Molecular typing and antimicrobial susceptibility profiles of *Campylobacter jejuni* isolates from north China

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To obtain the genotype and antimicrobial susceptibility profiles of *Campylobacter jejuni* isolates from north China, 93 *C. jejuni* isolates (56 isolates from patients with diarrhoea, 7 isolates from Guillain–Barré syndrome patients and 30 isolates from chicken stools) were selected for multilocus sequence typing (MLST), PFGE and drug resistance testing. A total of 49 sequence types (STs) were identified from the entire panel of 93 *C. jejuni* isolates. Fifty-six isolates belonged to 14 clonal complexes, while 37 isolates could not be assigned to any known clonal complex. The most frequently observed clonal complexes were ST-21 (11 isolates), ST-353 (10 isolates) and ST-443 (6 isolates). Fifty-three PFGE *Sma*I patterns were identified among 93 isolates. No erythromycin-, gentamicin- or streptomycin-resistant isolates were found among the 44 strains isolated in 2008. Resistance to nalidixic acid, levofloxacin and ciprofloxacin was observed in 100% (44/44) of the tested isolates. This study has shown the genetic characteristics of *C. jejuni* isolates in north China. In addition, overlapping clonal groups were defined by both MLST and PFGE for *C. jejuni* human and chicken isolates.

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INTRODUCTION

Campylobacter jejuni is a Gram-negative microaerobic bacterium. It is a major foodborne pathogen and causes human gastroenteritis throughout the world (Blaser *et al.*, 1983). In addition to the burden of disease due to gastroenteritis, *C. jejuni* infection is highly associated with the development of Guillain–Barré syndrome (GBS), an acute motor paralysis that may result from autoimmune antibodies against *C. jejuni* antigens (Nachamkin *et al.*, 1998; Sheikh *et al.*, 1998).

C. jejuni infections are common throughout China; however, data on the genetic characteristics of this pathogen are limited. In 2007, an outbreak of GBS was reported to be associated with the preceding *C. jejuni* infection in north China (Zhang *et al.*, 2010). Poultry are

usually considered to be the major reservoir for *C. jejuni* that causes human disease in developed countries (Wilson, 2002; Altekruuse *et al.*, 1994); however, a genetic correlation between the isolates from poultry and humans has not been reported in China. Subtyping methods used in bacteria provide an opportunity to better understand the population genetics, epidemiology and ecology of pathogens obtained from different sources. The aim of this study was to investigate the genotypes and antimicrobial susceptibility profiles of *C. jejuni* isolates from enteritis patients, GBS patients and poultry in north China.

METHODS

Bacteria and culture conditions. All 93 *C. jejuni* strains were collected from Beijing, Henan and Hebei in north China from 2003 to 2008. Among the 57 Beijing isolates, 35 were obtained from the stool specimens of patients with diarrhoea in one hospital and 22 strains were isolated from one chicken farm. Seven strains were obtained from stool specimens from GBS patients in one hospital in Shijiazhuang,

Abbreviations: GBS, Guillain–Barré syndrome; HS, heat-stable; MLST, multilocus sequence typing; ST, sequence type; UPGMA, unweighted pair group method with arithmetic averages.

Fig. 1. Histogram clonal complex distribution of 93 *C. jejuni* isolates from north China. CC, Clonal complex; no CC, does not belong to any known clonal complex. Grey bars, human isolates; black bars, chicken isolates.

Fig. 2. PFGE *Smal* dendrogram of 100 *C. jejuni* isolates. The levels of similarity were calculated using the Dice coefficient (1.2 % optimization and 1.5 % position tolerance) and UPGMA for the cluster analysis. Fifteen clonal groups were identified with an 80 % similarity cut-off level. The identification (ID), PFGE type, MLST type and clonal complex of the isolates are shown on the right.

Hebei province, and three strains were from conventionally reared poultry in the same region. Twenty-one strains were from stool specimens from patients with diarrhoea and five strains were isolated from chicken faeces from conventionally reared poultry in the same region in Henan. The PFGE and multilocus sequence typing (MLST) profiles of seven GBS outbreak-associated *C. jejuni* strains were added to this study panel (Zhang *et al.*, 2010).

