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A PCR-RFLP assay to detect and type cytolethal distending toxin (*cdt*) genes in *Campylobacter hyointestinalis*

Noritoshi HATANAKA[1)], Kazumasa KAMEI[1)], Srinuan SOMROOP[1)], Sharda Prasad AWASTHI[1)], Masahiro ASAKURA[1)], Naoaki MISAWA[2)], Atsushi HINENOYA[1)] and Shinji YAMASAKI[1)]*

[1)]Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58, Rinkuourai-kita, Izumisano, Osaka 598-8531, Japan
[2)]Faculty of Agriculture, University of Miyazaki, Japan

Abstract. Campylobacter hyointestinalis is considered as an emerging zoonotic pathogen. We have recently identified two types of cytolethal distending toxin (cdt) gene in C. hyointestinalis and designated them as Chcdt-I and Chcdt-II. In this study, we developed a PCR-restriction fragment length polymorphism (RFLP) assay that can differentiate Ch*cdt-I* from Ch*cdt-II*. When the PCR-RFLP assay was applied to 17 other *Campylobacter* strains and 25 non-*Campylobacter* strains, PCR products were not obtained irrespective of their *cdt* gene-possession, indicating that the specificity of the PCR-RFLP assay was 100%. In contrast, when the PCR-RFLP assay was applied to 35 *C. hyointestinalis* strains including 23 analyzed in the previous study and 12 newly isolated from pigs and bovines, all of them showed the presence of *cdt* genes. Furthermore, a restriction digest by EcoT14-I revealed that 29 strains contained both Ch*cdt-I* and Ch*cdt-II* and 6 strains contained only Ch*cdt-II*, showing 100% sensitivity. Unexpectedly, however, PCR products obtained from 7 *C. hyointestinalis* strains were not completely digested by EcoT14-I. Nucleotide sequence analysis revealed that the undigested PCR product was homologous to *cdtB* but not to Ch*cdt-IB* or Ch*cdt-IIB*, indicating the presence of another *cdt* gene-variant. Then, we further digested the PCR products with DdeI in addition to EcoT14-I, showing that all three *cdt* genes, including a possible new Ch*cdt* variant, could be clearly differentiated. Thus, the PCR-RFLP assay developed in this study is a valuable tool for evaluating the Ch*cdt* gene-profile of bacteria.

Key words: *cdt* genes, *C. hyointestinalis*, PCR-RFLP

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Campylobacter hyointestinalis was first identified from a pig with proliferative enteritis [6, 8] and has recently been isolated from both diseased and healthy animals and raw milk [4, 7, 9, 15]. Furthermore, this species was isolated from human diarrheal stool samples [5, 14, 16]. At present, *C. hyointestinalis* is considered to be an emerging zoonotic pathogen, and its clinical importance and pathogenic mechanism are under evaluation.

Cytolethal distending toxin (CDT), which is a genotoxin capable of directly damaging DNA in target cells, is considered to be a possible virulence factor of various Gram-negative bacteria including *C. hyointestinalis*. CDT consists of three subunits, CdtA, CdtB and CdtC, which are encoded by the genes *cdtA*, *cdtB* and *cdtC*, respectively [3]. The CDT holotoxin causes G_2/M cell cycle arrest, cytoplasmic distension and eventually cell death via apoptosis [3]. Although the pathogenic mechanism of CDT *in vivo* is not well understood, CDT produced by *Campylobacter jejuni* has been reported to cause panmural inflammation with mucosal denudation and necrosis affecting the jejunum, ileum and colon in mice [10].

