

Application of Pulsed-Field Gel Electrophoresis To Identify Potential Outbreaks of Campylobacteriosis in New Zealand

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Since 2002, New Zealand's incidence of campylobacteriosis has exceeded 300 cases per 100,000 people per annum. To evaluate genetic variation in human isolates, 183 *Campylobacter* isolates were collected from a single clinical laboratory in Christchurch: 77 during an 8-week period in spring, and the rest 3 months later over a second 8-week period in autumn. Isolates were identified to the species level and subtyped using Penner serotyping (*Campylobacter jejuni* only) and pulsed-field gel electrophoresis (PFGE) using both SmaI and KpnI. Approximately two-thirds of the isolates could be grouped into clusters of between 2 and 26 isolates with indistinguishable SmaI and KpnI patterns. Less than 10% of the isolates were of the same type between the two sampling periods. The epidemiological relevance of the PFGE clusters was supported by temporal clustering, some spatial clustering, and some statistically significant demographic similarities among cases in a cluster. Conversely, patient cases yielding isolates which did not cluster with isolates from other cases were more likely to report recent overseas travel and less likely to live within larger urban centers. To identify whether these clusters actually represent common-source outbreaks, however, would require the detailed, rapid, and reiterative epidemiological investigation of cases within a PFGE cluster. The combined and timely application of subtyping and epidemiological investigation would appear to be a promising strategy for understanding campylobacteriosis in New Zealand.

The identification and investigation of disease outbreaks—which can each be defined as two or more cases thought to be linked by a common exposure—has been invaluable for understanding and combating many diseases. Campylobacteriosis has emerged worldwide as a significant cause of gastric illness, and New Zealand has one of the highest rates of campylobacteriosis in the developed world, with 327.4 cases per 100,000 people notified in 2004 (1). While outbreaks of campylobacteriosis have been identified both in New Zealand and elsewhere (8, 12, 14, 36), they generally account for only a small proportion of cases and, as a consequence, campylobacteriosis has been described as predominantly a sporadic disease (23, 25) for which the investigation of outbreaks is of limited value (15, 23).

Most recognized cases of campylobacteriosis are caused by infection with *Campylobacter jejuni*, with a smaller proportion caused by *Campylobacter coli* (25). Species of *Campylobacter* can be carried by a range of animal species including farm animals, wild birds, and pets (6, 7, 9, 29) and spread via contaminated food, milk, water, and even flies (11). An increasing awareness has emerged of the importance of, first, identifying *Campylobacter* to the species level (17) and second, applying appropriate subtyping methodology (35). Penner serotyping of *C. jejuni* and *C. coli* has been used for many years (21), and a range of molecularly-based subtyping approaches have been developed, including pulsed-field gel electrophoresis (PFGE) (30), multilocus sequence typing (10), *fla* typing, and AFLP (26).

In this pilot study we sought first to evaluate the range of Penner serotypes and PFGE types present among notified human isolates from one defined geographical area in New Zealand and in two defined time periods. The null hypothesis was that all or most isolates would be different, limiting any potential application of subtyping to identify and delineate clusters of *Campylobacter* cases. Second, epidemiological data for notified cases subtyped in this study were examined in a preliminary evaluation of the potential significance or relevance of any clustering of isolates observed.

MATERIALS AND METHODS

Campylobacter isolates were obtained from 183 human fecal samples submitted to a clinical laboratory in Christchurch, New Zealand, which primarily processes fecal samples referred from community general practitioners. These isolates were temporally separated, with the first 77 collected over an 8-week period in spring 2002 (laboratory testing dates of 9 September to 13 November 2002, weeks 1 to 8), and the second set of 106 collected 15 weeks later in autumn 2003 (laboratory testing dates of 26 February to 17 April 2003, weeks 23 to 30). The isolates were obtained from fecal samples streaked onto charcoal cefoperazone deoxycholate agar that were incubated microaerobically at 37°C for 48 h. Colonies that were suggestive of *Campylobacter* were confirmed as gram-negative curved bacilli by Gram stain. Isolates were then restreaked on Columbia sheep blood agar, identified as either *C. jejuni* or *C. coli* using a multiplex PCR assay (37), and frozen at –80°C.

