Original Article

Genetic and Antibiotic Resistance Characteristics of Campylobacter jejuni Isolated from Diarrheal Patients, Poultry and Cattle in Shenzhen *

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Abstract

Objective To investigate genetic and antibiotic resistance characteristics of Campylobacter jejuni (C. jejuni) isolated from Shenzhen.

Methods Multilocus sequence typing and agar dilution methods were used to define the genotype and antibiotic resistance of C. jejuni, respectively.

Results In total, 126 C. jejuni strains were isolated. The prevalence of C. jejuni was 5.3% in diarrheal patients. The prevalence in poultry meat (36.5%) was higher than that in cattle meat (1.1%). However, the prevalence in poultry cloacal swabs (27.0%) was lower than that in cattle stool (57.3%). Sixty-two sequence types were obtained, among which 27 of the STs and 10 alleles were previously unreported. The most frequently observed clonal complexes were ST-21 (11.9%), ST-22 (10.3%), and ST-403 (7.1%). ST-21, ST-45, ST-354, ST-403, and ST-443 complexes overlapped between isolates from patients and cattle, whereas ST-45 and ST-574 complexes overlapped between isolates from patients and poultry. All C. jejuni were resistant to at least one antibiotic. The highest resistance rate was toward ciprofloxacin (89.7%), followed by tetracycline (74.6%), and nalidixic acid (69.0%).

Conclusion This is the first report of the genotypes and antibiotic resistance of C. jejuni in Shenzhen. Overlapping clonal complexes were found between isolates from patients and cattle, and between patients and poultry.

Key words: Campylobacter jejuni; Multilocus sequence typing; Antibiotic resistance; Poultry; Cattle; Diarrheal patients

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Biographical note of the first author: JU Chang Yan, female, born in 1982, MD, majoring in pathogenic microbiology.
INTRODUCTION

Campylobacter jejuni (C. jejuni) is one of the major causes of bacterial gastroenteritis in both developed and developing countries\cite{1-3}. People become infected by consuming contaminated food, particularly poultry meat, raw milk, and water\cite{4-9}. In addition to gastrointestinal illness, C. jejuni may also lead to autoimmune conditions known as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome\cite{8}. Zhang et al.\cite{9} reported an outbreak caused by C. jejuni in Jilin, China during 2007, in which 32 patients with GBS were recorded, indicating that Campylobacter is a threat to human health in China.

C. jejuni has a very wide distribution in animals. Poultry is considered as the major reservoir of Campylobacter. The prevalence of C. jejuni in chicken meat was reported as 13.7% (31/227) in Tianjin\cite{10} and 17.2% (52/302) in central China\cite{11}. In addition, Campylobacter can also inhabit cattle, pigs, ducks, dogs, and other wild and domestic animals\cite{12-15}, making it challenging to trace the sources and routes of transmission.

Antibiotic resistance of C. jejuni is an increasing concern\cite{16}. Infections by antibiotic resistant strains are usually associated with a longer illness duration, a higher risk of invasive disease, and higher healthcare costs\cite{17-19}. In Shanghai (Eastern China), more than 96% of the Campylobacter spp. isolates are resistant to quinolones and tetracyclines\cite{20}. In central China, even higher resistance of C. jejuni isolates from retail chicken meat has been reported: 100% resistance to ciprofloxacin and 90% resistance to tetracycline\cite{11}.

Shenzhen is the fastest-growing major city in China, with a highly concentrated population and high consumption of meat. Hence, studying the genetic and antibiotic resistance characteristics of C. jejuni in Shenzhen is important. However, there is little reported data about C. jejuni in Shenzhen.

The aim of this study was to characterize the genetic profile and antibiotic resistance of C. jejuni isolated from cattle, poultry, and patients with diarrhea in Shenzhen.

METHODS

The study was approved by the institutional review board of the Nanshan Center for Disease Control and Prevention. Written informed consent was obtained from each adult patient or from the children’s parents or guardians.

