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SHORT PAPER

Molecular epidemiology of nalidixic acid-resistant campylobacter isolates from humans and poultry by pulsed-field gel electrophoresis and flagellin gene analysis

T. L. WU[1]*, L. H. SU[1], J. H. CHIA[1], T. M. KAO[1], C. H. CHIU[2], A. J. KUO[1] AND C. F. SUN[1]

[1] *Department of Clinical Pathology, Chang Gung Memorial Hospital, Taoyuan, 333, Taiwan*
[2] *Department of Paediatrics, Chang Gung Children's Hospital, Taoyuan, 333, Taiwan*

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SUMMARY

To investigate the potential of poultry products as the source of human infections associated with quinolone-resistant campylobacters, 140 human and 75 poultry isolates of nalidixic acid-resistant campylobacters were collected between 1996 and 1998, and analysed by two molecular typing methods. By the analysis of restriction fragment length polymorphism of the flagellin gene, 33 distinct patterns were obtained, with 18 of which shared by both human (89%) and poultry (93%) isolates. By the pulsed-field gel electrophoresis of *Sma*I-restricted macrofragments, 105 different profiles were obtained, and 11 were found in both human (40%) and poultry (23%) isolates. When the two typing methods were combined, 112 unique genotypes were obtained, 11 of which were shared by both populations, including 53 (38%) human isolates and 14 (19%) poultry isolates. Although domestic poultry products are still important sources of the quinolone-resistant campylobacter infections in humans, there are other factors that might contribute to these increasing infections simultaneously. A more stringent policy in the use of antimicrobial agents in food animals can no longer be ignored.

Campylobacter species are one of the most common etiologic agents of bacterial gastroenteritis worldwide. Previous reports indicated that a variety of avian species could harbor commensal campylobacters and thus acted as potential reservoirs for infections in humans [1]. Contamination of poultry carcasses and other poultry products by campylobacters during transport and processing further contributed to the vast-transmission of infections [2] associated with this genus.

Recently there have been increasing numbers of reports regarding the multiresistance of campylobacter isolates [3], particularly to fluoroquinolones [3–5], in many countries, including Taiwan [6]. Species with cross resistance to nalidixic acid were also reported [3, 4, 6]. A relatively higher rate of antimicrobial resistance was observed among food isolates of animal origins compared to that of clinical isolates [4]. It is hypothesized that, other than foreign travel, the use of fluoroquinolones in both clinical practice and animal husbandry may have led to this consequence [5]. However, there has been no molecular evidence documented in the literature so far.

The purpose of this study was to determine the genetical relationship between nalidixic acid-resistant campylobacter isolates from humans and domestic poultry isolates. Two molecular typing methods, pulsed-field gel electrophoresis (PFGE) of *Sma*I-restricted macrofragments and the analysis of restriction fragment length polymorphism of the flagellin gene, *flaA*, amplified by the polymerase chain reaction (PCR-RFLP), were used to provide a more definite evidence whether poultry contamination may

* Author for correspondence: Department of Clinical Pathology, Lin-Kou Medical Centre, Chang Gung Memorial Hospital, No. 5, Fu-Hsing Street, Kweishan, Taoyuan 333, Taiwan.

have contributed to the clinical infections and the increasing resistance to fluoroquinolones associated with the bacterium.

A total of 215 nalidixic acid-resistant campylobacter isolates were included in this study. Among these, 140 human isolates, including 117 *C. jejuni* and 23 *C. coli*, were collected from the stool specimens submitted to the Clinical Microbiology Laboratory of Chang Gung Memorial Hospital between 1996 and 1998. The other 75 strains, including 51 *C. jejuni* and 24 *C. coli*, were poultry isolates. These consisted of 53 isolates from 102 domestic chicken products (52%) purchased from retail markets among Taipei and Taoyuan districts, and 22 isolates, kindly provided by the Food Research Institute in Hsin-Chu County, from the specimens collected among the farms and processing companies. Another four isolates of *C. jejuni* from the chicken products were susceptible to nalidixic acid, and therefore were not included in this study. Thus, both human and poultry isolates were from generally similar areas including northern and southern Taiwan.

Campylobacter species were isolated and identified according to standard methods [7]. The identity of all campylobacter isolates were confirmed by a PCR method previously described by Oyofo et al. [8] to the genus level. Isolates with controversial biochemical reactions were identified by a previously described multiprimer PCR method [9] to the species level.

The resistance to nalidixic acid was examined by a standardized disk-diffusion test [10]. The interpretive criteria for *Enterobacteriaceae* and quality-control guidelines established by the National Committee for Clinical Laboratory Standards were used [10].