All isolates were analyzed by PFGE using the standardized PulseNet protocol (30), with the *Salmonella* Braenderup H9812 strain restricted with XbaI as a size standard (16). Gels were made with 1% (wt/vol) SeaKem Gold agarose and electrophoresed for 18 h using an initial switch time of 6.8 s and a final switch time of 38.4 s for SmaI and an initial switch time of 5.2 s and a final switch time of 42.3 s for KpnI. PFGE profiles were analyzed and compared using BioNumerics version 4.0 (Applied Maths, Ghent, Belgium). Isolates were submitted to the PulseNet Aotearoa New Zealand *Campylobacter* database, where SmaI and KpnI pattern designations were assigned. PFGE clusters were defined as isolates with indistinguishable SmaI and KpnI patterns. These PFGE clusters were designated with a single letter (A through X) for PFGE clusters within either of the sampling periods, while PFGE cluster designations AA through EE were as-

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Spring 2002 sampling		PFGE-SmaI	PFGE-KpnI	Penner serotype	Cases		Percentages	
Cluster	PFGE Type				2002	2003	2002	2003
A	Sm0021:Kp0109			11	5	1	6%	1%
B	Sm0036:Kp0096			8,17	26	0	34%	0%
D	Sm0040:Kp0065			2	7	2	9%	2%
E	Sm0048:Kp0112			4 complex	2	0	3%	0%
F	Sm0063:Kp0101			1,44	2	0	3%	0%
G	Sm0081:Kp0018			2	2	0	3%	0%
Total clustered cases					44	3	57%	3%
Total Spring 2002 cases					77			
Autumn 2003 sampling		PFGE-SmaI	PFGE-KpnI	Penner serotype	Cases		Percentages	
Cluster	PFGE Type				2003	2002	2003	2002
H	Sm0001:Kp0016			untypeable	2	0	2%	0%
I	Sm0018:Kp0076			57	3	0	3%	0%
J	Sm0030:Kp0056			untypeable	3	0	3%	0%
K	Sm0037:Kp0104			2	3	0	3%	0%
L	Sm0038:Kp0037			1,44 (5); 23,36(1)	6	0	6%	0%
D	Sm0040:Kp0065			2	2	7	2%	9%
M	Sm0042:Kp0107			2	2	1	2%	1%
N	Sm0046:Kp0026			4 complex	5	0	5%	0%
O	Sm0050:Kp0083			4 complex	5	0	5%	0%
P	Sm0052:Kp0082			4 complex	2	0	2%	0%
Q	Sm0055:Kp0052			23,36	2	0	2%	0%
R	Sm0072:Kp0100			2	2	0	2%	0%
S	Sm0093:Kp0115			12	2	0	2%	0%
T	Sm0094:Kp0048			55	2	0	2%	0%
U	Sm0098:Kp0043			1,44	18	0	17%	0%
V	Sm0245:Kp0042			<i>C. coli</i>	14	0	13%	0%
X	Sm0050:Kp0039			2	2	0	2%	0%
Y	Sm0037:Kp0038			2	2	1	2%	1%
Total clustered cases					77	9	73%	12%
Total Autumn 2003 cases					106			

FIG. 1. PFGE clusters observed in the two sampling periods.

signed to isolates observed only once in both sampling periods. Heat-stable (HS) Penner serotypes were determined using a panel of 43 *C. jejuni* antisera produced in-house according to the method of Penner and Hennessy (28).

Isolates were matched to notified cases in the New Zealand EpiSurv notified diseases database using data provided by the clinical laboratory. Home addresses corresponding to the notified cases were mapped using ArcView version 8.2 (ESRI, Redlands, California). Christchurch City cases were defined as those whose patients had home addresses within a 15-km diameter of the city center. Odds ratios (OR) and 95% confidence intervals (CI) (3) were calculated for cases of the same PFGE type using, as the control group, cases of a different PFGE type from the same sampling period, unless otherwise specified.