Sample Collection

During March 1 to November 20, 2016, we enrolled patients who were outpatients presenting with acute diarrhea, which was defined as ≥ 3 passages of watery, loose, mucosal, or bloody-stools during a 24 h period. Stool samples from child patients (≤ 5 years old), adolescent patients (5-17 years old), and adult patients (≥ 18 years old) were collected from four medical facilities, designated as Hospital A, B, Health care C, and Maternal and children’s hospital D. A total of 437 patients with diarrhea were enrolled, including 173 child patients, 58 adolescent patients, and 206 adult patients. In total, 437 stool samples were collected.

The poultry meat samples were collected from three retail markets (n = 63), and the cattle meat samples were collected from a slaughterhouse (n = 30) and three retail markets (n = 63). The poultry cloacal swabs (n = 74) were collected from a poultry wholesaler, while the cattle stool samples (n = 103) were collected from a cattle farm (n = 64) and a slaughterhouse (n = 39).

For the stool samples, from both patients and cattle, approximately 0.5 g of stool was collected. Both the stool samples and the cloacal swabs were transferred in Cary-Blair medium at 4 °C within 8 h for bacteria isolation. Approximately 200 g of meat samples were collected in a sterile plastic bag, and transported to laboratory at 4 °C within 4 h for isolation.

Culture, Isolation, and Identification

Two Campylobacter isolation kits (ZC-CAMPY-001 and ZC-CAMPY-002, Qingdao Sinova Biotechnology Co. Ltd, Qingdao, China) were used to isolate Campylobacter from stool and meat samples, respectively\cite{21}. Briefly, the stool or cloacal swab samples were inoculated into enrichment broth. Each meat sample was placed in a sterile plastic bag containing enrichment media. The enrichment broth was then incubated at 42 °C in a microaerophilic atmosphere (5% O₂, 10% CO₂, and 85% N₂) for 48 h. Approximately 300 µL of the enrichment medium was then spotted onto the surface of a membrane filter from the kit, which was passed onto Karmali and Columbia agar plates. Suspected colonies were picked after incubation at 42 °C for 48 h in a microaerophilic atmosphere. Finally, Gram staining and biochemical tests were conducted. Species identification was performed using PCR, according to a previously described method\cite{22}.
Genotype and antibiotic resistance of Shenzhen C. jejuni isolates

Multilocus Sequence Typing

MLST was performed according to the Pubmlst protocol (https://pubmlst.org/campylobacter/info/primers.shtml).[13] Briefly, genomic DNA was extracted using a commercial kit (QIAamp DNA mini kit, QIAGEN, German), and seven housekeeping genes (aspA, glnA, gltA, glyA, pgm, tkt, and uncA) were amplified using PCR. The amplification reactions were performed in a 20 µL mixture containing 10 µL of 2x PCR master (Quanshijin Biotechnology Ltd. Corp., Beijing, China), 0.5 µL of forward and reverse primer, 1 µL of DNA template, and 7.5 µL of pure water. PCR was performed as 1 cycle of 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 2 min at 50 °C, and 1 min at 72 °C; and a final extension of 5 min at 72 °C. After amplification, 2 µL of the PCR product was subjected to electrophoresis through a 1.5% agarose gel. Then the PCR products were the purified and sequenced. The sequence was subjected to a BLAST search against the PubMLST database, after which the allele numbers were assigned and sequence types (ST) and clonal complexes (CC) were determined. Novel alleles and STs were submitted to the PubMLST database.

Antibacterial Susceptibility Testing

Commercial kits (Zhongchuang Biotechnology Ltd. Corp., Qingdao, China) were used to determine the minimum inhibition concentration (MIC) of all C. jejuni isolates. The agar dilution method was used,[12] and the breakpoints used for each antibiotic were those recommended by National Antimicrobial Resistance Monitoring System (NARMS-2014): Erythromycin (≥ 32 µg/mL), azithromycin (≥ 8 µg/mL), nalidixic acid (≥ 64 µg/mL), ciprofloxacin (≥ 4 µg/mL), gentamycin (≥ 8 µg/mL), streptomycin (≥ 16 µg/mL), chloromycetin (≥ 32 µg/mL), florfenicol (≥ 8 µg/mL), tetracycline (≥ 16 µg/mL), telithromycin (≥ 16 µg/mL), and clindamycin (≥ 8 µg/mL).

