

# PCR-Based Restriction Fragment Length Polymorphism (RFLP) Analysis of *Campylobacter jejuni* Isolates from Humans, Chickens and Dogs in Northern Taiwan

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**ABSTRACT.** Two hundred and twenty strains of *Campylobacter jejuni* (70 human, 51 canine and 99 chicken strains) were isolated from September 2003 to September 2004 in northern Taiwan. These strains were subtyped by PCR-RFLP analysis of the flagellin (*FlaA*) gene. On the basis of restrictive digest, six types were identified with *AfaI*, seven types with *MboI* and five types with *HaeIII*. With the combination of these three enzymes, 47 distinct PCR-RFLP patterns were observed—25 each from human and chicken isolates, and 9 from canine isolates. In human strains, the most frequently occurring types were Cj-28 (14.3%), Cj-17 (10%), Cj-16 (8.6%), Cj-37 (7.1%) and Cj-46 (7.1%). In canine strains, the most prevalent types were Cj-1 (33.3%), Cj-26 (19.6%), Cj-3 (15.7%), Cj-2 (9.8%) and Cj-10 (9.8%). In chicken strains, the most frequently occurring types were Cj-46 (40.4%), Cj-29 (9.1%), Cj-45 (7.1%) and Cj-41 (5.1%). The results suggest that poultry is a source, but not the sole source, of *C. jejuni* infection in humans. Two RFLP types, Cj-17 and Cj-37, frequently occurring in human isolates in this study have also been found to be prevalent in human isolates in Japan, China and the Czech Republic, indicating a possible international clonal spread.

**KEY WORDS:** *Campylobacter jejuni*, canine, chicken, human, PCR-RFLP analysis.

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*Campylobacter jejuni* and, to a lesser extent, *C. coli* are major causes of gastroenteritis in humans [12, 23]. Localized *Campylobacter* infections of a variety of organs have also been rarely linked with Guillain-Barré syndrome (GBS), reactive arthritis, Reiter's syndrome, hemolytic uremic syndrome (HUS), and septicemia [1, 2]. Recently, immunodeficiency states (for example, immunosuppressive drugs, acquired immunodeficiency syndrome and the expanding elderly group) have made *C. jejuni* infections even more significant in the human population [25]. The majority of cases of human campylobacteriosis in developed countries are most probably caused by consumption of undercooked poultry, raw milk, or untreated surface water [4, 15]. It has been reported that the main source of *Campylobacter* spp. infection is the consumption of contaminated poultry products [7, 24]. The organism is present in a high percentage of clinically normal farmed poultry [5, 6]. Living in a household with a dog has also been identified as a significant risk factor for acquiring this disease [15, 25]. Most infected dogs act as asymptomatic reservoirs, shedding *Campylobacter* in their stools and ultimately infecting other animals by contaminating the environment [11].

Because of the multiple sources of infection in humans, there is a need for epidemiological typing methods to determine the source of infection and route of transmission. During the past decade, traditional typing methods, such as bacteriophage typing and serotyping, have been replaced by newer techniques, such as plasmid fingerprinting, ribotyping, polymerase chain reaction (PCR)-based methods and pulsed-field gel electrophoresis (PFGE) [32]. Pulsed-field gel electrophoresis (PFGE) is a highly discriminatory and reproducible method for the typing of *C. jejuni* [14, 28, 29]. Unfortunately it is labor intensive and time consuming. An

alternative method that is simple, fast, cheap and has sufficient discriminatory power is PCR-restriction fragment length polymorphism (RFLP) of the highly variable flagellin gene (*flaA*) [3, 10, 21, 26, 34]

*C. jejuni*, and to a lesser extent *C. coli* and *C. lari*, is an important cause of infectious enteritis in Taiwan [18]. The isolation rate of *Campylobacter* species ranged from 1.9% to 5.6% in the patients with diarrhea or gastroenteritis in Taiwan [9, 18, 31]. It has been shown that campylobacter enteritis occurs throughout the year in Taiwan, but predominantly occurred in the winter [18, 33] and majority of the campylobacter enteritis patients were younger than 5 years [18, 33]. It has also been shown that *C. jejuni* isolates from humans were significantly more susceptible than chicken strains to erythromycin, clindamycin and ciprofloxacin [17], but to the best of the author's knowledge, no molecular comparisons of the *C. jejuni* human isolates and animal isolates have been conducted in Taiwan. The objectives of this study were to evaluate the genomic diversity by PCR-RFLP of *C. jejuni* strains from humans, chickens and dogs in northern Taiwan and to determine possible epidemiologic relationships.