RESULTS

Multiplex PCR analysis identified 168 of the isolates as *C. jejuni* and 15 isolates as *C. coli*. All of the *C. coli* isolates were recovered

in the second sampling period. SmaI PFGE patterns were generated for all 183 isolates, with 57 different patterns observed. Three of the isolates could not be restricted with KpnI, but of the remaining 180 isolates, 71 different KpnI patterns were generated. When combined, 77 different SmaI-KpnI combinations were observed. Forty-nine of the isolates produced SmaI-KpnI profiles which were observed only once in the study. The remaining isolates formed PFGE clusters of between 2 and 26 isolates (Fig. 1), with 57% of the isolates in weeks 1 to 8 and 73% of the isolates in weeks 23 to 30 forming PFGE clusters. Just nine PFGE subtypes representing 31 isolates were observed in both sampling periods (Fig. 2). Penner serotyping of the 168 *C. jejuni* isolates identified 17 different serotypes (Table 1). The

Cluster	PFGE Type	PFGE-SmaI		PFGE-KpnI		Penner serotype	Cases 2002	Cases 2003
		1000	2000	3000	4000			
A	Sm0021:Kp0109					11	5	1
D	Sm0040:Kp0065					2	7	2
M	Sm0042:Kp0107					2	2	1
Y	Sm0037:Kp0038					2	2	1
AA	Sm0018:Kp0077					Untypeable	1	1
BB	Sm0033:Kp0080					Untypeable, 3	1	1
CC	Sm0038:Kp0124					2	1	1
DD	Sm0056:Kp0050					23,36	1	1
EE	Sm0062:NOCUT					35	1	1

FIG. 2. PFGE types observed in both sampling periods.

four most common serotypes observed (2; 1,44; 8,17; and 4 complex) accounted for 71% of the isolates in this study. Except for serotypes 8,17 and 11, and those serotypes observed only once, all serotypes could be distinguished into multiple PFGE types, with relatively high diversity indices (Table 1). Among the PFGE groupings, except for two instances, all isolates within a cluster had the same Penner serotype.

It was possible to match 165 of the 183 isolates (90%) to notified cases in the New Zealand EpiSurv notified diseases database. Using the case notification date for comparison, these cases represented 29% of the total notified cases in this region for each sampling period. The ages of the patients in the cases ranged from 8 months to 80 years. Females comprised 51% of cases, and the ethnicity of patients in the cases was 92% European, 3% Maori, and 5% other.

Due to incomplete case histories, identifying common links between cases was difficult, and a common source between cases was not identified for any of the PFGE clusters. However, the epidemiological relevance of the PFGE clusters was supported by a number of similarities observed between cases in the three largest PFGE clusters, cases in some of the smaller

PFGE clusters, and, also, the unique isolates. Temporal clustering was observed for almost all of the isolates within a PFGE cluster (Fig. 3). For example, all 18 cases in the U cluster occurred within a 4-week period at the start of the autumn sampling period, and both cases in the F cluster were isolated in the same week (Fig. 3).

B cluster. One-third of the isolates (26 of 77) in the spring 2002 sampling period were of the same PFGE type (Sm0036:Kp0096) and all were Penner serotype HS:8,17 (Table 1). This PFGE type was not seen among any of the autumn 2003 isolates. Onset dates were recorded for 18 of these cases and ranged between 18 September 2002 and 5 November 2002. Two of the cases represented married patients, with an onset date one day apart, suggesting both are primary cases. Patients from eight of the cases did not respond to questionnaires, and the patient from one case was hospitalized. Home addresses were available for patients from 24 of the B cluster cases, who were more likely to reside within the Christchurch City region (OR = 6.2, 95% CI = 2.0 to 18.9). Ten of the cases were patients who reported contact with a dog and/or a cat (OR = 3.4, CI = 1.1 to 10.3).

U cluster. Eighteen cases in autumn 2003 had the PFGE profile Sm0098:Kp0043. All were serotype 1,44. Onset dates of the cases were between 15 February 2003 and 5 March 2003 (5 were of unknown onset date). Patients from three of the cases reported friends or family with symptoms, none of which were included in this study. The occupations of patients in 16 of the cases were available, and 4 were students at the same tertiary educational institute. None of the other patients whose cases were in the study listed their occupation as being at the same institute. Using “*Campylobacter* of subtype U” as the case definition and “student at this institute” as the occupation produces an odds ratio of 55 (95% CI = 2.8 to 1,075). Patients from five of the other cases with subtype U had occupations at similar tertiary institutes and hospitals. The ages of the patients in 13 of the cases in the U cluster were between 19 and 36 years, suggesting an age-related link between cases (OR = 11.7, CI = 3.4 to 40.4).