Briefly, fresh C. jejuni colonies were suspended in an NaCl solution (0.9%) to obtain a suspension with 0.5 McFarland turbidity. One hundred microliters of the suspension were diluted in 900 µL NaCl solution (0.9%) resulting in a concentration range of 10^5-10^7 colony forming units (cfu) per mL. Each well was dispensed with 2 µL of the suspension. The plates were incubated at 37 °C for 48 h in microaerophilic atmosphere. The MIC was defined as the lowest concentration of each antibiotic that could prevent visible growth of C. jejuni, and the MIC<sub>50</sub> was defined as the MIC required to inhibit the growth of 50% of the C. jejuni isolates. In addition, multi-drug resistance (MDR) was defined as resistance to three or more antibiotics in this study. C. jejuni ATCC33560 was used as the control.

Data Analysis

Statistical analysis was carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2016. The prevalence and MDR rate of C. jejuni isolated from different sources were compared using the Chi-square test (at a confidence level of 95%). The Simpson’s index (D), described previously,[11] was used to determine the genetic diversity of C. jejuni genotypes. MIC<sub>50</sub> values were calculated using Microsoft Excel 2016.

RESULTS

A total of 126 C. jejuni strains were isolated from samples taken in this study. Multilocus sequence typing and antibacterial susceptibility testing were successfully performed in all isolates.

Prevalence of C. jejuni from Poultry, Cattle, and Patients with Diarrhea

The prevalence of C. jejuni from the various sources is shown in Table 1. Statistically, the isolation rate from poultry meat was significantly higher than that from cattle meat (χ<sup>2</sup> = 36.222, P < 0.001). However, the isolation rate from poultry meat was significantly lower than that from cattle stool (χ<sup>2</sup> = 15.950, P < 0.001).

The patients with C. jejuni ranged in age from 7 months to 43 years old. Three strains were isolated from 7, 8, and 9 month-old infants. The overall prevalence of C. jejuni in the patients with diarrhea was 5.3% (23/437). Separately, the prevalence of C. jejuni was 4.0% (7/173) in child patients, 6.9% (4/58) in adult patients, and 3% (2/66) in elderly patients.

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of Samples</th>
<th>No. of Isolates</th>
<th>Prevalence of C. jejuni (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry meat</td>
<td>63</td>
<td>23</td>
<td>36.5</td>
</tr>
<tr>
<td>Cattle meat</td>
<td>93</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Cattle feces</td>
<td>103</td>
<td>59</td>
<td>57.3</td>
</tr>
<tr>
<td>Poultry cloacal swab</td>
<td>74</td>
<td>20</td>
<td>27.0</td>
</tr>
<tr>
<td>Patients’ feces</td>
<td>437</td>
<td>23</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Table 1. Prevalence of C. jejuni in Poultry, Cattle, and Diarrheal Patients in Shenzhen
in adolescent patients, and 5.8% (12/206) in adult patients (≥ 18 years old). No statistically significant difference was found in the prevalence of *C. jejuni* between the different age groups ($\chi^2 = 0.955$, $P = 0.620$).

**Multilocus Sequence Typing**

We identified 62 STs among the 126 isolates (Table 2). Among them, 27 STs were not reported previously, and 10 novel alleles were found in this study: GlyA734, 735, 736, 737, and 738; pgm894, 896, 897, and 898; and tkt699. Isolates from patients with diarrhea had the highest proportion of new STs (12/23, 52%), followed by those isolated from poultry sources (22/43, 51.0%), and those from cattle sources (7/60, 11.7%).

