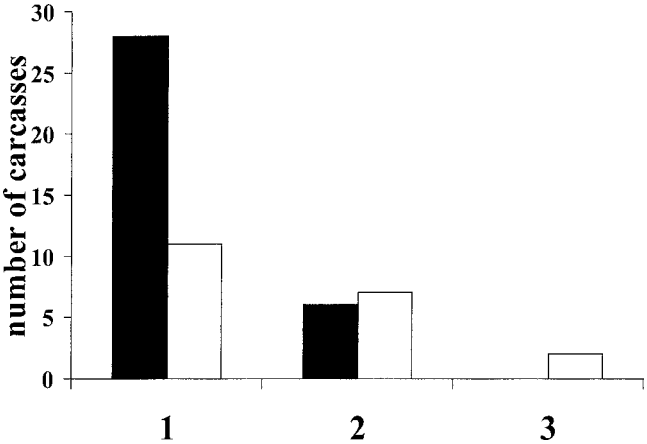

FIGURE 3. *Carcasses having PFGE patterns shared by* Campylobacter *isolates obtained from chickens purchased (1) at the same store, (2) at different stores selling chickens from the same producer, and (3) at different stores selling chickens from different producers. Solid, purchased at different times; open, purchased at the same time.*

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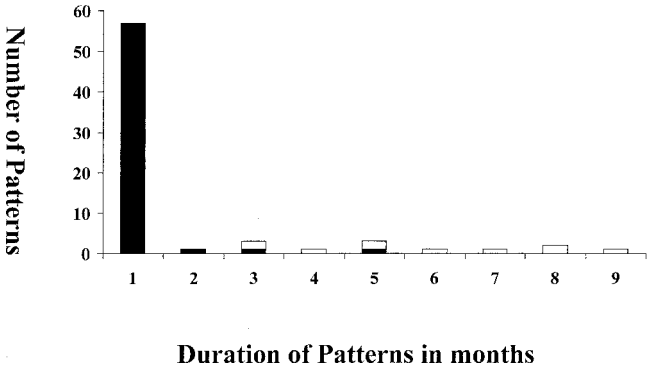

FIGURE 4. *The temporal distribution of* Campylobacter *with indistinguishable PFGE patterns from retail chicken carcasses. Duration is maximum time span over which a pattern was encountered. Solid, patterns that persisted in consecutive months; open, patterns that persisted for several months that were interrupted by months in which they did not appear.*

terns that appeared more than once and ranged from 1 to 9 months. Most patterns appeared only once ($n = 57$); others appeared several times throughout the year.

The diversity of patterns was studied by comparing one image of each pattern in a composite dendrogram. When such comparisons of the 70 patterns were made, the isolates were divided into two large groups that shared less than 2% homology. One group contained 13 of the 17 *C. coli* patterns and 6 of the 51 *C. jejuni* patterns. The PFGE patterns of these 13 *C. coli* and 6 *C. jejuni* shared 35 to 95% homology. The other group consisted of the PFGE patterns of 45 *C. jejuni,* 4 *C. coli,* and 2 *Campylobacter* spp. that shared homology ranging from 12 to 84%. The dendrogram enabled a preliminary discrimination of 13 of 17 (76%) of the *C. coli* and 45 of 51 (88%) of the *C. jejuni.* These data are in agreement with empirical observations that visual analysis of the PFGE pattern distinguished *C. coli* isolates from those of other *Campylobacter* spp.

Isolates from humans. Forty-eight different PFGE patterns were displayed by isolates from the 54 cases of campylobacteriosis submitted to the Arkansas Department of Health. All clinical isolates were *C. jejuni.* Three pairs of clinical isolates shared common *Sma*I and *Kpn*I PFGE patterns. Four of the clinical isolates displayed *Sma*I and *Kpn*I PFGE patterns indistinguishable from four different PFGE patterns of *C. jejuni* isolated from poultry.