## MATERIALS AND METHODS

**Bacterial strains:** Seventy strains of *Campylobacter jejuni* were isolated from fecal specimens of patients treated for enteritis in Mackay Memorial Hospital (Taipei City) during the period from September 2003 to June 2005. Fifty-one strains of *C. jejuni* were isolated from rectal swab cultures collected from impounded dogs in six public animal shelters located in northern Taiwan. Each shelter was visited twice at least three months apart and all the dogs in the

shelters were sampled on each occasion. Another 99 strains of *C. jejuni* were isolated from a poultry processing plant that received broilers, mainly from northern Taiwan. The processing plant was visited twice at least three months apart and 20 chickens from each farm were sampled on each occasion.

**Culture and identification:** The bacteria were cultured on cefoperazone vancomycin amphotericin B (Campy CVA) agar plates (BBL, Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 42°C under microaerophilic conditions (10% CO<sub>2</sub>, 5% O<sub>2</sub>, and 85% N<sub>2</sub>). Preliminary identification of *Campylobacter* was based on phenotypic characteristics: colonial appearance, microscopic morphology, motility, oxidase and catalase reactions. The polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis for the *flagellin A* (*flaA*) gene previously described by Nishimura *et al.* [22] was used to further confirm and subtype *C. jejuni*.

**PCR-RFLP analysis:** The genomic DNA of *Campylobacter* colonies was extracted using a commercial Spin-Clean™ Genomic DNA Purification Kit (MBI Fermentas GMBH, St. Leon-Rot, Germany). For each PCR, 5 µl of the prepared DNA sample was added to 50 µl reaction mix containing 1.5 mM MgCl<sub>2</sub>, 200 µM each deoxynucleotide, 0.2 µM each primer, 1 Unit Taq polymerase (BerTaq polymerase, Bertec Corporation, Columbus, OH) and 5 µl 10X buffer (Viogene, Sunnyvale, CA). The two commercially synthesized primers were a 20 bp forward primer 5'-TAC-TACAGGAGTTCAAGCTT-3' and a 20 bp reversed primer 5'-GTTGATGTAAGTTGATTTTG-3'. Both primers have been described previously [22] and gave the predicted product of 702 bp representing the variable (V1) region. The PCR cycle used consisted of an initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 51°C for 35 sec, and an extension step at 72°C for 1 min; and final extension at 72°C for 10 min. PCR was performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA). PCR products (10 µl) were added in 10 µl reaction buffer

(Takara, Takara Shuzo Co., Ltd., Shiga, Japan) with 3 U of restriction enzymes *AfaI*, *MboI* and *HaeIII* (Takara, Japan). The reaction mixture was incubated at 37°C for 5 hr. The products of the digest were analyzed on 3% agarose gel (Promega, Madison, WI). One hundred bp DNA Ladder (Promega) was used as a standard size marker.

## RESULTS

The comparison of RFLP patterns of 70 *C. jejuni* isolates from humans, 51 from dogs and 99 from chickens revealed an expected high heterogeneity among strains. Based on restriction digest, six types of RFLP patterns were detected with *AfaI*, seven with *MboI* and five with *HaeIII* (Fig. 1). Combination of these enzymes revealed 47 types (Cj-1 to Cj-47), of which 25 occurred in human isolates, 11 in canine isolates and 25 in poultry isolates. The number of strains exhibiting each *flaA* RFLP pattern is listed in Table 1.

The most frequently detected type in human strains was Cj-28 (14.3%), followed by Cj-17 (10%), Cj-16 (8.6%), Cj-37 (7.1%) and Cj-46 (7.1%). Further types were detected only in one to four strains. In *C. jejuni* canine strains, nine patterns were found, the most prevalent being Cj-1 (33.3%), followed by Cj-26 (19.6%), Cj-3 (15.7%), Cj-2 (9.8%) and Cj-10 (9.8%). Further types were detected only in one strain. In *C. jejuni* poultry strains, 25 patterns were found, the most frequently occurring being Cj-46 (40.4%), followed by Cj-29 (9.1%), Cj-45 (7.1%) and Cj-41 (5.1%). Further types were detected only in one to four strains.