Recently, we have identified two types of *cdt* gene clusters in *C. hyointestinalis*, namely, the *cdt-I* (Ch*cdt-I*) and *cdt-II* (Ch*cdt-II*) genes from *C. hyointestinalis* strains 022 [17] and ATCC35217^T [12], respectively. The toxins encoded by these genes (ChCDT-I and ChCDT-II, respectively) both induced cell distention and death in HeLa cells. However, the homologies between these two ChCDTs were only 25.0, 56.0 and 24.8% in their CdtA, CdtB and CdtC subunits, respectively. Since there was a low homology between ChCDT-I and ChCDT-II, particularly regarding their CdtA and CdtC subunits, which are responsible for binding to receptor molecules, it is possible that their target cells might differ. Thus, ChCDT-I and ChCDT-II may have different pathogenic mechanisms *in vivo*. Therefore, it is important to analyze the distribution of these two *cdt* gene-variants in *C. hyointestinalis* to understand the difference in pathogenesis between ChCDT-I and ChCDT-II. In this study, we have developed a PCR-

*Correspondence to: Yamasaki, S., Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58, Rinkuourai-kita, Izumisano, Osaka 598-8531, Japan. e-mail address: shinji@vet.osakafu-u.ac.jp

restriction fragment length polymorphism (PCR-RFLP) assay for the detection and differentiation of Ch*cdt-I* and Ch*cdt-II* in *C. hyointestinalis*. The specificity and sensitivity of the PCR-RFLP assay were evaluated, and the presence and types of *cdt* genes in 35 *C. hyointestinalis* strains, including 12 strains newly isolated from pigs and bovines, were successfully determined by the PCR-RFLP assay.

MATERIALS AND METHODS

Bacterial strains and growth media

Thirty-five strains of *C. hyointestinalis*, which are described in Table 1, were examined in this study. Among them, 23 strains were known to possess Ch*cdt-I* and/or Ch*cdt-II* [12], and the other 12 strains were newly isolated from pigs and bovines. A total of 42 bacterial strains other than *C. hyointestinalis* including 17 strains of 11 other *Campylobacter* species and 25 strains of 20 non-*Campylobacter* species were also included in this study (Table 1).

Campylobacter, *Helicobacter* and *Arcobacter* spp. were grown on blood agar [blood agar base No. 2 (Oxoid Ltd., Basingstoke, U.K.) supplemented with 5% (v/v) defibrinated horse blood (Nippon Bio Supp. Center, Tokyo, Japan)] under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) at 37°C for 2 days or more. *Vibrio* spp. were cultured using TCBS agar (NISSUI Pharmaceutical Co., Ltd., Tokyo, Japan), and other bacteria were grown in Luria–Bertani broth (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) at 37°C overnight.

DNA preparation

Template DNAs for PCR were prepared by the boiling method as previously reported [1, 11]. Briefly, bacterial colonies grown on appropriate agar plates were suspended in 500 *µl* of TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM ethylenediaminetetraacetic acid). Bacterial suspensions were boiled for 10 min, kept on ice for 10 min and centrifuged at 20,000 ×*g* for 10 min. Then, the supernatants were collected and used as DNA templates for PCR. As positive controls for the PCR and RFLP assays, pET28a Ch*cdt-IB* and pET28a Ch*cdt-IIB,* which carry the Ch*cdt-IB* and Ch*cdt-IIB* genes of *C. hyointestinalis* strains 022 and ATCC35217^T, respectively, were used [12, 17].

PCR amplification assay

PCR was performed with ChCdt-BF (5′-GCTACTTGGAATATGCAAGG-3′) and ChCdt-BR (5′-TGGTTCTCTATTRAAATCWCC-3′) primer set using an Applied Biosystems Veriti® Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Each PCR mixture contained 0.5 and 0.15 *µ*M of ChCdt-BF and ChCdt-RF primers, respectively, 1 *µl* of DNA template, 0.2 mM of each dNTP, 1 × r*Taq* DNA polymerase buffer and 0.5 U of r*Taq* DNA polymerase (Takara Bio Inc., Otsu, Japan) in a total volume of 20 *µl*. The samples were subjected to an initial denaturation step at 94°C for 3 min followed by 30 cycles of amplification, each cycle consisting of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec. A final extension step at 72°C for 3 min was included. PCR products were analyzed by electrophoresis using a 2% PrimeGel™ Agarose LE gel (Takara Bio Inc.), and bands were visualized with ultraviolet (UV) light after staining with ethidium bromide (1 *µg*/m*l*). Images were captured on a ChemiDoc system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.).