Eighty-six isolates belonged to 13 clonal complexes, while 40 isolates could not be assigned to any known clonal complex. The most frequently observed clonal complexes were ST-21 (11.9%), ST-22 (10.3%), and ST-403 (7.1%).

Separately, 27 STs were obtained from 43 poultry isolates, and 18 STs were obtained from 60 cattle isolates. The calculated Simpson’s index of the STs from the poultry isolates was 0.977, versus 0.912 from the cattle isolates.

| Table 2. ST Type of 126 C. jejuni Isolates from Shenzhen |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Poultry Source  |                  | Cattle Source  | Patients Source |
|                                  | ST              | Clone Complex   | Number of     | ST              | Clone Complex   | Number of     | ST              | Clone Complex   | Number of     |
|                                  |                 |                 | Isolates      |                 |                 | Isolates       |                 |                 | Isolates       |
| 464                              | 464             | 464             | 3              | 464             | 464             | 19             | 21             | 7              | 51              | 443             | 1              |
| 2145                             | 574             | 1               | 2              | 22              | 22              | 13             | 45             | 45             | 1              | 403             | 403             | 2              |
| 2895                             | 574             | 2               | 45             | 45              | 45              | 2              | 51             | 51              | 2              | 1811            | 21              | 1              |
| 4324                             | NA             | 1               | 51             | 443             | 443             | 1              | 61             | 61              | 6              | 1919            | 52              | 1              |
| 6493                             | NA             | 2               | 61             | 61              | 61              | 6              | 113            | 460             | 1              | 2131            | NA              | 1              |
| 6607                             | NA             | 3               | 113            | 460             | 460             | 1              | 257            | 257             | 6              | 2132            | NA              | 1              |
| 6909                             | 574             | 2               | 257            | 257             | 257             | 6              | 403            | 403             | 5              | 4265            | 52              | 1              |
| 6913                             | 464             | 1               | 354            | 354             | 354             | 3              | 583            | 45              | 3              | 8003            | 403             | 1              |
| 6962                             | NA             | 1               | 583            | 45              | 45              | 3              | 2089           | NA              | 2              | 8880            | 574             | 2              |
| 7456                             | NA             | 1               | 2089           | NA              | NA              | 2              | 5799           | 443             | 1              | 8901            | 52              | 1              |
| 8089                             | NA             | 2               | 5799           | 443             | 443             | 1              | 6500           | 21              | 1              | 8904            | 45              | 1              |
| 8847                             | NA             | 1               | 6500           | 21              | 21              | 1              | 45             | 2              | 8905            | 354             | 1              |
| 8877*                            | 45             | 2               | 7469           | 464             | 464             | 1              | 7695           | 21              | 1              | 8906            | 52              | 1              |
| 8879                             | NA             | 2               | 7695           | 21              | 21              | 1              | 574            | 1               | 2              | 8907*           | NA              | 1              |
| 8880                             | 574             | 1               | 8896*          | NA              | NA              | 2              | 8898           | 21              | 4              | 8908            | NA              | 1              |
| 8881*                            | NA             | 1               | 8898           | 21              | 21              | 4              | 8934           | 464             | 1              | 8909            | NA              | 1              |
| 8883*                            | NA             | 2               | 8934           | 464             | 464             | 1              | 4              | 8910            | 21              | 1              |
| 8884                             | NA             | 4               | 4              | 8911*           | 362             | 1              | 8935*          | 403             | 1              |
| 8886                             | NA             | 1               | 2              | 8935*           | 403             | 1              |
| 8887                              | NA             | 2               | 2              | 8935*           | 403             | 1|
| 8889                              | NA             | 2               | 2              | 8935*           | 403             | 1|
| 8892                              | NA             | 1               | 2              | 8935*           | 403             | 1|
| 8893*                             | NA             | 1               | 2              | 8935*           | 403             | 1|
| 8894*                             | NA             | 1               | 2              | 8935*           | 403             | 1|
| 8895                              | NA             | 1               | 2              | 8935*           | 403             | 1|
| 8936                              | NA             | 1               | 2              | 8935*           | 403             | 1|
| Total                             | 43             | 60              | 23             |

Note. *NA, not assigned; ^New STs are given in bold; ^ST contains the new allele unreported previously.
Moreover, ST-21, ST-45, ST-354, ST-403, and ST-443 overlapped between isolates from patients and cattle, while ST-45 and ST-574 overlapped between isolates from patients and poultry. In addition, ST-45 was the only clonal complex found in all three sources.

