Original Article

Isolation and Characterization of Campylobacter Strains from Diarrheal Patients in Central and Suburban Bangkok, Thailand

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SUMMARY: Campylobacter-induced diarrhea is increasingly recognized worldwide. However, little information is available regarding the Campylobacter strains associated with diarrheal patients in Thailand. In this study, we attempted to isolate Campylobacter strains from diarrheal patients in Thailand and to characterize the species using a cytotoxical distending toxin (cdt) gene-based C. jejuni, C. coli, and C. fetus-specific multiplex PCR assay. Campylobacter species were also confirmed using 16S rRNA gene sequencing and hipO gene detection. From 2,500 diarrheal stool specimens, 76 Campylobacter-like organisms were isolated and identified via conventional culture methods. Among these 76 organisms, 73 were identified as Campylobacter species (43 C. jejuni, 29 C. coli, and 1 C. fetus) via multiplex PCR, whereas 3 remained unidentified. Two Campylobacter-like organisms yielded 2 amplicons corresponding to cdt genes from C. jejuni and C. coli. Subsequently, C. jejuni and C. coli were re-isolated from each sample. The third isolate was identified as C. hyointestinalis via 16S rRNA gene sequencing. To our knowledge, this is the first report on the isolation of C. hyointestinalis from a diarrheal patient in Thailand. These data indicate that C. jejuni (58%) and C. coli (40%) are prevalent among diarrheal patients in Thailand.

INTRODUCTION

Campylobacters species (spp.) are Gram-negative, highly motile, and spiral-shaped bacteria that grow under microaerobic conditions. At least 25 species are currently recognized in the genus Campylobacter, among which C. jejuni and C. coli are the most frequently isolated in association with human diseases in developed countries (1). In the United States of America (USA) and Japan, more than 90% of Campylobacter spp. isolated from diarrheal patients are C. jejuni, whereas the C. coli isolation rate is less than 5% (2,3). However, it is not well known which Campylobacter spp. are prevalent among diarrheal patients in Thailand.

Symptoms of infections caused by C. jejuni and C. coli can vary from asymptomatic to watery or bloody diarrhea. C. fetus has also been associated with human diseases, particularly sepsis. However, outbreaks of food poisoning caused by C. fetus occurred in Osaka and Tokyo, Japan in 2004 and 2009, respectively, in individuals who consumed raw beef liver or Yukke (raw beef). In addition, Campylobacter spp. are also been associated with other clinical conditions such as bacteremia, Guillain–Barré syndrome (GBS), hemolytic–uremic syndrome, pancreatitis, and reactive arthritis (4). It has been estimated that C. jejuni infections, but not C. coli infections precede GBS in 20–50% of cases in developed countries (5).

Although the virulence mechanisms of Campylobacter spp. are not well understood, 4 major virulence properties, motility, adherence, invasion and toxin production, have been considered through analogies with other enteric pathogens (6,7). Among these mechanisms, cytotoxical distending toxin (CDT) is the most well characterized toxin in Campylobacter spp. CDT is encoded by 3 closely linked genes termed cdtA, cdtB, and cdtC. CDT is a unique cytotoxin that enters the nucleus and degrades genomic DNA via the DNase-I activity of the CdtB subunit resulting in cell death. cdt genes have been reported in several Campylobacter spp., such as C. jejuni, C. coli, C. fetus, C. upsaliensis, and C. lari (8).

Accurate identification of Campylobacter strains at a species level, particularly C. jejuni and C. coli, is important when defining the disease spectrum, tracing the sources of infection, and determining the route of transmission. However, conventional methods for Campylobacter spp. isolation and identification from stool specimens require approximately 1 week and sometimes lead to the misidentification of C. jejuni as C. coli or vice versa because the level of hippuricase activity, which is the only discriminative marker for these species (9), is very weak in some C. jejuni strains or false positive hippuricase activity may be detected in C. coli strains (9,10). Furthermore, the incidence of C. fetus infection may be underestimated because the selective media and growth temperature (42°C) currently used in
laboratories are suitable for *C. jejuni* and *C. coli* but not for *C. fetus*. Therefore, various genetic methods have been developed to detect and identify *Campylobacter* spp. (11–14). We also developed a cdt gene-based species-specific multiplex PCR to identify *C. jejuni*, *C. coli*, and *C. fetus* (15).