The bacteria were cultured on Skirrow selective medium [Columbia Agar Base (Oxoid CM0331), supplemented with 5 % sheep blood and *Campylobacter* selective supplement (Oxoid SR0117)] at 42 °C in a microaerobic atmosphere (5 % O₂, 10 % CO₂ and 85 % N₂) for 72 h.

Bacterial identification was performed using Gram stain, oxidase and catalase tests, and a *C. jejuni*-specific PCR according to previously published reports (Klena *et al.*, 2004; Wang *et al.*, 2002).

MLST and PFGE analysis. MLST was performed by sequencing seven housekeeping gene loci (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*) using previously described primers (Dingle *et al.*, 2001). Briefly, each of the seven gene fragments was amplified by PCR with Pyrobest DNA Polymerase (TaKaRa Bio), purified, and sequenced with an ABI Prism 377XL DNA Sequencer (Applied Biosystems). The sequence data for each of the seven MLST loci were compared with sequences in the MLST database (www.pubmlst.org/campylobacter) to deter-

mine the allele number. The sequence type (ST) of each strain was assigned from the profiles of the seven alleles in the MLST database.

PFGE was performed according to the standard PulseNet PFGE protocol for *C. jejuni* (http://www.cdc.gov/pulsenet/protocols/campy_protocol.pdf). All the isolates in this study were analysed by digestion with *Sma*I and 47 isolates were analysed by digestion with *Kpn*I. PFGE cluster analysis was performed using BioNumerics (Version 4.1; Applied Maths) and the Dice coefficient was determined using the unweighted pair group method with arithmetic averages (UPGMA). Isolates were assigned to the same PFGE macrorestriction profile (mrp) when they clustered at greater than 95 % similarity (1.2 % optimization and 1.50 % position tolerance).

Penner serotyping. Thirty-four *C. jejuni* isolates were selected for serotyping with the heat-stable (HS) serotyping method of Penner using a commercial 25 Penner HS antisera set (*Campylobacter* Antisera Seiken Set; Denka Seiken) as previously described (Rautelin & Hanninen, 1999).

Antibiotic susceptibility test. The MICs for 44 isolates isolated in 2008 (24 isolates from patients with diarrhoea and 20 isolates from chicken faeces) of 10 antibiotics, gentamicin, chloramphenicol, erythromycin, tetracycline, streptomycin, ampicillin, metronidazole, nalidixic acid, levofloxacin and ciprofloxacin, were determined using E-test (AB Biodisk) according to the manufacturer's instructions and the Global Salm-Surv laboratory protocol training course. Briefly, freshly cultured bacteria were suspended in saline and adjusted to a 1.0 McFarland turbidity standard concentration. The bacterial suspension was streaked with a swab over the surface of a 15 cm Mueller–Hinton agar plate containing 5 % sheep blood by rotating the plate by 60° after each streak. The inoculation was completed by running the swab around the rim of the agar. The E-test strips were then applied to the agar surface using forceps. The plates were incubated at 37 °C in a microaerobic atmosphere for 48 h. The MIC was read at the point of the intersection between the growth zone edge and the E-test strip. The MIC breakpoints used in this study were those recommended for *Enterobacteriaceae* with a resistance MIC greater than or equal to 16 µg ml⁻¹ for streptomycin, gentamicin, ampicillin, chloramphenicol, tetracycline, nalidixic acid, ciprofloxacin and levofloxacin. The resistance breakpoint for erythromycin was set as MIC greater than or equal to 8 µg ml⁻¹, and MIC greater than or equal to 1 µg ml⁻¹ was designated resistant. For metronidazole, an MIC greater than or equal to 256 µg ml⁻¹ was designated highly resistant. *C. jejuni* ATCC 33560, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as control strains. In addition, the erythromycin-resistant *Campylobacter coli* strain WHO C-9.1 (MIC greater than 32 µg ml⁻¹) from the Global Salm-Surv External Quality Assurance System was set as the erythromycin-resistant control.