**Antibacterial Susceptibility Testing**

The MICs of the quality control strain (*C. jejuni* ATCC 33560) were within the reference quality control range. Overall, all the isolates were resistant to at least one antibiotic. The highest resistance rate was detected for ciprofloxacin (89.7%), followed by tetracycline (74.6%), and nalidixic acid (69.0%). The resistance rates of *C. jejuni* from different sources are shown in Figure 1.

The MIC₅₀ s of nalidixic acid and tetracycline to all *C. jejuni* isolates were ≥ 64 µg/mL, while the MIC₅₀ s of erythromycin, azithromycin, gentamycin, telithromycin, and clindamycin to all *C. jejuni* isolates were ≤ 2 µg/mL. For the different sources, *C. jejuni* from poultry had higher or equal MIC₅₀ s compared with those from patients or cattle against all 11 antibiotics.

For multidrug resistance (MDR), 88.4% of *C. jejuni* isolated from poultry were resistant to four to nine antibiotics. Among them, one strain was resistant to nine antibiotics, including erythromycin, azithromycin, nalidixic acid, ciprofloxacin, gentamycin, streptomycin, chloromycetin, telithromycin, and clindamycin. This strain was isolated from a chicken cloacal swab from a poultry wholesaler. Similarly, 73.9% of isolates from patients with diarrhea were resistant to four to eight antibiotics. The most prevalent resistance pattern was combined nalidixic acid, ciprofloxacin, florfenicol, and tetracycline resistance (30.4%). In particular, 65.2% of isolates from patients were resistant to florfenicol. In contrast, all *C. jejuni* from cattle were resistant to less than five antibiotics. None of the isolates from cattle were resistant to erythromycin, azithromycin, gentamycin, telithromycin, or clindamycin.

Statistically, the MDR rate among the isolates from poultry (97.6%, 42/43) was significantly higher than that from cattle (60.0%, 36/60) ($\chi^2 = 19.343, P < 0.001$). No statistical significance was observed between the MDR rate of isolates from poultry and patients (91.3%, 21/23) ($\chi^2 = 1.401, P = 0.236$).

**DISCUSSION**

*C. jejuni* is one of the major causes of gastroenteritis worldwide. However, there is limited information about the genetic and antibiotic resistance characteristics of *C. jejuni* in Shenzhen, which makes tracking the origin of transmission challenging. In this study, we conducted multilocus sequence typing and antibiotic susceptibility testing for *C. jejuni* from poultry, cattle, and patients with diarrhea in Shenzhen.

Several studies of *Campylobacter* prevalence in children have been conducted previously. For instance, Wang et al. [24] reported no *Campylobacter*...
infections in patients with diarrhea in Beijing, either in children (under 5 years, 0/1422) or adults (0/1047). Zhu et al.\textsuperscript{[25]} reported that 2.9% of children with diarrhea were infected by \textit{Campylobacter} in Wuhan (Southwest China). Our study revealed a \textit{C. jejuni} prevalence of 4.0% (7/173) in children with diarrhea (≤ 5 years old) and 5.8% (12/206) in adults with diarrhea (≥ 18 years old). The discrepancy in prevalence may be caused by regional differences or the isolation methods that we adopted, which was a standard enhanced filtration based method incorporating a commercial kit.