Stability of PFGE patterns. No change was observed in the PFGE patterns of three of the four original isolates during the 50 passages in vitro. After passage 30, two new PFGE patterns were observed in one isolate (Fig. 5). All of the subisolates obtained after day 30 displayed one of two patterns (variants A and B), and none displayed the original pattern. Twenty-one subisolates displayed the variant A pattern, and nine displayed the variant B pattern, which, according to MAF-1 (minimum/maximum autocorrelation factors) statistical software analysis, share a 70% identity with the original isolate. After 30 passages, the largest *Sma*I fragment in the original pattern was no longer present, and a smaller fragment appeared in both the variant

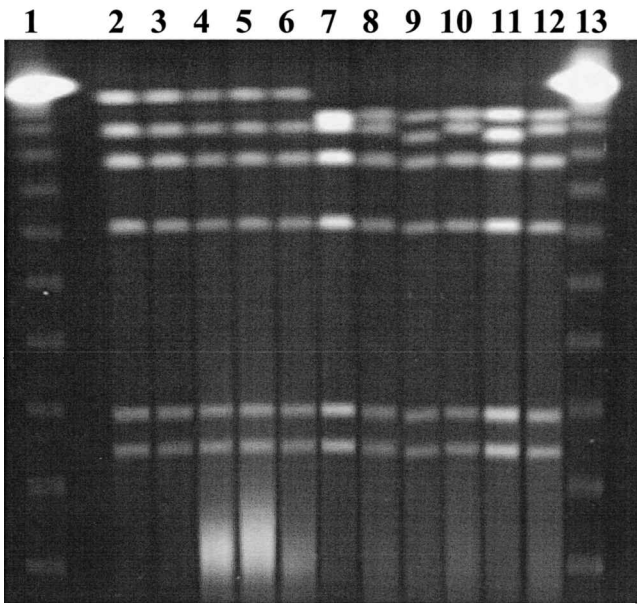

FIGURE 5. *PFGE profiles of a* Campylobacter *isolate that underwent change during in vitro serial passage. Lanes 1 and 13, λ phage DNA size standard; lane 2, PFGE pattern of original isolate; lanes 3 and 4, PFGE pattern demonstrated by all colonies after 10 passages; lanes 5 and 6, PFGE pattern demonstrated by all colonies after 20 passages; lanes 7 and 8, PFGE pattern demonstrated by 9 of 10 colonies after 30 passages; lanes 9 and 10, DNA PFGE pattern demonstrated by the two types of colonies after 40 passages; lanes 11 and 12, PFGE pattern demonstrated by the two types of colonies after 50 passages.*

A and B patterns. All of the other bands were identical to those of the original isolate. Variant B differed from the original pattern and the variant A pattern in that a second fragment was slightly smaller than in the original isolate. The size of the largest fragment in the original pattern was approximately 580 kb; the size of the newly generated fragment in variants A and B was approximately 485 kb, a deletion of approximately 90 to 100 kb.

DISCUSSION

To more accurately assess the diversity of PFGE patterns on a carcass, a direct isolation procedure was developed that eliminated the preenrichment step. The long preenrichment step provides an opportunity for rapidly growing strains to be selected and perhaps overgrow slower-growing strains, whereas the direct plating procedure would be expected to provide a better representation of the population of organisms present on a carcass. The preenrichment procedure would provide for the recovery of bacteria that were not recovered by the direct method used in this study, at the risk of their overgrowth obscuring some of those detected in the direct procedure. Alternatively, the direct procedure may cause injury to some *Campylobacter* spp.; consequently, these are not recovered by the direct method. Therefore, the diversity of *Campylobacter* spp. present on a single carcass observed in this study probably underestimates the actual diversity. Although up to 100 CFU were recovered from each carcass, it is probable that

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many organisms remained on the carcass after the rinse procedure.

Like the PFGE patterns of the human isolates, those of poultry isolates show extensive polymorphism. In the present study, 59% of the PFGE patterns were encountered on a single carcass only. Among the 54 clinical *Campylobacter* isolates, four had PFGE patterns indistinguishable from those of isolates obtained from the poultry carcasses. Two of the clinical isolates that matched those obtained from carcasses were obtained from patients within 1 month after they were isolated from poultry.

