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Original Article

Evaluation of a Cytolethal Distending Toxin (*cdt*) Gene-Based Species-Specific Multiplex PCR Assay for the Identification of *Campylobacter* Strains Isolated from Diarrheal Patients in Japan

S. M. Lutful Kabir, Ken Kikuchi[1]**, Masahiro Asakura, Sachi Shiramaru, Naoki Tsuruoka[2], Aeko Goto[2], Atsushi Hinenoya, and Shinji Yamasaki*

Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531; [1]Department of Infection Control Science, Faculty of Medicine, Juntendo University, Tokyo 113-8421; and [2]Clinical Laboratory, Tokyo Women's Medical University Hospital, Tokyo 162-8666, Japan

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SUMMARY: We have developed a cytolethal distending toxin (*cdt*) gene-based species-specific multiplex PCR assay for the detection and identification of *Campylobacter jejuni*, *C. coli*, and *C. fetus*. The applicability of this assay was evaluated with 325 *Campylobacter* strains isolated from diarrheal patients in Japan and the results were compared with those obtained by other genetic methods, including *hipO* gene detection and 16S rRNA gene sequencing. Of the 325 strains analyzed, 314 and 11 were identified as *C. jejuni* and *C. coli*, respectively, by combination of *hipO* gene detection and 16S rRNA gene sequencing. When the multiplex PCR assay was employed, 309, 310, and 314 strains were identified as *C. jejuni* on the basis of *cdtA*, *cdtB*, and *cdtC* gene-specific primers, respectively. Similarly, 11, 11, and 10 strains were identified as *C. coli* on the basis of *cdtA*, *cdtB*, and *cdtC* gene-specific primers, respectively. Sequence analysis of the *cdt* gene region of 6 strains (5 *C. jejuni* and 1 *C. coli*) which did not yield specific PCR products in any of the *cdt* gene-based multiplex PCR assays revealed deletions or mutations of the *cdt* genes. Pulsed-field gel electrophoresis indicated that *C. jejuni* and *C. coli* strains were genetically diverse. Taken together, these findings suggest that the *cdtC* gene-based multiplex PCR seems to be a particularly simple and rapid method for differentiating between species of *Campylobacter* strains, such as *C. jejuni* and *C. coli*. However, combination of these multiplex PCR assays will allow more accurate identification.

INTRODUCTION

Campylobacteriosis is increasingly being recognized as one of the most important food-borne gastroenteritis in humans in industrialized nations (1). Among more than 17 species currently reported in the genus *Campylobacter* (2), *C. jejuni* and *C. coli* are most frequently isolated from human gastroenteritis cases in Japan and USA. In Japan, *C. jejuni* is associated with 94% of *Campylobacter*-related enteritis cases, while *C. coli* and *C. fetus* are associated with 4.0 and 1.3%, respectively (3). *C. fetus* is usually implicated in sepsis in animals and humans, and rarely causes gastroenteritis in humans. However, an outbreak of *C. fetus*-related food poisoning due to consumption of raw beef liver and Yukke (raw beef) was reported in Osaka, Japan in 2004. Furthermore, campylobacters have also been associated with other clinical conditions, such as Guillain-Barré syndrome, hemolytic-uremic syndrome, pancreatitis, and reactive arthritis (4).

The accurate identification of *Campylobacter* spp., particularly *C. jejuni* and *C. coli*, is crucial in order to be able to define their disease spectrum and to determine their sources of infection and route of transmission. However, conventional identification methods for *Campylobacter* spp., particularly *C. jejuni* and *C. coli*, are tedious and time-consuming as these organisms are slow-growing and have fewer informative biochemical characteristics. Indeed, it is sometimes problematic to differentiate *C. jejuni* from *C. coli* by conventional biochemical methods because of the extensive similarity between these two species (5). Hippuricase activity is the only marker known to be able to discriminate between the two (6). However, this activity is sometimes very weak in *C. jejuni*, and hippuricase-negative strains of this species are also well known (7), thus meaning that *C. jejuni* can be misidentified as *C. coli*. False-positive results for non-*C. jejuni* species have also been described (5). Several PCR-based assays targeting the 16S or 23S rRNA gene, or other species-specific genes, have been developed to facilitate the differentiation of *C. jejuni* from *C. coli* (5,8–13). However, the ability of these assays to acurately identify *Campylobacter* spp. has not yet been fully confirmed.

Cytolethal distending toxin (CDT), which is one of

*Corresponding author: Mailing address: Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1, Rinku Ourai-Kita, Izumisano, Osaka 598-8531, Japan. Tel & Fax: +81-72-463-5653, E-mail: shinji@vet.osakafu-u.ac.jp

**Present address: Graduate School of Medicine, Juntendo University, Tokyo 113-8421, Japan

the best characterized virulence factors in *Campylobacter* spp., is encoded by three linked genes known as *cdtA*, *cdtB*, and *cdtC* (14). Furthermore, the *cdt* gene clusters in *C. jejuni*, *C. coli*, and *C. fetus* have been demonstrated to be universally disseminated in each species in a species-specific manner (15). Recently, a *cdt* gene-based species-specific multiplex PCR assay has been developed for the detection and identification of *C. jejuni*, *C. coli*, and *C. fetus* (16). Subsequent evaluation of the *cdt* gene-based multiplex PCR assays with *Campylobacter*-like strains isolated from poultry and diarrheal patients in Thailand indicated that (17, unpublished observation) targeting of the *cdtB* gene appeared to be most appropriate. However, some mutations on the *cdtA*, *cdtB*, and *cdtC* genes of *C. jejuni* and *C. coli* were also reported (18–20). It is therefore important to evaluate such *cdt* gene-based multiplex PCR assays with a large number of *Campylobacter* strains isolated from various origins, especially clinical isolates.