RESULTS

All of the isolates were identified as *C. jejuni* using the biochemical test and confirmed by PCR. In total, 49 STs were identified from the 93 isolates. Among them, 56 isolates were assigned to 34 STs that belonged to 14 clonal complexes. Thirty-seven isolates, which were assigned to 15 STs, did not belong to any known clonal complex. The most commonly identified clonal complex was ST-21 (eleven isolates, including five human isolates and six chicken isolates), followed by ST-353 (ten isolates, including nine human isolates and one chicken isolate) and ST-443 (six isolates). The most frequently occurring clonal complex in human isolates was ST-353 (nine

isolates). Eight chicken isolates belonged to ST-2274 and were not assigned to any known clonal complex. For the GBS patient isolates, three belonged to the ST-22 clonal complex, two belonged to the ST-443 clonal complex, and the remaining two isolates belonged to the ST-45 and ST-354 clonal complexes, respectively. The clonal complex distribution is shown in the histogram (Fig. 1).

Fifty-six profiles were determined from 100 *C. jejuni* isolates when PFGE was performed with a *Sma*I restriction digestion (Fig. 2). There were 15 groups of closely related isolates (greater than 80 % similarity in banding patterns) that were assigned a profile group number (S1–S15) (Fig. 2). The most common profile group was S12, which included seven isolates from chicken stools and four isolates from diarrhoea patient stools. Five groups had both human and chicken isolates. Eight profiles showed less than 70 % similarity with the other groups. Six of the eight GBS patient isolates (including the GBS outbreak-associated strain ICDCCJ07001) had identical profiles. Two of these isolates (HB-CJGZHANX and HB-CJGZHANGX) belonged to the S4 group, while four of them (HB-CJGZHANGB, HB-CJGXWM, HB-CJGLICH and HB-CJGLUL) had less than 80 % similarity with any of the groups. Forty-one profiles were identified from the 47 isolates by *Kpn*I digestion analysis (Fig. 3). Eight groups were identified that had isolates possessing over 80 % similarities with each other and were designated K1–K8. Two GBS isolates (HB-CJGLXCH and HB-CJGZHANX) had identical profiles and were closely related to another GBS isolate (HB-CJGZHANGB). Two other GBS isolates (HB-CJGZHANGX and HB-CJGLUL) also had a similar profile. Another GBS isolate (HB-CJGXWM), which had an identical *Sma*I digestion profile to another three GBS isolates, did not show any profile similarities with these GBS isolates in the *Kpn*I digestion pattern. Similar to the *Sma*I digestion analysis, the GBS isolate HB-CJGLICH did not show any significant similarity with the other GBS isolates based on the *Kpn*I digestion pattern (Fig. 3).

Nine serotypes were found among 25 isolates. Five isolates belonged to HS:6 and HS:7, while four isolates were from HS:3. Two GBS isolates belonged to HS:19 and another three GBS isolates belonged to HS:41, HS:37 and HS:3, respectively. Nine isolates could not be typed using the serotyping kit.

All 44 *C. jejuni* strains tested for antibiotic susceptibility were resistant to nalidixic acid, ciprofloxacin and levofloxacin. From these strains, 84 % (37/44), 73 % (32/44) and 59 % (26/44) of them were resistant to tetracycline, metronidazole and ampicillin, respectively. All tested isolates were susceptible to erythromycin, gentamicin and streptomycin. The MIC₅₀ and MIC₉₀ of each antibiotic are shown in Table 1.

DISCUSSION

Bacterial subtyping methods have not only improved the ability to detect and track outbreaks, but they also provide

Fig. 3. PFGE *KpnI* dendrogram of 47 *C. jejuni* isolates. The levels of similarity were calculated with the Dice coefficient (1.2 % optimization and 1.5 % position tolerance) and UPGMA for the cluster analysis. Eight clonal groups were identified with an 80 % similarity cut-off level. The ID, PFGE type, MLST type and clonal complex of the isolates are shown on the right.

tools to track the sources of bacterial contamination. Furthermore, the use of subtyping methods provides an opportunity to better understand the population genetics, epidemiology and ecology of pathogens from different sources.