Poultry are considered as the major reservoir of \textit{Campylobacter} spp. The prevalence of \textit{C. jejuni} in poultry is believed to be higher than that in cattle. However, in the present study, the prevalence of \textit{C. jejuni} in poultry cloacal swabs was only 27.0%, versus 57.3% in cattle feces (Table 1). This unexpected result may have been caused by the sampling method. For poultry, cloacal swabs were collected, whereas feces were collected from cattle. Compared with feces, swabs carry lower sample volume, which may have lead to a lower isolation rate of \textit{C. jejuni} in poultry.

Moreover, in this study, the prevalence of \textit{C. jejuni} in cattle meat (1.1%) was significantly lower than that in poultry meat (36.5%) (Table 1). In addition to the different prevalence of \textit{C. jejuni} in poultry and cattle, fecal contamination during evisceration may have contributed to this discrepancy. Poultry feathers are plucked and then birds are eviscerated. During evisceration, the intestines can rupture, leading to contamination of the skin of the carcass. For cattle, after evisceration, the hide is removed; therefore, contamination of the carcass is less likely. Since the skin on poultry carcasses is retained and the hide of cattle is not, the risk of fecal contamination is higher in poultry than in cattle.

Ma et al.\textsuperscript{[10]} reported that the most prevalent clonal complexes were ST-354 and ST-21 in \textit{C. jejuni} from retail chickens in Tianjin. Zhang et al.\textsuperscript{[26]} also reported that the ST-21 complex was the most common clonal complex of \textit{C. jejuni} among human isolates and chicken isolates in north China. Conversely, in our study, no ST-21 complexes were found in the poultry isolates. In addition, 51.0% of STs from poultry isolates were newly reported. Our results suggested that the \textit{C. jejuni} isolated from poultry in Shenzhen may be independent to other areas, although sampling bias cannot be excluded.

Islam et al.\textsuperscript{[27]} reported that the ST-403 complex was the most prevalent lineage among 49 \textit{C. jejuni} strains recovered from patients with enteritis or GBS. In the present study, we found nine \textit{C. jejuni} isolates that belonged to the ST-403 complex; four of them were isolated from patients and were resistant to five to eight antibiotics, while five of them were isolated from cattle and were resistant to three antibiotics. The strong antibiotic resistance of ST-403 complex isolates from patients requires further research.

In a previous study, the ST-45 complex was identified from raw or undercooked meat, as well as from contact with dogs or cats\textsuperscript{[28]}. In addition, the ST-45 complex has been found in penguins on the Antarctic\textsuperscript{[29]}, implying that this complex has a wide host range and is environmentally well adapted. In our study, we identified eight isolates that could be assigned to the ST-45 complex; four of them were from cattle, three were from poultry, and one was from a patient. The ST-45 complex was the only clonal complex that overlapped with all three sources in this study, suggesting that the ST-45 complex is widespread in Shenzhen.

A high prevalence of resistant \textit{C. jejuni} has been reported in China previously\textsuperscript{[11,20,26]}, and ciprofloxacin, nalidixic acid, and tetracycline were the most commonly reported resistance pattern. Similarly, our study found the highest resistant rate occurred for ciprofloxacin (89.7%), followed by tetracycline (74.6%), and nalidixic acid (69.0%). However, 65.2% of \textit{C. jejuni} from patients were resistant to florfenicol in this study, which has been barely reported before. Florfenicol is only permitted for veterinary use; therefore, further investigation into the reason for this high resistance to florfenicol is needed.

More research is needed to track the origin of transmission. Nevertheless, we believe this to be the first report on the genetic and antibiotic resistance of \textit{C. jejuni} in Shenzhen. Based on the genetic characteristics of \textit{C. jejuni} isolates identified in this study, our data may facilitate the development of pathogen tracking in Shenzhen.

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AUTHOR CONTRIBUTIONS

ZHANG Mao Jun, Duan YONG Xiang, LU Jing Rang, and JU Chang Yan participated in the design and coordination of the study, analyzed the data, and wrote the manuscript. MA Yan Ping, YU Mu Hua, CHEN Hui, LIU Chu Yun, GU Yi Xin, and FU Yan Yan participated in the sample collection, performed the experiments, and analyzed the data.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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