In the present study, the applicability of *cdt* gene-based multiplex PCR was further tested with 325 *Campylobacter* strains isolated from diarrheal patients in Japan. For further evaluation, the results obtained were compared with other genetic methods, such as *hipO* gene detection and sequencing of the 16S rRNA gene.

MATERIALS AND METHODS

Bacterial strains and media: A total of 325 *Campylobacter* strains isolated from stool specimens of diarrheal patients aged between 9 months and 85 years who visited the Tokyo Women's Medical University Hospital, Japan during the period 1997–2005 were used in this study. These strains were identified as *Campylobacter* spp., *C. jejuni*, or *C. coli* by culture methods, microscopy, and biochemical tests (catalase, oxidase, and hippuricase activity) at the same hospital. *C. jejuni* ATCC 43432^T, *C. coli* ATCC 33559^T, and *C. fetus* ATCC 27374^T were used as reference strains. All *Campylobacter* strains were grown on blood base agar no. 2 (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood (Nippon Bio-Supp. Center, Tokyo, Japan) under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C for 2 days. *Escherichia coli* C600, grown in Luria-Bertani (LB) broth at 37°C overnight, was used as a negative control for the multiplex PCR assay.

Chemicals and enzymes: Chemicals were purchased from either Nacalai Tesque (Kyoto, Japan), Wako Pure Chemicals (Tokyo, Japan), or Sigma Chemical Co. (St. Louis, Mo., USA). Ex *Taq* DNA polymerase was purchased from Takara Bio Inc. (Shiga, Japan). An ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit was purchased from Applied Biosystems (Foster, Calif., USA). SeaKem LE agarose for the analysis of PCR products was obtained from FMC Bioproducts (Rockland, Maine, USA). Molecular weight markers for agarose gel electrophoresis were purchased either from Takara Bio Inc. or New England Biolabs Inc. (Beverly, Mass., USA).

DNA preparation: Template DNA was prepared by the boiling method, as described by Hoshino et al. (21). Briefly, a loopful of bacteria collected from an agar plate was suspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and the resulting suspension boiled for 10 min then centrifuged at 12,800 × *g* for 5 min. A 2-μl portion of supernatant was used as the PCR template.

***hipO* gene PCR:** The hippuricase gene (*hipO*) was amplified by PCR using the primers and conditions listed in Table 1 (8). The PCR reaction contained the appropriate concentration of primer sets (Table 1), 0.2 mM each of dNTP mixture (dATP, dCTP, dGTP, and dTTP), 1 × Ex *Taq* DNA polymerase buffer, and 1.0 U of Ex *Taq* DNA polymerase in a 40-μl reaction volume. Amplification was performed using an Applied Biosystems GeneAmp PCR 9700 (Applied Biosystems). PCR products were analyzed by 1.5% agarose gel electrophoresis and the bands were visualized with UV light after staining with ethidium bromide (1 μg/ml). Images were captured on a Bio-Rad Gel Doc system (Bio-Rad Laboratories, Hercules, Calif., USA).

Sequencing of the 16S rRNA gene: The PCR primers and conditions used during amplification of the 16S rRNA gene are described in Table 1. The reaction mixture was as described above, and the PCR product was purified using a QIAquick PCR Purification Kit according to the manufacturer's instructions (QIAGEN GmbH, Hilden, Germany). The purified DNA was subjected to a cycle sequencing reaction using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the primers used for 16S rRNA gene amplification and four additional primers that can specifically bind the inner region of the 16S rRNA gene (Table 1). Nucleotide sequences were determined using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed using the DNA Lasergene software package (DNASTAR, Madison, Wis., USA). Homology searches were performed against all sequences in the GenBank database using the BLAST search engine, which is available from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Multiplex PCR: The multiplex PCR assay used to detect the *cdtA*, *cdtB*, and *cdtC* genes of *C. jejuni*, *C. coli*, and *C. fetus* was performed using the gene-specific primers and PCR conditions (16) are summarized in Table 1. All reactions contained appropriate concentrations of three primer sets, 0.2 mM each of dNTP mixture, 1 × Ex *Taq* DNA polymerase buffer, and 1.0 U of Ex *Taq* DNA polymerase in a 40-μl reaction volume. PCR products were analyzed by 2% agarose gel electrophoresis. Bands were visualized and images captured as described above.

Nucleotide sequence analysis of the entire *cdt* gene cluster: The entire *cdt* gene cluster was amplified by PCR using the primer set described in Table 1. The amplified PCR products were purified using a QIAquick PCR Purification Kit and sequenced using a series of primers as described previously (15). A summary of the sequencing strategy, together with the corresponding primer locations for *Cj-cdtABC*, is provided in Fig. 1. The sequences obtained were analyzed as described above.