From these results, it is evident that the majority of the prevalent types (occurring in > 5% of isolates) were different among human, canine and poultry strains. The only overlapping types were Cj-41, which accounted for 5.7% of the human strains and 5.1% of the poultry strains, and Cj-46, which constituted 7.1% of the human strains and 40.4% of the poultry strains. Of the 25 types of *C. jejuni* detected in humans, only six types occurred, with various frequencies, in both human and dog, and only seven types occurred, with various frequencies, in both human and poultry. Thirteen

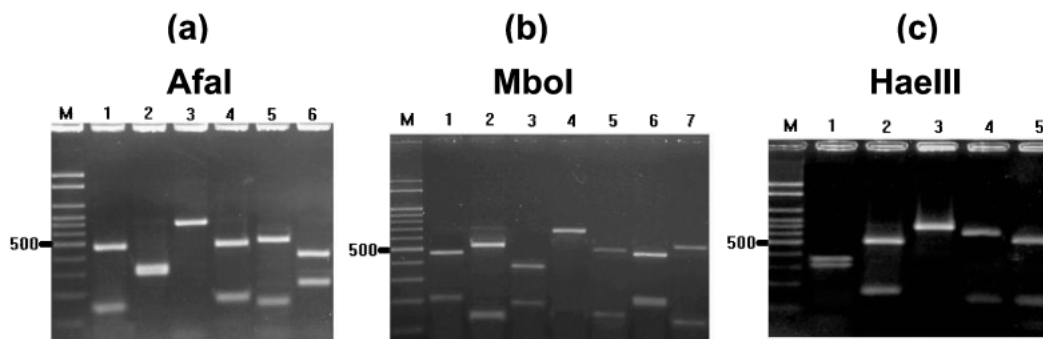


Fig. 1. Representative restriction endonuclease digests of the PCR-amplified, 702 bp flagellin A region of *Campylobacter jejuni*. RFLP patterns obtained by *AfaI*, six patterns (a), *MboI*, seven patterns (b) and *HaeIII*, five patterns (c) are shown. (a) Lane M: 100 bp DNA Ladder; lane 1–6: *AfaI* 6 patterns, 1–6, respectively. (b) Lane M: 100 bp DNA Ladder; lane 1–7: *MboI* 7 patterns, 1–7, respectively. (c) Lane M: 100 bp DNA Ladder; lane 1–5: *HaeIII* 5 patterns, 1–5, respectively.

Table 1. RFLP typing scheme profiles of *Campylobacter jejuni* isolates from human, dog and chicken

Restriction pattern number ( <i>AfaI</i> - <i>MboI</i> - <i>HaeIII</i> )	RFLP type	Source of strains			Total (n=220)
		Human (n=70)	Dog (n=51)	Chicken (n=99)	
(1-1-1)	Cj-1	0	17 (33.3%)	0	17 (7.7%)
(1-4-1)	Cj-2	0	5 (9.8%)	0	5
(1-6-1)	Cj-3	1	8 (15.7%)	0	9
(1-6-5)	Cj-4	0	1	0	1
(2-4-3)	Cj-5	4 (5.7%)	0	0	4
(2-5-3)	Cj-6	1	0	0	1
(2-7-3)	Cj-7	3	1	0	4
(3-1-2)	Cj-8	0	0	1	1
(3-2-2)	Cj-9	0	1	0	1
(3-2-3)	Cj-10	0	5 (9.8%)	0	5
(3-3-2)	Cj-11	0	0	2	2
(3-3-3)	Cj-12	0	0	2	2
(3-3-4)	Cj-13	0	0	4	4
(3-3-5)	Cj-14	0	0	4	4
(3-4-2)	Cj-15	4 (5.7%)	0	4	8
(3-4-3)	Cj-16	6 (8.6%)	0	0	6
(3-4-4)	Cj-17	7 (10%)	1	2	10
(3-4-5)	Cj-18	2	0	1	3
(3-5-3)	Cj-19	0	0	1	1
(3-5-4)	Cj-20	1	0	0	1
(3-6-2)	Cj-21	2	0	0	2
(3-6-3)	Cj-22	4 (5.7%)	1	0	5
(3-6-4)	Cj-23	1	0	0	1
(3-6-5)	Cj-24	1	0	0	1
(3-7-2)	Cj-25	1	0	0	1
(3-7-3)	Cj-26	1	10 (19.6%)	0	11 (5%)
(3-7-4)	Cj-27	1	0	1	2
(4-4-3)	Cj-28	10 (14.3%)	1	3	14 (6.4%)
(4-5-3)	Cj-29	0	0	9 (9.1%)	9
(4-6-3)	Cj-30	0	0	3	3
(4-6-4)	Cj-31	0	0	2	2
(5-2-3)	Cj-32	1	0	0	1
(5-3-2)	Cj-33	0	0	1	1
(5-3-3)	Cj-34	0	0	1	1
(5-3-4)	Cj-35	0	0	1	1
(5-4-2)	Cj-36	0	0	1	1
(5-4-3)	Cj-37	5 (7.1%)	0	0	5
(5-4-4)	Cj-38	1	0	1	2
(5-6-3)	Cj-39	1	0	0	1
(5-7-2)	Cj-40	1	0	0	1
(5-7-3)	Cj-41	4 (5.7%)	0	5 (5.1%)	9
(5-7-4)	Cj-42	0	0	1	1
(5-7-5)	Cj-43	0	0	1	1
(6-4-2)	Cj-44	2	0	0	2
(6-4-3)	Cj-45	0	0	7 (7.1%)	7
(6-4-4)	Cj-46	5 (7.1%)	0	40 (40.4%)	45 (20.5%)
(6-5-4)	Cj-47	0	0	1	1