V cluster. Fourteen cases in autumn 2003 had the PFGE profile Sm00245:Kp0042 and represented 14 of the 15 *C. coli* isolates identified (none were identified from the 2002 isolates). Two of these cases were not notified, and the patients from three did not respond to questionnaires. Isolates in this

TABLE 1. Variation in PFGE types observed among the *C. jejuni* Penner serotypes

Serotype	Count	%	PFGE types	Diversity
2	39	23	21	0.54
1,44	29	17	8	0.28
8,17	26	15	1	0.04
4c	26	15	15	0.58
11	6	4	1	0.17
23,36	6	4	5	0.83
12	3	2	2	0.67
35	3	2	2	0.67
37	3	2	3	1.00
57	3	2	3	1.00
3	2	1	2	1.00
9	2	1	2	1.00
21	2	1	2	1.00
5	1	1	1	1.00
15	1	1	1	1.00
45	1	1	1	1.00
55	1	1	1	1.00
Untypeable	14	8	10	0.71
Total	168		81	

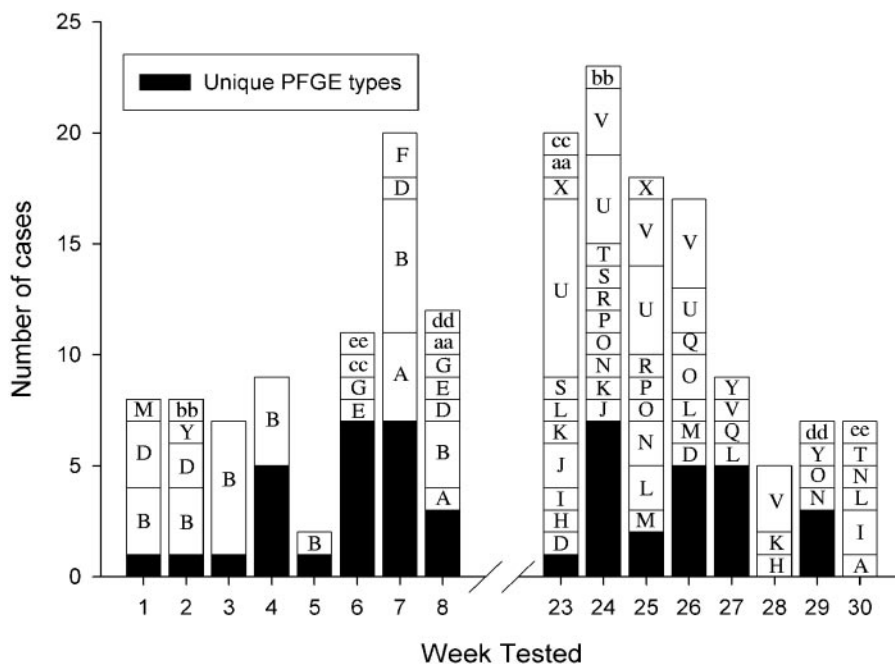


FIG. 3. Temporal clustering of *Campylobacter* isolates.

cluster had onset dates between 20 February 2003 and 11 March 2003. Eight of the 12 cases providing age data were from patients more than 37 years old (OR 2.4, CI 0.7 to 8.9). Patients from four of the nine cases reported recreational water contact, with three different swimming pools and one stream (OR 5.2, CI 1.1 to 23.6).

Smaller clusters. For many of the two- to three-case clusters, data were only available for one of the cases, making any interpretation impossible. For several of the small clusters, patients from the cases had similar ages. For example, cases in Q and I clusters (two each) were for patients both over 55 (OR = 16.9, CI = 0.8 to 364), R cluster case patients were aged 19 and 22 (OR = 80, CI = 3.4 to 1,889), and the patients from Y cluster cases were 9 and 12 years old (OR = 99, CI = 4.1 to 2,392). Patients from four of the six isolates in the D cluster lived in rural towns (OR = 1.9, CI = 0.3 to 11.0) as did those for four of the five cases in cluster A (OR = 3.9, CI = 0.4 to 36.6). Within the Christchurch City area some suggestive, although not conclusive, clustering of PFGE types was observed, with, for example, the patients from the two cases in cluster F living within 2.5 km of each other.

Unique isolates. Isolates which did not cluster with any others were more common from cases of patients not residing in the Christchurch City area (OR = 1.7, CI = 0.9 to 3.4). A response was recorded for patients from 150 of the cases regarding their recent travel, of which all 4 cases with travel beyond Australia and New Zealand had unique types of *Campylobacter* isolates (OR = 19, CI = 1.0 to 368).