Nucleotide sequence accession numbers: The *cdt* gene sequences of *C. jejuni* and *C. coli* analyzed in this study have been registered with the DDBJ (strain/accession no.; *C. jejuni*: K40/AB562904, K109/AB545927, K114/

Table 1. Primers and conditions used for the various PCR and sequence analysis

Primer	Sequence (5′–3′)	Target/ purpose	PCR condition (30 cycles)			Amplicon size (bp)	Primer conc. (μM)	Reference
			Denaturation	Annealing	Extension			
CjspAU2	AGGACTTGAACCTACTTTTC	*Cj cdtA*	94°C, 30 s	55°C, 30 s	72°C, 30 s	631	0.25	16
CjspAR2	AGGTGGAGTAGTTAAAAACC							
CcspAU1	ATTGCCAAGGCTAAAATCTC	*Cc cdtA*				329	0.5	
CcspAR1	GATAAAGTCTCCAAAACTGC							
CfspAU1	AACGACAAATGTAAGCACTC	*Cf cdtA*				489	0.5	
CfspAR1	TATTTATGCAAGTCGTGCGA							
CjSPBU5	ATCTTTTAACCTTGCTTTTGC	*Cj cdtB*	94°C, 30 s	56°C, 30 s	72°C, 30 s	714	0.125	16
CjSPBR6	GCAAGCATTAAAATCGCAGC							
CcSPBU5	TTTAATGTATTATTTGCCGC	*Cc cdtB*				413	0.15	
CcSPBR5	TCATTGCCTATGCGTATG							
CfSPBU6	GGCTTTGCAAAAACCAGAAG	*Cf cdtB*				553	0.25	
CfSPBR3	CAAGAGTTCCTCTTAAACTC							
CjspCU1	TTTAGCCTTTGCAACTCCTA	*Cj cdtC*	94°C, 30 s	55°C, 30 s	72°C, 30 s	524	0.25	16
CjspCR2	AAGGGGTAGCAGCTGTTAA							
CcspCU1	TAGGGATATGCACGCAAAAG	*Cc cdtC*				313	0.5	
CcspCR1	GCTTAATACAGTTACGATAG							
CfspCU2	AAGCATAAGTTTTGCAAACG	*Cf cdtC*				397	0.5	
CfspCR1	GTTTGGATTTTCAAATGTTCC							
Cj-CdtH1	CTCTTGACCAATCAACGC	*Cj-cdtABC*	94°C, 30 s	55°C, 30 s	72°C, 180 s	2,985	0.4	15
Cj-CdtH2	AAGCTATAGGCCACATACC							
Cc-CdtH1	AAGGCTATAGAATGCCTCC	*Cc-cdtABC*	94°C, 30 s	55°C, 30 s	72°C, 180 s	3,048	0.4	15
Cc-CdtH2	TTACTGCACTTGCCATAGC							
HIP400F	GAAGAGGGTTTGGGTGGTG	*hipO*	94°C, 30 s	66°C, 30 s	72°C, 45 s	735	0.4	8
HIP1134R	AGCTAGCTTCGCATAATAACTTG	gene						
16S9F	GAGTTTGATCCTGGCTC	16S rRNA	94°C, 30 s	55°C, 30 s	72°C, 90 s	1,530	0.4	17
16S1540R	AAGGAGGTGATCCAGCC	gene						
Cj2U	CCAAAGGGTCTTTCCAAG	Sequence	NA	NA	NA	NA	NA	This study
Cj3U	AATGGGATCAATCCTCGC	for entire						
Cj4U	GCATACAAGCACCTATTAC	*Cj-cdtABC*						
Cj5U	TGCAACAAGGTGGAACAC							
Cj6U	TTCCAACTAGCGCAACTC							
Cj7U	AACGCTTTGGAATAGCCC							
Cj2R	GTACTAAATCAGCACCGC							
Cj3R	CCAAACCGTTAAAGCTGC							
Cj4R	GATGAGCCTTGCAAATTCC							
Cj5R	AACTGTAGTAGGTGGAGG							
Cj6R	ATCTCCAGTAGGAGTTGC							
Cj7R	GGTAGCAGCTGTTAAAGG							
Cc2U	CATGCTCCTTAGTGCTTG	Sequence	NA	NA	NA	NA	NA	This study
Cc3U	ATAATGCCGTCAAAGGCC	for entire						
Cc4U	GGAGACTTTATCCGATGAC	*Cc-cdtABC*						
Cc5U	TAGAGTGGATGTAGGAGC							
Cc6U	TGACTTAAGAGCACGCAC							
Cc7U	AATGGTGGCAATCAATGC							
Cc2R	TTACAGGTGGGACAAGTG							
Cc3R	TAAAGCTGCCAAGCTCTTG							
Cc4R	TAGGAGCGCCTATATACC							
Cc5R	AGCATCATTGCCTATGCG							
Cc6R	TTCATCCAAGCTAGGCTC							
Cc7R	GCTTCGATTATAGCAGGG							
16S520F	GAGTTTGATCCTGGCTC	Sequence	NA	NA	NA	NA	NA	This study
16S1199F	GCAACGAGCGCAACCC	for						
16S741R	GTATCTAATCCTGTTTGC	*Cj*- and *Cc*-						
16S1240R	CCATTGTAGCACGTGT	16S rRNA						

Cj, *C. jejuni*; Cc, *C. coli*; Cf, *C. fetus*; NA, not applicable.