types were solely detected in human strains, five solely in canine strains, and 17 only in poultry strains.

## DISCUSSION

Various restriction endonucleases have been chosen for the RFLP analysis of the *flaA* genes, such as *DdeI* [21], *HinfI* [26] and *EcoRI/PstI* [3]. Nishimura *et al.* [22] intro-

duced the used of three restriction endonucleases, *AfaI*, *MboI* and *HaeIII*. With the combination of these three enzymes, 25 *C. jejuni* types were found in human strains from China and Japan [22]. Later, 22 types were found in the Czech Republic, including 20 types in human strains and nine types in chicken strains [27]. In this study, we found many more types—a total of 47 types, with 25 in human strains, 11 in strains from stray dogs, and 25 in strains from

chickens. The finding of extensive genetic diversity in *C. jejuni* isolates is in accordance with previous reports.

Global spread of a major clone of HS-19 serotype *C. jejuni* has been documented [20]. Similarly, two RFLP types (Cj-17 and Cj-37) identified in this study might also spread internationally, since Cj-17 RFLP type (restriction pattern 3-4-4) accounts for 31.9% of Japanese human isolates [22], 28% of those in the Czech Republic [27], 11.8% of those in China [22] and 10% of Taiwanese human isolates, while Cj-37 (restriction pattern 5-4-3) was found in 19% of Czech [27], 17.4% of Japanese [22], 10.6% of Chinese [22] and 7.1% of Taiwanese human isolates, respectively.

Although Cj-17 RFLP type (restriction pattern 3-4-4) was common in human isolates from four countries, it only accounts for 2.0% and 1.8% of Taiwanese and Czech poultry isolates [27] and 2.0% of Taiwanese canine isolates. The Cj-37 molecular type (restriction pattern 5-4-3) was the most prevalent type in Czech poultry isolates (34%) [27] but not found in Taiwanese poultry and canine isolates. In Taiwan the most prevalent molecular type of poultry isolates, Cj-46 (pattern 6-4-4), accounted for 7.1% of the human isolates and was not found in canine isolates. The results indicate that poultry might be a source of human infection, but that sources other than poultry exist and that human *C. jejuni* infections are from multiple sources.

It is alarming that the most prevalent RFLP type of *C. jejuni* in stray dogs was Cj-1 (pattern 1-1-1); because it was shown by Nishimura *et al.* [22] that this molecular type is significantly associated with the HS-19 serotype. Most of *C. jejuni* isolates from Guillain-Barré syndrome (GBS) patients belong to the HS-19 serotype [13, 16, 30, 35]. In our study, we did not find the Cj-1 (pattern 1-1-1) type in human isolates. This may be because the bacteria were isolated from enteritis, not GBS, patients. This molecular type was also not found in human strains from Czech patients suffering from enteritis; nor, as in the present study, was it found in poultry strains [27]. On the other hand, the Cj-1 (pattern 1-1-1) type accounts for 5.8% and 10.6% of the human *C. jejuni* isolates from diarrheic patients in Japan and China, respectively [22]. In a study in northern China, *C. jejuni* infection was a risk factor for GBS [19], but an association between previous *C. jejuni* infection and GBS was not found in a study in southern Taiwan [8]. Thus the significance of the predominant Cj-1 RFLP type in stray dogs as a public health hazard for residents in Taiwan may need further study.

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