RFLP assay

PCR products (2 to 5 *µl*; 100 to 200 *ng*) were digested with either 4 U of EcoT14-I or 2 U of EcoT14-I and 2 U of DdeI (New England Biolabs Inc., Ipswich, MA, U.S.A.) under 1 ×H buffer or 1 ×K buffer (Takara Bio Inc.) in a final volume of 10 *µl* at 37°C overnight. Then, the digested PCR products were analyzed by 3% PrimeGel™ Agarose LE gel electrophoresis as described above.

Detection limit of the PCR-RFLP assay

The *C. hyointestinalis* strains, ATCC35217^T, 022 and 3197, were cultured on blood agar under anaerobic conditions at 37°C for 2 days, and the colonies that grew were suspended in sterile phosphate-buffered saline. The density of each bacterial culture was adjusted to OD_{600}=1.0 and then 10-fold serially diluted in phosphate-buffered saline, and DNA templates were prepared from 100 *µl* of each dilution by the boiling method as described above. Then, PCR assays were carried out with these DNA templates. Furthermore, to determine the viable bacterial count, 100 *µl* of each dilution was spread on blood agar plates in triplicate and cultured at 37°C for 2 days under anaerobic conditions, and then, the number of colonies on each plate was counted.

Colony hybridization assay

The distribution of Ch*cdt-IB* and Ch*cdt-IIB* in 12 newly isolated strains of *C. hyointestinalis* was examined by colony hybridization assay as described previously [11], with minor modifications. In brief, Ch*cdt-IB* and Ch*cdt-IIB* gene-probes were prepared by PCR with each primer set (Table S1) using *C. hyointestinalis* strains 022 (Ch*cdt-IB*) and ATCC35217^T(Ch*cdt-IIB*) as DNA templates, and the obtained PCR products were purified from agarose gel using the Wizard SV® Gel and PCR clean-up system (Promega Corporation, Madison, WI, U.S.A.). Strains were grown on a nitrocellulose membrane (GE Healthcare U.K. Ltd., Buckinghamshire, U.K.) overlaid on blood agar plates under anaerobic conditions at 37°C overnight. Colonies were lysed, and DNA was denatured *in situ* by the alkaline lysis method followed by UV cross-linking with a UV crosslinker (CX-2000; UVP LLC, Upland, CA, U.S.A.). The processed nitrocellulose membranes were hybridized with each gene probe, and radioactivity was visualized using an FLA-7000 biomolecular imager (GE Healthcare U.K. Ltd.).

Table 1. Bacterial strains used in this study and the distribution of Ch*cdt-I* and Ch*cdt-II* genes