AB545928, K170/AB545929, K328/AB545930; *C. coli*: K168/AB562905).

Pulsed-field gel electrophoresis (PFGE): PFGE was performed to determine the genetic diversity of the *Campylobacter* strains using the rapid protocol (22), as described previously (17). PFGE data were analyzed using the Fingerprinting II software (Bio-Rad Laboratories), applying the Dice similarity index and the UPGMA method. PFGE patterns were considered to be clonally related if they had a similarity coefficient higher than 85 and 90% (usually corresponding to a difference of no more than 3 bands) for *C. jejuni* and *C. coli* strains, respectively. Moreover, those *C. jejuni* and *C. coli* strains which showed identical *Sma*I PFGE patterns were also analyzed by digesting intact genomic DNA with *Kpn*I to verify the *Sma*I-typing results, as described by On et al. (23).

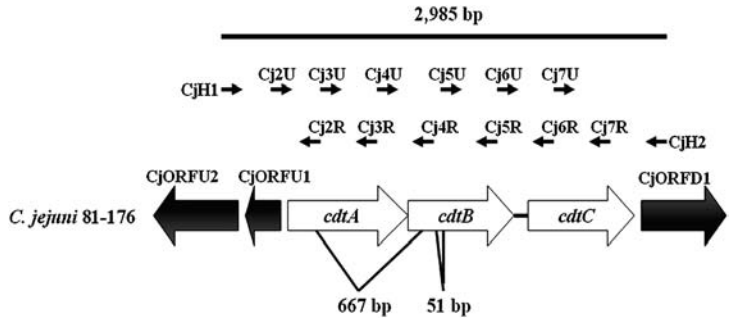

Fig. 1. Schematic representation of the location of the *cdt* gene cluster in the genome of *C. jejuni* 81-176, illustrating the locations of the 667- and 51-bp deletions in strains K109, K114, K170, and K328. The straight line indicates the region amplified by PCR for the analysis of the *cdt* gene cluster of *C. jejuni* strains as shown in Table 1. Summary of the sequencing strategy with corresponding primer locations (arrows) used to complete 2,985-bp region is shown. The open arrow indicates each *cdt* gene cluster. The closed arrow indicates each open reading frame (ORF) located in the flanking region of each *cdt* gene cluster. The numbers, 667 bp and 51 bp, indicate the size of the *cdt* genes deletion. CjORFU2, CjORFU1, and CjORFD1 indicate genes coding for cytochrome oxidase, hypothetical protein, and L-lactate permease, respectively. The *cdt* gene cluster of *C. coli* strains was also determined similarly by using a series of sequence primer as shown in Table 1.

RESULTS

Species identification of *Campylobacter* strains by *hipO* and 16S rRNA genes: Of the 325 *Campylobacter* strains isolated from diarrheal patients in Japan, 218, 104, and 3 strains were identified as *C. jejuni*, *Campylobacter* spp., and *C. coli*, respectively, using conventional methods. These strains were further identified up to species level by genetic methods, such as *hipO* gene detection and sequencing of the 16S rRNA gene, as shown in Table 2. A total of 314 of these strains generated a 735-bp fragment of the *hipO* gene, whereas the remaining 11 did not yield a PCR product. The absence of *hipO* gene in these 11 strains was further confirmed by colony hybridization (data not shown). They were subsequently subjected to 16S rRNA gene analysis and were identified as *C. coli*.

Evaluation of the *cdt* gene-based multiplex PCR for species identification: When the multiplex PCR assay (16) targeting the *cdtA*, *cdtB*, or *cdtC* gene was used, these genes from *C. jejuni* were specifically amplified from 309, 310, and 314 of the 325 *Campylobacter* strains, respectively (Table 2). Similarly, *cdtA*, *cdtB*, and *cdtC* genes of *C. coli* were amplified from 11, 11, and 10 strains, respectively, using this assay (Table 2). Five strains (K40, K109, K114, K170, and K328) did not yield any specific PCR product of the *cdtA* gene, and all except strain K40 failed to produce the desired PCR products of the *cdtB* gene. Interestingly, this K40 strain was identified as *C. jejuni* by *cdtB* and *cdtC* gene-specific primers, and the other 4 strains (K109, K114, K170, and K328) were identified as *C. jejuni* by *cdtC* gene-specific primers (Fig. 2). One strain (K168), which did not yield any specific PCR product when using *cdtC* gene-specific primers (Table 2 and Fig. 2), was identified as *C. coli* using *cdtA* and *cdtB* gene-specific primers (Fig. 2). Each reference strain also produced the desired specie-specific DNA fragment when subjected to *cdt* gene-based multiplex PCR (Fig. 2). None of the strains tested produced a specific band corresponding to the *cdtA*, *cdtB*, and *cdtC* genes of *C. fetus* (data not shown). These findings suggest that the *cdtC* gene-based multiplex PCR is more accurate and reliable than *cdtA* and *cdtB* gene-based multiplex PCR for differentiating the species of *Campylobacter* strains used in this study.