To examine the prevalence of *Campylobacter* spp. in Thailand and to compare the clonal relationship between human and poultry isolates reported previously, in this study, we attempted to isolate *Campylobacter* spp. from diarrheal patients and to identify the involved species using genetic methods, including cdt gene-based multiplex PCR and pulsed-field gel electrophoresis (PFGE) DNA fingerprint analysis.

**MATERIALS AND METHODS**

**Isolation, bacterial strains, and culture conditions:** A total of 2,500 stool specimens were collected from diarrheal patients in Thailand, including children and adults, at Bamrasnaradura Infectious Diseases Institute between 2001 and 2002 and were inoculated onto modified CCDA medium (Oxoid, Basingstoke, UK) supplemented with a CCDA selective supplement (Oxoid). The agar plates were incubated at 37°C for 48 h under microaerobic conditions (10% CO₂, 5% O₂, and 85% N₂) using a microaerophilic generating sachet (CampyGen™; Oxoid, CN35A). *Campylobacter*-like organisms were isolated and identified using conventional culture methods and microscopy to confirm the presence of spiral Gram-negative rods, distinctive colony morphology, positive catalase and oxidase reactions, and growth at 37°C or 42°C. *C. jejuni* ATCC 33560, *C. coli* ATCC 33559, and *C. fetus* ATCC 27374 were used as reference strains. *Escherichia coli* strain C600 grown in Luria–Bertani broth was used as a negative control for multiplex PCR and dot-blot hybridization. *C. jejuni* strain 81–176, *C. coli* strain C01-243, and *C. fetus* strain C01-187 were used to prepare DNA probes for dot-blot hybridization. All *Campylobacter* strains were grown on Skirrow medium (blood agar base No. 2 [Oxoid]) supplemented with 5% (vol/vol) lysed horse blood (Nippon Bio-Supp. Center, Tokyo, Japan) and *Campylobacter* selective supplements (Skirrow; Oxoid) at 37°C or 42°C for 48 h under microaerobic conditions (10% CO₂, 5% O₂, and 85% N₂) and were subsequently used for further analysis.

**Chemicals and enzymes:** Chemicals were purchased from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemicals (Tokyo, Japan), or Sigma–Aldrich Co. (St. Louis, MO, USA). Ex Taq polymerase and *Sma*I restriction enzyme were purchased from Takara Bio Inc. (Shiga, Japan). A digoxigenin (DIG)-based DNA labeling and detection kit was purchased from Roche Diagnostic GmbH (Mannheim, Germany). The BigDye terminator v.1.1 cycle sequencing kit was purchased from Applied Biosystems Inc. (Foster City, CA, USA). Pulsed-field certified agarose and low melt preparative grade agarose were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). SeaKem Gold agarose was obtained from FMC Bioproducts (Rockland, ME, USA). Molecular weight markers for agarose gel electrophoresis were purchased from Takara Bio Inc.

**PCR:** The cdtB gene-based species-specific multiplex PCR assay for the detection of *C. jejuni*, *C. coli*, and *C. fetus* cdtB genes and PCR with cdtB common primers for the detection of *C. jejuni*, *C. coli*, and *C. fetus* cdtB genes were performed as described previously (15, 16). PCR for hipO gene detection and 16S rRNA gene amplification was performed as described previously (17, 18). The PCR primers and conditions used in this study are summarized in Table 1. Briefly, template DNA for PCR assays was prepared via a previously described boiling method (19). A loopful of bacteria collected from an agar plate was suspended in 1 ml of TE buffer (10 mM Tris–HCl, 1.0 mM EDTA [pH 8.0]), followed by boiling for 10 min and centrifugation at 12,800 g for 5 min; 2 μl of the supernatant was subsequently used as a PCR template. All reactions contained appropriate concentrations of the primer sets (15), 0.2 mM of each dNTP mixture, 1 × Ex Taq DNA polymerase buffer, and 1.25 U of Ex Taq DNA polymerase in a 50 μl-reaction volume. Amplification was performed on a GeneAmp PCR 9700 thermal cycler (Applied Biosystems Inc.). PCR products were analyzed by 1.5% or 2.0% agarose gel electrophoresis, and bands were visualized with UV light after staining with ethidium bromide. Images were captured on a Bio-Rad Gel Doc system (Bio-Rad Laboratories, Inc.).

**DNA preparation:** Bacterial chromosomal DNA was purified and evaluated with respect to quantity and quality as described previously (18).