DISCUSSION

Subtyping analysis. The most important finding of this study was that up to two-thirds of the isolates examined can be grouped into clusters of two or more isolates with indistin-

guishable PFGE profiles. Less than 30% of the notified cases in the time periods examined were actually part of this study, suggesting that examination of isolates from all cases would produce both additional and larger clusters of PFGE types. The definition of a subtype cluster used in this study was indistinguishable PFGE patterns with both SmaI and KpnI restriction enzymes. The combination of Penner serotyping and PFGE (usually with SmaI) has been used and recommended by a number of researchers (9, 27, 31). While Penner serotyping did break down some SmaI types in this study, KpnI digestion provided better discrimination both to identify differences and confirm similarities (Fig. 4A). Penner serotyping provided no additional level of discrimination beyond that which SmaI and KpnI digestion provided.

Digestion with KpnI was almost as discriminatory as SmaI and KpnI combined, suggesting that digestion with KpnI alone could be an effective approach, a conclusion also supported by Michaud et al. (24). In addition, the cost of the KpnI enzyme is less than 30% of the cost of SmaI, reducing the overall consumables cost of PFGE with KpnI to almost half that of PFGE with both SmaI and KpnI. However, even among the limited number of isolates in this study, isolates with indistinguishable or similar KpnI patterns can be further subgrouped when analyzed with SmaI (Fig. 4B). Internationally, most *Campylobacter* PFGE data have been generated using SmaI as the primary enzyme (PulseNet, CampyNet), perhaps partly because KpnI is a more difficult enzyme with which to achieve reproducible results. SmaI digestion, while less discriminatory, is sufficient in many cases to demonstrate that isolates are different. We believe that to demonstrate similarity, or that isolates are indistinguishable, digestion with two enzymes is essential, a finding also supported by other researchers (20, 27, 31). With sufficient international data, a reconsideration of