Nucleotide sequence analysis of the entire *cdt* gene cluster: In order to verify the results of the multiplex PCR assay, the entire *cdt* gene clusters of 6 strains which did not produce PCR products when using any of the *cdt* gene-based multiplex PCR assays were amplified and sequenced as described in Materials and Methods section. Two out of these 6 strains yielded specific PCR

Table 2. Summary of identification of species of *Campylobacter* strains isolated from diarrheal patients in Japan

Biochemical identification	Conventional genetic identification methods			*cdt* gene-based multiplex PCR		
	hipO	16S rRNA gene sequence	Conclusion	*cdtA*	*cdtB*	*cdtC*
Campylobacter spp. (97)	+	ND	*C. jejuni*	*C. jejuni*	*C. jejuni*	*C. jejuni*
Campylobacter spp. (6)	−	*C. jejuni*/*C. coli*	*C. coli*	*C. coli*	*C. coli*	*C. coli*
Campylobacter spp. (1)	−	*C. jejuni*/*C. coli*	*C. coli*	*C. coli*	*C. coli*	—
C. jejuni (212)	+	ND	*C. jejuni*	*C. jejuni*	*C. jejuni*	*C. jejuni*
C. jejuni (1)	+	ND	*C. jejuni*	—	*C. jejuni*	*C. jejuni*
C. jejuni (4)	+	ND	*C. jejuni*	—	—	*C. jejuni*
C. jejuni (1)	−	*C. jejuni*/*C. coli*	*C. coli*	*C. coli*	*C. coli*	*C. coli*
C. coli (3)	−	*C. jejuni*/*C. coli*	*C. coli*	*C. coli*	*C. coli*	*C. coli*
Positive%				98.50%	98.80%	99.70%

ND, not done.

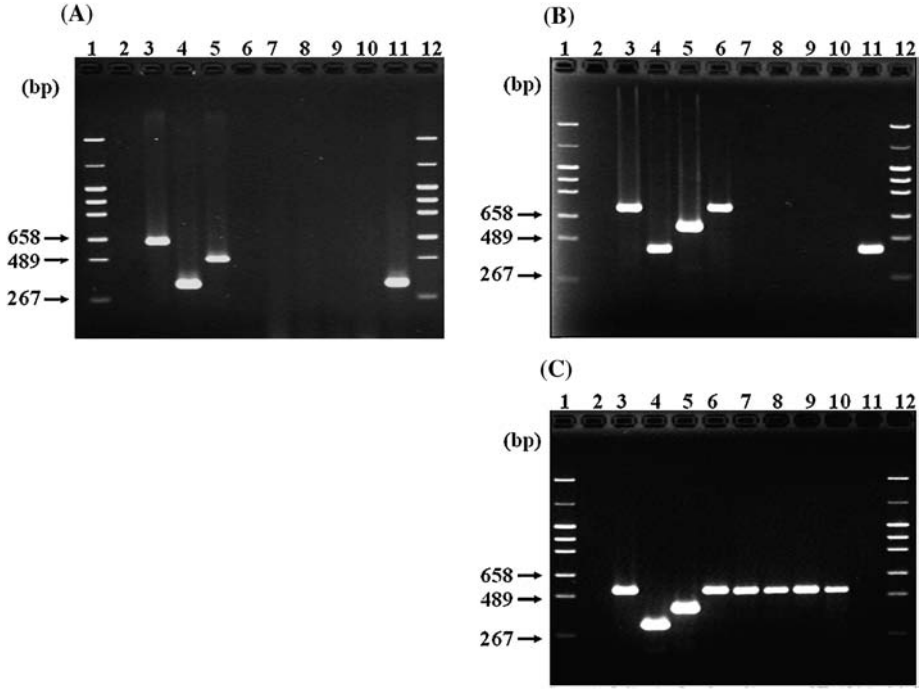

Fig. 2. Detection of the species of *Campylobacter* strains by (A) *cdtA*, (B) *cdtB*, and (C) *cdtC* gene-based multiplex PCR assay. Lanes: 1 and 12, pHY marker; 2, *Escherichia coli* C600; 3, *C. jejuni* ATCC 43432; 4, *C. coli* ATCC 33559; 5, *C. fetus* ATCC 27374; 6, K40; 7, K109, 8, K114; 9, K170; 10, K328; 11, K168.