C. jejuni isolated from 197 human cases and from poultry in Finland over a 3-year period demonstrated similar diversity *(7).* Although the prevalence of *Campylobacter* spp. in poultry was quite low compared to the present study, the predominant genotypes in humans were also found in chickens. Analysis of human and chicken isolates that shared PFGE patterns with amplified fragment length polymorphism and ribotyping demonstrated extensive congruence between the strains within the PFGE groups, indicating genetic relatedness of strains colonizing chickens and infecting humans *(8).*

For molecular epidemiologic data to be useful in traceback studies, PFGE patterns must show enough polymorphism to distinguish different strains but not so much that each isolate displays a different pattern. Ideally, such variation should identify isolates from a common source, which may be identified in terms of time and place (i.e., when and where the common source existed). In the present study, 57 of 70 (81%) patterns observed on poultry were encountered only within a 1-month period, and 16 of 57 (28%) were patterns shared by more than one carcass. According to this study, when patterns were shared, they were most likely to be from isolates obtained from carcasses purchased at the same store at the same time. A weaker link existed between patterns of isolates obtained from carcasses purchased at the same store at various times or from carcasses purchased at the same time from different stores selling chickens from the same producer. Only two of the patterns were shared by isolates obtained from carcasses from different producers (independent of time of purchase). The data support the conclusion based on epidemiologic data, i.e., that most cases of human campylobacteriosis are sporadic *(4).* The PFGE patterns of both the isolates from humans and from retail poultry carcasses demonstrate extensive variation. This variation and the fact that many carcasses are contaminated with more than one PFGE-definable strain confound efforts of traceback. These factors have contributed to the lower priority of *C. jejuni* for PulseNet *(18).*

In the present study, more than one strain of *Campylobacter* spp. was found on 67% of the infected carcasses, and up to six strains were found on a single carcass. The chicken may have been superinfected with more than one strain in the brooder house, or it may have been contaminated postslaughter. Even though the postslaughter stages of washing and chilling may reduce the level of organisms *(13),* they also offer an opportunity for cross-contamination. The presence of more than one strain on a carcass could be the result of genetic instability of organisms. If this was the case, then organisms contaminating the carcass should be clonally related. Analysis of *C. jejuni* isolates from poultry flocks indicated the presence of more than one PFGE-distinguishable strain on a carcass and suggested that isolates with different PFGE patterns were clonally related *(21).* The instability of PFGE patterns has been demonstrated in *Campylobacter* spp. during passage through newly hatched chick intestines *(6).* Attempts to reproduce evidence for the instability of PFGE patterns of these isolates were unsuccessful during 10-day in vitro serial culture *(6).* More recently, PFGE patterns of *C. jejuni* from chicken flocks and from humans that were isolated 20 years ago were compared to recent poultry isolates. The data indicated that the PFGE patterns of some clones of *C. jejuni* remained stable over a 20-year period *(12).* In the present study, the PFGE pattern of one of four isolates did change during serial culture over 50 passages. The enzyme *Sma*I identifies a rare restriction site in the *Campylobacter* genome, and changes in the PFGE pattern may reflect changes in the restriction sites themselves or changes in the DNA lying between restriction sites. Changes in restriction fragment length can reflect actual changes in the restriction site caused by nucleotide substitution at a site, causing the loss or addition of a site, or it may reflect insertions, deletions, or inversions caused by transposons, bacteriophage, or insertion sequences that alter the size of a fragment. For these reasons, changes in the PFGE pattern may reflect only a very limited region of the *Campylobacter* genome, and changes in other regions may go undetected. Although the present study demonstrates that PFGE patterns of *Campylobacter* spp. can undergo change, this alone does not account for the extensive pattern variation observed in isolates obtained from individual carcasses.

In summary, the data indicate extensive variability in the PFGE patterns of *Campylobacter* spp. isolated from humans and from poultry carcasses. The diversity of the PFGE patterns is consistent with the fact that most cases of campylobacteriosis are sporadic and that the variation of PFGE patterns present on possible sources of infection (e.g., retail poultry carcasses) makes traceback studies difficult. In spite of such difficulties, PFGE provides a useful tool for linking a particular strain of *Campylobacter* to its source.

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