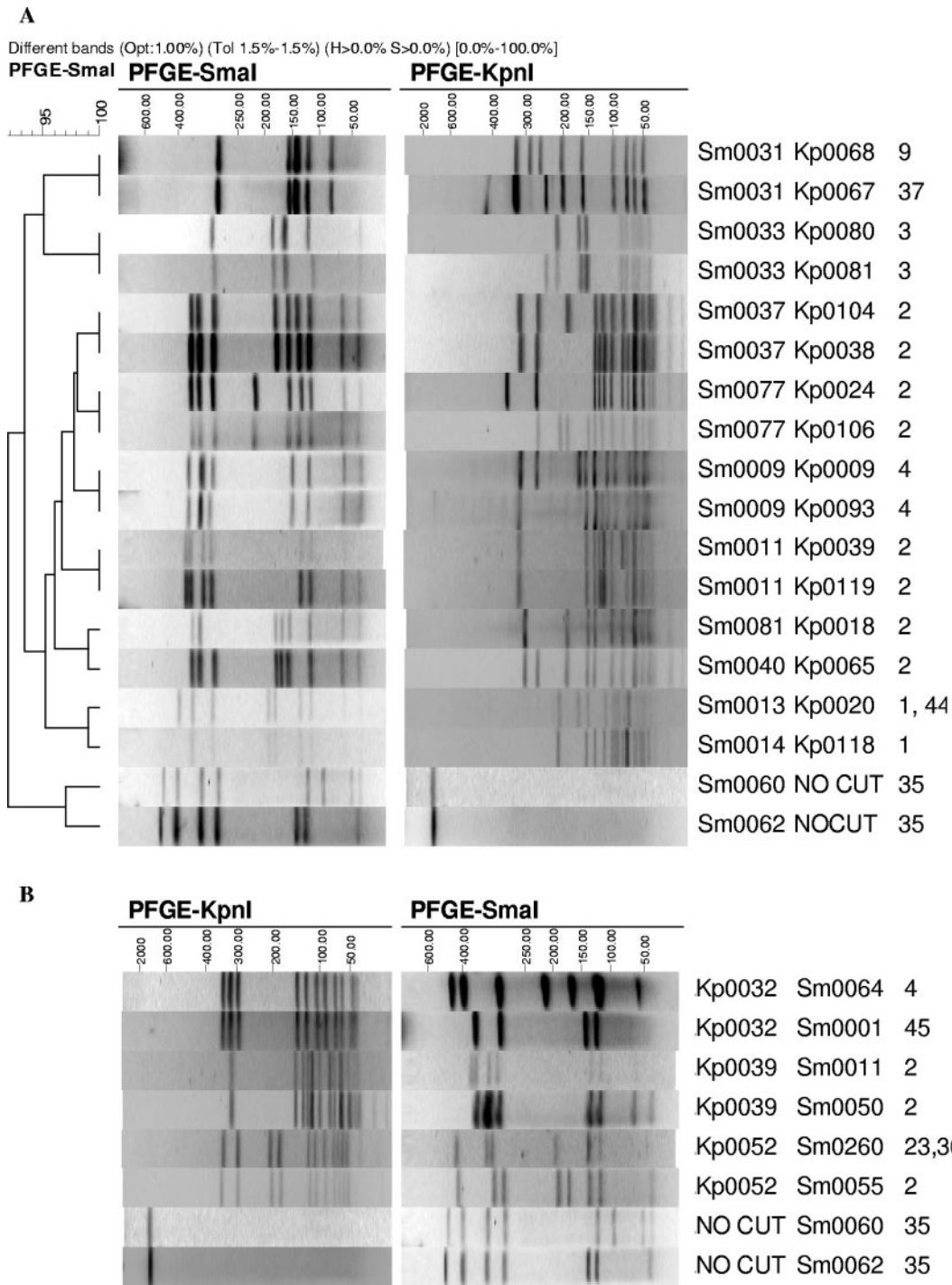


FIG. 4. PFGE profiles of selected isolates. (A) Illustration of the value of KpnI to distinguish SmaI patterns and to confirm relatedness among similar isolates. (B) Shown, conversely, are isolates indistinguishable using KpnI that can be distinguished using SmaI digestion.

using KpnI as the primary enzyme could be made in the future and useful comparisons with existing data still made.

Epidemiological analysis. In this study, 90% of isolates could be linked retrospectively to notified campylobacteriosis cases, which is a rate 10% higher than that reported previously in the Auckland area of New Zealand (33). The epidemiological information associated with these notified cases was ob-

tained retrospectively from data submitted to the New Zealand EpiSurv databases. These data are entered by local public health units based primarily on general practitioner notifications and postal questionnaires filled out by the patients in the cases themselves. No effort was made in this study to improve the quality of this initial data collection, nor to follow up potential clusters of isolates. Consequently the epidemiological

data associated with these notified cases were very incomplete with, for example, only 75% of the notified cases in this study having an onset date recorded. Identifying actual common sources of *Campylobacter* between cases in a cluster was not possible, and this study was not designed for that objective. Examination of the cases did, however, identify a number of commonalities between cases in a PFGE cluster, including temporal and spatial linkages. The use of geographic information system mapping could be especially useful, particularly if, in addition to home addresses, the place of work or school, main shopping areas, and restaurants frequented were also mapped. A number of demographic features were also shared by some cases in a PFGE cluster, including ages and, interestingly for the U cluster, occupations.

The value of subtyping is aptly illustrated when the correlation observed between subtype U cases and the occupation category (at a specific university) is considered without subtyping. In a situation where the case definition was simply campylobacteriosis, there would be patients from four cases that attend this university and 85 that do not. If a case-control study were to be conducted on the 89 cases, then we would require 89 matching controls. Based on a population in the study area of 450,000 and a university population of 10,000, then at least two of the controls would, by chance, be university students. An odds ratio in this case would be just 2.0 (95% CI = 0.4 to 11.5), rather than 51 (95% CI = 2.6 to 1024), which the more specific case definition of subtype U provides. If actual common sources are identified, subtyping will be essential to support investigative suspicions.

No *C. coli* isolates were identified in the first sampling period, but 15 were recovered in the second sampling period, 14 of which were of the same PFGE type. The suggested association of this cluster with recreational water contact is interesting, since *C. coli* is often associated with water (7, 13, 19). Pigs and sheep have also been identified to have relatively higher prevalence of *C. coli* (7). Recreational water contact has been previously identified as a source of campylobacteriosis both in New Zealand (4, 34) and overseas (32). The patients from the four cases involved had recreational contact with four different rivers, lakes, or swimming pools. Therefore, if recreational water contact was a source, it would suggest that this *C. coli* type is very common in New Zealand. We have insufficient data on *C. coli* PFGE types in New Zealand to make that assessment. Alternatively, recreational water contact may in fact be a surrogate of a shared lifestyle, which is reflected in some other unidentified common source for the cases in this cluster.