**Dot hybridization:** Dot-blot hybridization assays were performed as described previously (20), with some modifications. Briefly, heat-denatured genomic DNA (300 ng) was applied to a GeneScreen Plus (DuPont, Boston, MA, USA). The membranes were hybridized with cdt gene probes under highly stringent conditions as described previously (18). The cdtA, cdtB, and cdtC gene probes for *C. jejuni*, *C. coli*, and *C. fetus* were amplified by PCR using the primer sets described above. For this purpose, genomic DNA was purified from *C. jejuni* 81–176, *C. coli* C01-243, and *C. fetus* C01-187 and used as templates. The sequences of these cdt genes have previously been confirmed (16). The *hipO* gene used as a probe was also amplified via PCR from purified genomic DNA of *C. jejuni* strain 81–176 (template) and a primer set that essentially have been described previously (21). PCR products were purified as described above and labeled and detected using a DIG DNA labeling and detection kit (Roche Diagnostic GmbH). After hybridization, the membranes were washed twice with 2 × SSC containing 0.1% SDS for 15 min per wash at room temperature, followed by washing twice with 0.2 × SSC containing 0.1% SDS for 30 min per wash at 65°C. DNA–probe hybridization was visualized by p-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer’s instructions.

**Sequencing of 16S rRNA gene:** 16S rRNA gene sequence analysis was performed as described previously (18).

**PFGE:** Rapid-protocol PFGE was performed to examine the genetic diversity of the *Campylobacter* strains isolated in this study (22) with some modifications. Briefly, intact agarose-embedded chromosomal DNA was prepared from *C. jejuni* and *C. coli* strains, and PFGE was performed using a contour-clamped homo-
strains, which were identified as C. jejuni/coli by 16S rRNA gene sequence (Table 2). The hipO PCR results were also confirmed by dot-blot hybridization. It is noteworthy that the reference strains C. jejuni ATCC 33560 and C. coli ATCC 33559 were correctly identifiable as C. jejuni and C. coli through a similar procedure (data not shown). Taken together, these results concluded that 45, 31, 1, and 1 strains isolated from 76 stool specimens were C. jejuni, C. coli, C. fetus, and C. hyointestinalis, respectively (Table 2). To the best of our knowledge, this is the first report regarding the isolation of C. hyointestinalis from a diarrheal patient in Thailand.

**Table 2. Summary of the species identification of 78 Campylobacter strains isolated from diarrheal patients in Thailand**

<table>
<thead>
<tr>
<th>16S rRNA gene sequence</th>
<th>hipO</th>
<th>Multiplex PCR</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni/C. coli (76)</td>
<td>+ (45)</td>
<td>C. jejuni (45)</td>
<td>C. jejuni (45)</td>
</tr>
<tr>
<td>C. jejuni/C. coli (31)</td>
<td>- (33)</td>
<td>C. coli (31)</td>
<td>C. coli (31)</td>
</tr>
<tr>
<td>C. hyointestinalis (1)</td>
<td>- (1)</td>
<td>C. hyointestinalis (1)</td>
<td></td>
</tr>
<tr>
<td>C. fetus (1)</td>
<td>- (1)</td>
<td>C. fetus (1)</td>
<td>C. fetus (1)</td>
</tr>
</tbody>
</table>

The number in parenthesis indicates the number of strains.

**DNA fingerprinting of C. jejuni and C. coli strains by PFGE:** PFGE was employed to investigate the genetic diversity of the 45 C. jejuni and 31 C. coli strains identified in this study. Smal-digested genomic DNA from C. jejuni generated an average of 6–9 restriction fragments whereas Smal-digested genomic DNA from C. coli produced 9–13 fragments (Figs. 1 and 2). Using 80% genetic similarity as the cutoff, 45 C. jejuni and 31 C. coli strains were classified into 22 pulsotypes and 10 subtypes and 14 pulsotypes and 7 subtypes, respectively (Figs. 1 and 2). These data indicate genetic diversity among the C. jejuni and C. coli strains isolated in this study.

**Fig. 1. UPGMA dendogram based on 22 Smal-PFGE patterns of 45 C. jejuni clinical isolates.** The column to the right of the PFGE patterns corresponds (from left to right) to the strain number, year of isolation, and pulsotypes. Twenty-two pulsotypes were classified (HJ1-HJ22). Subtypes a and b were observed in pulsotypes HJ3, HJ7, HJ11, HJ14, and HJ18.