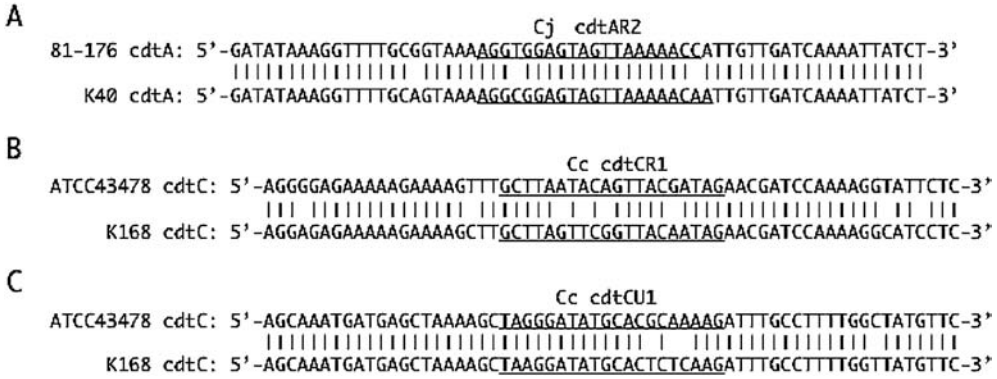

Fig. 3. Alignments of DNA sequences showing primer binding sites used for *cdtA* and *cdtC* gene-based multiplex PCR in the PCR negative strains of *C. jejuni* and *C. coli*, respectively. (A) Alignment of primer (CjcdtAR2) binding site and its flanking regions of *cdtA* sequences of *C. jejuni* strains 81-176 (Ac. no. U51121) and K40 (Ac. no. AB562904). (B) Alignment of primer (CccdtCR1) binding site and its flanking regions of *cdtC* sequences of *C. coli* strains ATCC43478 (Ac. no. AB274794) and K168 (Ac. no. AB562905). (C) Alignment of primer (CccdtCU1) binding site and its flanking regions of *cdtC* sequences of *C. coli* strains ATCC43478 and K168. Underlines indicate the sequence of primer binding site.

products of expected sizes (2,985 bp and 3,048 bp), and ClustalW alignment to the published *cdtABC* sequences of *C. jejuni* 81-176 (accession no. U51121) and *C. coli* ATCC43478 (accession no. AB274794) revealed mutations in the primer binding site of the *cdtA* and *cdtC* genes of these strains, respectively (Fig. 3). A smaller product of about 2,250 bp could also be amplified from these 4 strains (K109, K114, K170, and K328) using specific primers designed from the nucleotide sequence data. ClustalV alignment to the published *cdtABC* sequence of *C. jejuni* 81-176 revealed a 667-bp deletion between *cdtA* and *cdtB* and a separate 51-bp deletion within the *cdtB* gene of these 4 strains, as shown in Fig. 1. This result is consistent with the smaller amplicon size obtained upon PCR amplification of the entire *cdt* gene cluster region. Since the binding sites of the *C. jejuni*-specific *cdtA* reverse primer and *cdtB* forward primer are within the deleted 667-bp region of the entire *cdt* gene cluster of these unusual strains (data not shown), the findings support those of the multiplex PCR assay used in this study and suggest that *cdtC* gene-based multiplex PCR may be more accurate and reliable than either a *cdtA* or *cdtB* gene-based assay for differentiating *C. jejuni* and *C. coli*.

Genetic diversity of *C. jejuni* and *C. coli* strains based on PFGE analysis: PFGE was employed to investigate the genetic diversity of 50 *C. jejuni* and 11 *C. coli* strains identified in this study, all of which were randomly

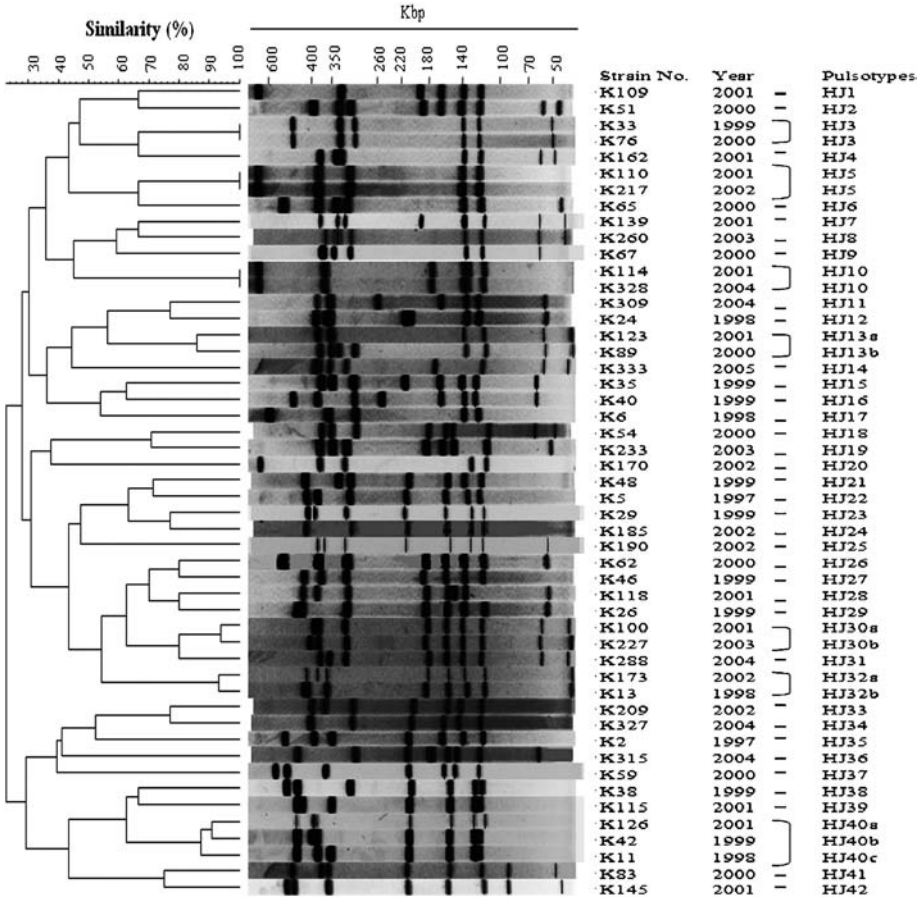

Fig. 4. UPGMA dendrogram of *Sma*I PFGE patterns of 50 *C. jejuni* strains isolated from diarrheal patients in Japan. The band position tolerance of 1.00% was used.