Case reports for many of the cases noted a number of food risk factors and suspected sources including particular foods eaten and restaurants frequented. Except in a very broad sense (e.g., chicken eaten) there were no direct matches in risk exposures for cases in a PFGE cluster. The specific risk factors noted do, however, provide numerous clues or starting points for secondary interviews of cases where the use of specific questions could more effectively identify or eliminate suspected sources. This type of approach has proven useful in interviews to identify sexual partners (5), is part of the cognitive interview technique used in criminal investigations (22), and is being applied to food-borne disease investigations (18).

PFGE cluster N (Sm0046:Kp0026) is indistinguishable from a familial common-source outbreak linked to precooked sau-

sages distributed by a particular butcher in Christchurch (14). These outbreak isolates were recovered 1 month after the final N cluster isolates, i.e., outside the sampling period of this study. It is possible that this described outbreak was actually larger and occurred over a longer time than reported by Graham et al. (14).

Michaud et al. (23) examined by KpnI PFGE 183 isolates of the 201 reported cases of campylobacteriosis in Quebec, Canada, over a 15-month period (rate, 63.1 cases per 100,000 people). They found 55% formed KpnI PFGE clusters of between 2 and 11 isolates (Dice similarity of 0.9) but found few epidemiological links and concluded that "molecular typing identifies relatively few additional cases representing potential common-source clusters." Hedberg et al. (15) reported that, of the 941 cases of campylobacteriosis reported among Minnesota residents in 1994, subtyping of 673 of these by PFGE identified 248 distinct PFGE patterns, 74% of which were represented by only one or two isolates. Most (87%) isolates could not be linked by time, geographic location, or PFGE type. They concluded that the large diversity of PFGE patterns limits the usefulness of PFGE for outbreak detection. In comparison, we found an apparently higher number of isolates belonging to subclusters. The rate of campylobacteriosis in the New Zealand study area is at least five times higher than in either Quebec or Minnesota. Whether this is due to a greater number of common-source outbreaks (and hence the higher proportion of isolates in clusters that we found) or whether other factors resulted in a higher proportion of cases being captured by the laboratory and surveillance system is an unresolved question. Whatever the explanation, New Zealand's high number of cases may, with the application of subtyping, make the identification of common-source outbreaks more practical. In this study, however, we were no more successful in identifying common sources than the researchers in the two studies cited above. We would suggest that this is not because those common sources do not exist, but because of limitations in the quality, quantity, and timeliness of the epidemiological data that were collected. For any disease, finding epidemiological links between cases is often a difficult and resource-intensive exercise. This is particularly so for *Campylobacter* cases which, although typically having an incubation period of 2 to 5 days, may have an incubation period extending up to 11 days (2, 25), with additional time delays until medical examination is sought, samples are analyzed, notifications occur, and investigations begin. The multitude of potential exposures and sources for *Campylobacter* infection complicates things further. Together with imperfect recall by the patients in cases, the possibility that some cases may be secondary to those with the primary exposure and the possibility that a case's patient may not have consumed the primary contaminated product but may have handled it or been exposed through cross-contamination will make identifying common sources difficult. Indeed, some clusters of types may not even have a common source if they represent a stable, endemic type, although this pilot study produced little evidence of this in New Zealand. If, as we suggest, PFGE clusters represent potential common-source outbreaks, finding common epidemiological linkages will be a difficult, but not impossible, task.

This study demonstrates that for New Zealand *Campylobacter* isolates, PFGE analysis is able to cluster isolates that

potentially represent common-source outbreaks. Despite limited and incomplete epidemiological information for each case, there was temporal, spatial, and demographic support for this hypothesis. We now propose genotyping all human isolates and targeted isolates from potential sources in defined temporal and spatial areas, in conjunction with prompt and reiterative investigation of clusters of cases. This approach may help identify and quantify the actual causes and sources of the campylobacteriosis in New Zealand and therefore provide support for appropriate interventions.

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