Flagellin gene analysis was performed according to the procedure described by Nachamkin et al. [11] with minor modifications. Crude DNA lysate was prepared by proteinase K treatment and followed by sonication. The PCR amplification was then performed in a Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT, USA). After digested overnight with 10U of *Dde*I (New England Biolabs, Beverly, MA, USA) at 37 °C, the products were loaded onto a 4% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME, USA) and electrophoresed at 120 V for 5 h in 0·5× Tris-borate-EDTA buffer (Amresco, Solon, OH, USA).

The procedures of restriction endonuclease digestion and pulsed-field gel electrophoresis described previously by Yan et al. [12] were performed with some modifications. Bacterial colonies grown overnight on Charcoal Cefoperazone Deoxycholate agar plates were harvested and cast into gel plugs. The

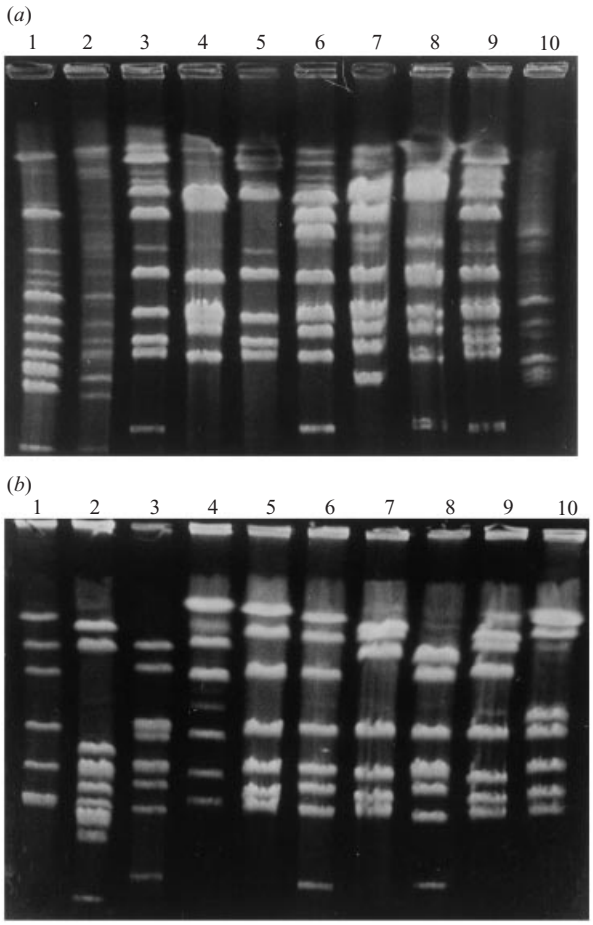

Fig. 1. Polymorphism of *Sma*I PFGE restriction profiles from campylobacter isolates that showing the same *flaA* RFLP patterns 3 (*a*) and 2 (*b*). (*a*) 1, 2, 10, *C. coli*; 3–9, *C. jejuni*; (*b*) 1, 3–10, *C. jejuni*; 2, *C. coli*.

plugs were treated in lysis solution (6 mM Tris-HCl [pH 7·6], 1 M NaCl, 100 mM EDTA [pH 7·5], 0·5% Brij, 0·2% deoxycholate, 0·5% sodium lauroyl sarcosine, 30 μg of RNase [DNase free] per ml, 1 mg of lysozyme per ml) at 37 °C for 24 h, and further incubated in ESP buffer (0·5 M EDTA [pH 9–9·5], 1% sodium lauroyl sarcosine, 500 μg of proteinase K per ml) at 50 °C for 24 h. After being washed thoroughly, thin slices of the DNA plugs were cut and incubated overnight with 10 U of *Sma*I (New England Biolabs, Beverly, MA, USA) at 25 °C. The plugs were then loaded into a 1% agarose gel prepared and run in 0·5× TBE buffer (Amresco, Solon, OH, USA). PFGE was carried out with a CHEF Mapper XA System (Bio-Rad Laboratories) at 14 °C. An auto-algorithm mode was chosen with the running molecular weights ranging from 40–400 kb. The gel was stained with ethidium bromide, and photographed with UV illumination. Any differences between PFGE profiles of

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Table 1. *Details of the 11 genotypes, their respective* flaA *RFLP patterns and* Sma*I PFGE restriction profiles that shared by both human and poultry isolates*

Genotype	*flaA* RFLP patterns	*Sma*I PFGE restriction profiles	Isolate no.	
			Human	Poultry
1	1	1	8	2
2	4	24	7	1
3	4	25	9	1
4	5	30	4	2
5	6	33	1	1
6	7	40	1	1
7	8	44	5	1
8	11	60	3	1
9	13	61	1	2
10	13	64	12	1
11	15	69	2	1

strains were considered significant, and types were arbitrarily defined accordingly.

During the preliminary collection for poultry isolates, 53 (52%) were obtained from 102 domestic chicken products, and 93% of the isolates were resistant to nalidixic acid. The results are compatible to those reported elsewhere [4] that poultry products were seriously contaminated with campylobacters, and the rate of antimicrobial resistance was very high which cannot be overlooked.