PFGE has proven to be a useful and discriminatory tool for the investigation of outbreaks of gastroenteritis caused by *C. jejuni* (Fitzgerald *et al.*, 2001). In addition, since MLST

indexes the variation in housekeeping genes, it has also been a useful method for investigating the DNA sequence diversity in *C. jejuni* (Dingle *et al.*, 2001; Sopwith *et al.*, 2006). In this study, PFGE was shown to be a more powerful tool than MLST by providing greater discrimination between sequences (53 STs from 100 isolates vs 56 PFGE patterns from 100 isolates). Overlapping clonal groups were defined by both MLST and PFGE for human and

Table 1. MIC₅₀ and MIC₉₀ (µg ml⁻¹) of 10 antibiotics for 44 *C. jejuni* isolates

Antibiotic	MIC range	MIC ₅₀	MIC ₉₀
Gentamicin	0.125–>256	0.75	1.5
Chloramphenicol	0.25–>256	1.5	32
Erythromycin	0.125–4	1	3
Tetracycline	0.125–>256	>256	>256
Streptomycin	0.1–8	1	2
Ampicillin	0.25–>256	16	>256
Metronidazole	0.064–>256	48	>256
Nalidixic acid	>256	>256	>256
Levofloxacin	>32	>32	>32
Ciprofloxacin	>32	>32	>32

chicken isolates. Although our collection may not be large enough to be representative of the diversity in *C. jejuni*, we identified the ST-21 complex as the most common Chinese lineage, which included most human and chicken isolates. This predominant clonal complex was corroborated by the dominant PFGE pattern group S12 (Fig. 2). The ST-21 complex is the prevalent complex in the general population structure of *C. jejuni* and is widespread across multiple hosts. This complex has previously been shown to be associated with infections in humans and with livestock and environmental sources, such as chickens, cattle and contaminated milk and water. As a result, it has been shown to be frequently associated with environmental and foodborne transmission (Clark *et al.*, 2005; Sopwith *et al.*, 2006; Dingle *et al.*, 2001). Based on our results, the transmission and infection sources of *C. jejuni* in China may share the same mode as in other countries.

Serotyping with the Penner HS method has been successfully used in the past to identify the subtype of *C. jejuni* that is associated with GBS (Kuroki *et al.*, 1993). In the present study, 34 *C. jejuni* strains from GBS patients and patients with diarrhoea were selected for the serotyping analysis. However, we observed poor discriminatory ability with this test. The Penner HS test was only able to subtype 74 % (25/34) of the isolates, and eight of them had cross-reactivity between antigens. With the exception of the three untyped isolates, two GBS isolates were from serotype HS:19 and the other three were serotypes HS:41, HS:37 and HS:3, respectively. HS:19 and HS:41 *C. jejuni* strains were previously shown to be highly associated with GBS (Rees *et al.*, 1995; Goddard *et al.*, 1997). In this study, two GBS-associated isolates were HS:19 and belonged to ST-22. These results were consistent with previous reports that only found this serotype in GBS-related strains (Islam *et al.*, 2009). One GBS isolate was HS:41 and ST-362 and has been reported as the second most prevalent complex found in GBS strains. Four GBS outbreak-associated *C. jejuni* isolates were shown to have the same PFGE and MLST profiles, which indicated that the same colony strain was responsible for the infection.

High resistance rates to quinolones, tetracycline and metronidazole were found in both human and chicken

isolates in the recently isolated strains in this study. Therefore, the selective pressure of antibiotics on *C. jejuni* strains in the environment in China needs to be considered in greater detail.

Since the number of isolates in this study was relatively small and isolates were only obtained from four regions in north China, we acknowledge that there may be a selective bias in this study. However, we believe this to be the first report on the molecular characterization of *C. jejuni* isolates from both humans and chickens in China. Based on the subtyping and molecular characterization of *C. jejuni* isolates in this study, our data may facilitate the development of pathogen tracking and *C. jejuni* infection prevention in China.

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