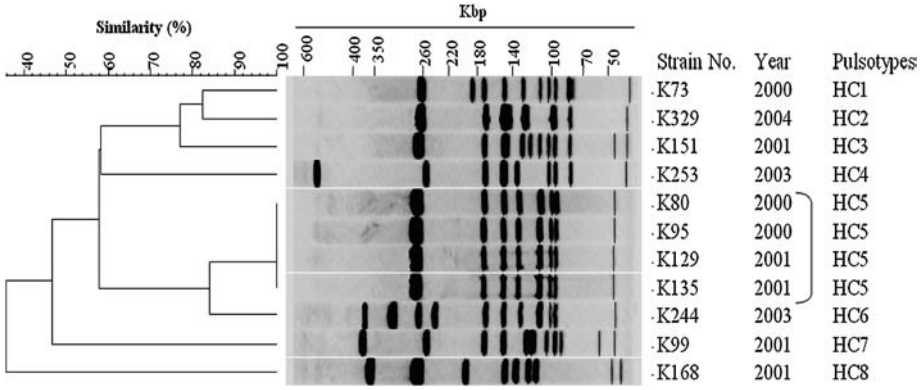

Fig. 5. UPGMA dendrogram of *Sma*I PFGE patterns of 11 *C. coli* strains isolated from diarrheal patients in Japan. The band position tolerance of 1.00% was used.

selected. Thus, the genomic DNA of *C. jejuni* and *C. coli* strains was digested with *Sma*I, as described previously (22,23). The *Sma*I-digested genomic DNA of the 50 *C. jejuni* strains generated an average of between 5 and 9 fragments, while the *Sma*I-digested genomic DNA of the 11 *C. coli* strains produced between 7 and 11 fragments (Figs. 4 and 5). Taking a genetic similarity of 85 and 90% as the cutoff, the 50 *C. jejuni* and 11 *C. coli* strains were classified into 42 pulsotypes and 4 subtypes, and 8 pulsotypes, respectively (Figs. 4 and 5). Furthermore, those *C. jejuni* and *C. coli* strains with the same *Sma*I PFGE patterns were analyzed by *Kpn*I digests. The typing results are summarized in Table 3. *Kpn*I PFGE analysis confirmed the results of the *Sma*I PFGE analysis for the *C. jejuni* strains in pattern II (Table 3). The *Campylobacter* strains showing *Sma*I PFGE patterns I, III, and IV could, however, be subtyped by *Kpn*I PFGE analysis (Table 3). These findings suggest that the *C. jejuni* and *C. coli* strains used in this study were genetically diverse.

DISCUSSION

Campylobacters are considered to be the most fre-

Table 3. Summary of PFGE analysis of *Campylobacter* strains isolated from diarrheal patients in Japan having the same *Sma*I PFGE profiles

Species	Strain no.	Year of isolation	*Sma*I[1)]	*Kpn*I[1)]
C. jejuni	K33	1999	I	i
(n = 6)	K76	2000	I	ia[2)]
	K110	2001	II	ii
	K217	2002	II	ii
	K114	2001	III	iii
	K328	2004	III	iiia[3)]
C. coli	K80	2000	IV	iv
(n = 4)	K95	2000	IV	iv
	K129	2001	IV	iv
	K135	2001	IV	iva[2)]

[1)]: All molecular types are arbitrarily defined.
[2)]: Subtype due to difference of two bands.
[3)]: Subtype due to difference of one band.

quently isolated food-borne bacteria associated with acute diarrhea in humans (24,25). However, as conventional methods for the detection and identification of *Campylobacter* spp. are laborious, time-consuming, and problematic, the development of a simple, rapid, and reliable method for the species identification of *Campylobacter* strains is a matter of great importance. In order to achieve this goal, we have recently developed a *cdt* gene-based species-specific multiplex PCR for the identification of *C. jejuni*, *C. coli*, and *C. fetus* (16). This *cdt* gene-based multiplex PCR assay was subsequently evaluated with 34 and 78 *Campylobacter*-like organisms isolated from poultry and human patients in Thailand, respectively (17, unpublished observation), and the results indicated that *cdtB* gene-based multiplex PCR appeared to be more reliable than either *cdtA* or *cdtC* gene-based multiplex PCR. However, the number of strains analyzed was rather low and the strains were geographically biased. Further evaluation with a large number of strains isolated from different sources, in particular clinical isolates, was therefore required for a proper validation of the *cdt* gene-based multiplex PCR as a simple, rapid, and reliable method for the routine identification of *C. jejuni*, *C. coli*, and *C. fetus*. In this study, the applicability of the *cdt* gene-based multiplex PCR was further evaluated with 325 *Campylobacter* strains isolated from diarrheal patients in Japan and compared with genetic methods such as *hipO* gene detection and 16S rRNA gene sequencing. Our results clearly indicate that species identification by *cdt* gene-based multiplex PCR correlates well with the collective results of the other genetic methods and is much simpler, more convenient, and may be more reliable than biochemical methods.