From the PCR-RFLP analysis, 33 distinct *flaA* patterns were found. Five prevailing ones were found among 76 (54%) human isolates, while 6 were found among 41 (55%) poultry isolates. Among the 22 *flaA* patterns identified in poultry isolates, 18 ($n = 70$, 93%) were also identified in human isolates ($n = 125$, 89%). The data supported the notion that poultry products are important food reservoirs of campylobacters for the infection in humans [13]. Using the same PCR-RFLP method as ours, a recent report also demonstrated a close association between the poultry isolates and the quinolone-resistant campylobacter infections in humans [5]. Compared to only 13 poultry isolates analysed in their study [5], our data involving 140 human isolates and 75 poultry isolates derived from a compatible geographical area and time period provide a more solid evidence for this relationship.

Highly diverse results were noted when the *Sma*I-restricted genomic DNA fragments were analysed by PFGE (Fig. 1). A total of 105 unique PFGE profiles were obtained from the 215 campylobacter isolates. Six profiles were prevalent among 53 (38%) human isolates, whereas another 3 were predominant among 16 (21%) poultry isolates. In contrast to the high similarity shown by *flaA* RFLP patterns, only 11 PFGE profiles were shared by both human ($n = 56$, 40%) and poultry ($n = 17$, 23%) isolates. Although PFGE was shown to provide a higher discrimination than the PCR-RFLP of the *flaA* gene [14] or other associated methods [12], recent reports have doubted the validity in using PFGE with a single restriction enzyme to delineate the genetic relatedness in campylobacters due to the insufficient fragments produced as well as the possibility of genomic rearrangement [15, 16]. In the present study, 21 (64%) of the 33 *flaA* RFLP patterns could be further separated into 2–13 different *Sma*I PFGE restriction profiles. Two ultimate examples were seen in *flaA* patterns 2 and 3, which were subsequently separated into 12 and 13 different *Sma*I PFGE restriction profiles, respectively (Fig. 1). Furthermore, 7 (7%) of the 105 *Sma*I PFGE restriction profiles could be further discriminated into two distinct *flaA* RFLP patterns. Our results appear to provide further evidence that the efficacy of PFGE for epidemiological studies of campylobacters is of some limitations.

A recent report illustrated that a combined genotyping system, consisting of the PCR-RFLP of the *flaA* gene and PFGE, may provide a more powerful tool for epidemiological investigations of campylobacters [17]. In the present study, 112 genotypes were obtained when both typing methods were combined. Among them, 65 genotypes were found only in human isolates, while another 36 were found only in poultry. The remaining 11 genotypes were shared by both populations, including 53 (38%) human isolates and

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14 (19%) poultry isolates (Table 1). The data implied that nearly 40% of human isolates may derive from poultry sources. On the other hand, since approximately 60% of the human isolates were possibly not associated with poultry isolates, there may be other factors that contributed to the increasing infections associated with campylobacters. Previous reports have indicated that the increase in infections associated with quinolone-resistant campylobacters was related to foreign travel [5]. Other food animals, such as pigs and cattle, also played a role as the reservoirs for this bacterium [18, 19]. Further epidemiological studies are needed to provide new explanations.

The development of resistance to ciprofloxacin [20] during therapy for *C. jejuni* infections has been recognized in several studies in the early 90s. As the resistance to nalidixic acid correlated well with that to ciprofloxacin [5], the nalidixic acid-resistant campylobacters were collected and analysed in this study to check whether there is a relationship between poultry products and the increasing quinolone-resistance in human infections. Our data suggested that quinolone-resistant campylobacters that caused human infections are at least in part derived from contaminated poultry products.

On the other hand, the false identification of *Campylobacter* species on the basis of susceptibility determined by disk diffusion with nalidixic acid has raised lots of concerns for years [21]. To avoid false identification, a multiprimer PCR [9] was adopted in the present study to verify the campylobacters to the species level more precisely. Without this effort, some of the *C. jejuni* isolates would have been falsely identified as *C. coli* or *C. lari*, and *vice versa*. It is therefore suggested that molecular methods, such as the multiprimer PCR used in this study, should be used to confirm the species of campylobacters in addition to the controversial biochemical testings. This is particularly important for epidemiological surveillance study of *Campylobacter* species.

Microbial resistance to antibiotics has become a global problem. The inappropriate use of antibiotics both in human medicine and in animal husbandry and agricultural industry may lead to the selection of antibiotic resistance in bacterial populations. The transmission of resistant bacteria from agricultural environments to humans may worsen such problems. By the molecular fingerprinting methods, the present study provided evidence that domestic poultry products are important sources of human infections associated with quinolone-resistant campylobacters. A more stringent policy in the use of antimicrobial agents in food animals is required.

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