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DISCUSSION

In this study, 76 Campylobacter-like organisms were initially isolated from patients with diarrhea (mostly children younger than 5 years) in Thailand and were further characterized for species identification and DNA fingerprinting. Seventy-three isolates were successfully identified to represent 43, 29, and 1 C. jejuni, C. coli, and C. fetus isolates, respectively, via cdt gene-based multiplex PCR, 16S rRNA gene sequencing, and hipO gene detection. However, 2 isolates were found to harbor mixtures of C. jejuni and C. coli according to cdt gene-based multiplex PCR; both C. jejuni and C. coli were successfully re-isolated from these 2 samples, indicating that cdt gene-based multiplex PCR is useful for detecting the co-existence of 2 or 3 Campylobacter spp., as reported by Asakura et al (15). However, 1 strain, which was identified to be a Campylobacter-like organism in Thailand using conventional culture methods, did not produce any specific products during cdtB gene-based species-specific multiplex PCR. We have recently developed a PCR assay that can amplify the cdtB genes of at least 3 Campylobacter spp., including C. jejuni, C. coli, and C. fetus, from a common primer set (16). This PCR assay successfully amplified a specific amplicon indicating that the unidentified strain might be a CDT-producing Campylobacter strain. Subsequently, this strain was identified as C. hyointestinalis via 16S rRNA gene sequencing. To our knowledge, this is the first report regarding the isolation of C. hyointestinalis from a diarrheal patient in Thailand. Finally, out of 78 isolated strains, 45, 31, and 1 were identified as C. jejuni, C. coli, and C. fetus, respectively, via cdtB gene-based multiplex PCR, whereas 1 strain was identified as C. hyointestinalis by 16S rRNA gene sequencing (Table 2). In South Africa, the Cape Town Protocol (Filter method) was used to determine that in diarrheal patients, C. jejuni was most prevalent species (n = 6,006) among Campylobacters spp. and related bacteria such as Helicobacter and Arcobacter. However, the C. jejuni isolation rate was 40%, followed by C. concisus (25%), C. upsaliensis (24%), C. coli (3.0%), and C. hyointestinalis (0.95%) (23). On the other hand, when 1,906 and 2,855 Campylobacter spp. and related bacteria were isolated from stool specimens of diarrhea in Belgium (24) and France (25), and examined, 77.2% and 78.9%, respectively, were C. jejuni and 18.1%, respectively, were C. coli, a slight difference in comparison to the rates reported in the USA (2) and Japan (3). However, the present study demonstrated a
40% isolation rate of *C. coli* in Thailand, which is quite high. Previous studies in northern Thailand failed to isolate *Campylobacter* spp. from healthy nonfarm residents; however, isolates were obtained from 5 and 18% of farm workers and children with diarrhea, respectively (26). In particular, the *C. coli* isolation rate is high among farm workers (66%) and children with diarrhea (38%) (26), indicating the prevalence of *C. coli* among *Campylobacter* spp. in Thailand. However, we cannot exclude the possibility that different isolation protocols caused this difference. As Mak-im et al. reported the detection of *Campylobacter* spp. in 3.5% of stool specimens from healthy children in Thailand (27), further studies are needed to discretely analyze whether *Campylobacter* spp. are an important etiologic agent of diarrhea in children.

PFGE analysis revealed that the *C. jejuni* and *C. coli* strains analyzed in this study were genetically diverse, indicating the prevalence of various genotypes of *C. jejuni* and *C. coli* in Thailand. It is of interest to note that some of these strains exhibited nearly identical PFGE patterns to those obtained from previously reported Thai poultry strains (18), as shown in Fig. 3. These *Campylobacter* strains were also isolated between 2001 and 2002, when human isolates were also obtained, and the poultry samples were also collected from same place near Bangkok where the human isolates were obtained as at 3 other places in and near Bangkok. These data suggest that in Thailand, *Campylobacter* strains might be transmitted between poultry and humans.

In conclusion, these data indicate that both *C. jejuni* and *C. coli* are prevalent among diarrheal patients in Thailand. To the best of our knowledge, this is the first report describing the isolation of *C. hyointestinalis* from a diarrhea patient in Thailand. The *cdt* gene-based species-specific multiplex PCR assay is a simple, rapid, and reliable method for *C. jejuni*, *C. coli*, and *C. fetus* detection and identification.

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**Conflict of interest** None to declare.

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