The routine detection of *Campylobacter* spp. in most clinical laboratories is based on a culture method using selective media and subsequent phenotypic identification (26). However, phenotypic identification can be challenging because of the special growth requirements and asaccharolytic nature of campylobacters and the fact that they possess very few distinguishing biochemical characteristics (27). The only biochemical test for discriminating between *C. jejuni* and *C. coli* is based on hippurate hydrolysis activity. However, this test is time-consuming, cumbersome, and the results are sometimes difficult to interpret when the enzymatic activity is impaired under the methodological conditions (7,28). Genetic methods could therefore represent a better alternative. Detection of the *hipO* gene by PCR is now considered to be superior for the confirmation of *C. jejuni* as this gene is highly conserved among this species (29). In this study, detection of the *hipO* gene in the 325 *Campylobacter* strains allowed 314 of them to be diagnosed as *C. jejuni*. As the 16S rRNA gene sequence has been widely utilized for the species identification of many bacteria, including *Campylobacter* (30,31), this methodology is not effective for discriminating between *C. jejuni* and *C. coli* (10,31). Thus, the remaining 11 strains were identified as *C. jejuni*/*C. coli* on the basis of their 16S rRNA gene sequence.

In reality, the detection of *hipO* gene is more convenient and reliable than a biochemical test for hippuricase activity (8,28). However, mutations of the *hipO* gene, such as deletions or mutations in the primer binding sites, have been found to result in no PCR amplification (29) or a smaller than expected PCR product (9). A single methodology such as *hipO* detection alone is therefore not sufficient to obtain accurate results, and a combination of two methodologies is recommended. A number of simple and rapid identification methods for *Campylobacter* spp. have been developed on the basis of species-specific genes (12,20,32–36). However, these methods typically require the use of hybridization probes, multiple restriction enzymes, or sophisticated equipment, which are beyond the means of many diagnostic laboratories.

This and previous studies have identified *cdt* genes in at least 560 strains, including 429 *C. jejuni*, 113 *C. coli*, and 21 *C. fetus* analyzed in a species-specific manner (15–17, unpublished observation). Such genes may therefore be an ideal target for detecting and discriminating between *Campylobacter* spp. However, deletions or mutations in the *cdt* gene cluster have been reported (18–20). Martinez et al. (20) demonstrated that the *cdtC* gene could be detected in all strains tested, although this was not the case for *cdtA* and *cdtB* genes. Likewise, Samosornsuk et al. (17) also reported *cdtA* or *cdtC* gene-based multiplex PCR-negative strains. Since the *cdtB* gene has the highest inter- and intraspecies sequence similarity among the *cdtA*, *cdtB*, and *cdtC* genes, we previously concluded that *cdtB* gene-based multiplex PCR might be the best option among *cdtA*, *cdtB*, and *cdtC* gene-based multiplex PCR assays on the basis of the preliminary evaluations of Samosornsuk et al. (17, unpublished observation). On the other hand, AbuOun et al. (19) have reported a 667-bp deletion between *cdtA* and *cdtB* and a separate 51-bp deletion within the *cdtB* genes of 3 strains isolated from campylobacteriosis patients in the United Kingdom in 2000. In this study, 2 strains (one *C. jejuni* and one *C. coli*) did not yield specific PCR products upon *cdtA* and *cdtC* gene-based multiplex PCR, respectively, due to mutations in the primer binding sites (Fig. 3). In addition, 4 *C. jejuni* strains did not yield specific PCR products upon *cdtA* and *cdtB* gene-based multiplex PCR due to a 667-bp deletion between *cdtA* and *cdtB* and a separate 51-bp deletion within the *cdtB* genes (Fig. 1), which are

exactly identical to those reported by AbuOun et al. (19). Interestingly, 2 of these 4 *C. jejuni* strains, which were isolated from epidemiologically unrelated diarrheal patients, were genetically related despite the fact that they were isolated at different time points (Table 3). These findings suggest that the same type of deletion can occur in strains that are genetically unrelated, isolated in time, and presumably come from different geographical regions. Thus, *cdt* genes may be a good target, although targeting only one such gene (*cdtA*, *cdtB*, or *cdtC*) may not be sufficient.

In conclusion, *cdt* gene-based species-specific multiplex PCR is a simple, rapid, and reliable method for the routine identification of *C. jejuni* and *C. coli*. Indeed, *cdtC* gene-based multiplex PCR may be more accurate and reliable than *cdtA* or *cdtB* gene-based multiplex PCR. However, a combination of *cdtC* and *cdtB* or *cdtA* gene-based multiplex PCR will likely provide more accurate and reliable results for the species identification of *C. jejuni*, *C. coli*, and *C. fetus*. Further evaluation with various *Campylobacter* strains of different origins (animal, environmental, and human) will be required to properly evaluate this methodology.

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Conflict of interest None to declare.

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