Bacterial species	Strain	Origin	Chcdt-*I*	Chcdt-*II*	Reference	Bacterial species	Strain	Origin	*cdt*
C. hyointestinalis	022	Clinical	+	+	[12]	*C. coli*[a]	ATCC33559	Pig	Cc*cdt*
	ATCC 35217^T	Pig	-	+	[12]	*C. coli*[a]	ATCC43478	Marmoset	Cc*cdt*
	1-1	Pig	+	+	[12]	*C. coli*[a]	CO1-017	Clinical	Cc*cdt*
	10-1	Gorilla	+	+	[12]	*C. consisus*	ATCC33237	Human	-
	2003	Bovine	+	+	[12]	*C. consisus*	ATCC51562	Human	-
	2030	Bovine	+	+	[12]	*C. curvus*	ATCC35224	Human	-
	2032	Bovine	+	+	[12]	*C. fetus*[a]	ATCC27374	Sheep	Cf*cdt*
	2033	Bovine	+	+	[12]	*C. helveticus*	ATCC51209	Cat	Chel*cdt*
	2034	Bovine	+	+	[12]	*C. hominis*	ATCCBAA-381	Human	-
	2035	Bovine	+	+	[12]	*C. jejuni*[a]	ATCC33560	Bovine	Cj*cdt*
	2037	Bovine	+	+	[12]	*C. jejuni*[a]	ATCC43432	Human	Cj*cdt*
	2038	Bovine	+	+	[12]	*C. jejuni*[a]	B86	Bovine	Cj*cdt*
	2039	Bovine	+	+	[12]	*C. jejuni*[a]	CO2-037	Clinical	Cj*cdt*
	3014	Bovine	+	+	[12]	*C. lari*[a]	JCM2530	Larus argintatus	Cl*cdt*
	3158	Bovine	+	+	[12]	*C. mucosalis*	ATCC49352	Pig	-
	3197[b]	Bovine	+	+	[12]	*C. upsaliensis*[a]	ATCC43954	Dog	Cu*cdt*
	3477	Bovine	+	+	[12]	*C. ureolyticus*	ATCC33387	Human	-
	3535[b]	Bovine	+	+	[12]	*Arcobacter butzuleri*	ATCC49616	Clinical	-
	3839[b]	Bovine	+	+	[12]	*A. cryaerophilus*	ATCC43157	Pig	-
	3857[b]	Bovine	+	+	[12]	*A. skirrowii*	ATCC51132	Unknown	-
	87-4[b]	Monkey	+	+	[12]	*Citrobacter freundii*	ATCC43864	Unknown	-
	94-6	Elephant	+	+	[12]	*Enterobacter cloacae*	NBPC 12935	Unknown	-
	SS	Pork	-	+	[12]	*Escherichia coli*[a]	ATCC11775	Human	-
	130206DCC11[b]	Bovine	+	+	This study	*Escherichia coli*[a]	ATCC25922	Clinical	-
	130206DCC12	Bovine	+	+	This study	*Escherichia coli*[a]	GB1371	Clinical	Ec*cdt-I*
	130325D2aC1	Bovine	+	+	This study	*E. albertii*[a]	19982	Clinical	Ea*cdt*
	141007D1C1	Bovine	+	+	This study	*Haemophilus ducreyi*[a]	ATCC700724	Human	Hd*cdt*
	141007D2C1[b]	Bovine	+	+	This study	*Helicobacter fennelliae*[a]	ATCC35684	Human	Hf*cdt*
	S1C-1	Pig	-	+	This study	*H. hepaticus*[a]	ATCC51448	Mouse	Hh*cdt*
	S2 TDNEFB-1	Pig	+	+	This study	*H. pylori*	ATCC43504	Human	-
	S2CB-1	Pig	-	+	This study	*H. pylori*	ATCC43629	Human	-
	S3C-1	Pig	+	+	This study	*Klebsiera oxytoca*	ATCC8724	Unknown	-
	S4TVB-1	Pig	-	+	This study	*K. pneumoniae*	ATCC13883	Unknown	-
	S5C-1	Pig	+	+	This study	*Providencia alcalifaciens*[a]	GTC02020	Unknown	Pa*cdt*
	S5TDNPFB-1	Pig	-	+	This study	*P. heimbachae*	ATCC35613	Penguin	-
						P. rettgeri	ATCC29944	Human	-
						P. rustigianii	ATCC33673	Human	-
						P. stuartii	ATCC29914	Human	-
						Salmonella enterica	ATCCBAA-664	Unknown	-
						Vibrio cholerae	VcN16961	Unknown	-
						Vibrio cholerae	VcBT-AP32541	Unknown	-
						Vibrio cholerae	Vc129	Unknown	-

a) Bacterial species known to possess *cdt* genes, b) Undigested PCR product was observed from these strains, +: Indicates that the gene was identified by Ch*cdt-B* gene-based PCR-RFLP assay, -: Indicates that the gene was not identified by Ch*cdt*-B gene-based PCR-RFLP assay.

Sequencing of cdt genes and PCR products

To determine the entire nucleotide sequence of the *cdt* gene cluster in 12 newly obtained *C. hyointestinalis* strains (Table 1), PCR was performed with a combination of forward primers targeting upstream of *cdtA* or *cdtB* and reverse primers targeting downstream of *cdtC* (Table S2). Each PCR mixture contained 0.5 μM of each primer, 1 μl of DNA template, 0.2 mM of each dNTP, 1 ×Ex *Taq*® DNA polymerase buffer and 1.0 U of Ex *Taq*® DNA polymerase in a total volume of 20 μl. The samples were subjected to an initial denaturation step of 3 min at 94°C followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 90 sec to 2 min, and then a final extension step at 72°C for 3 to 5 min. If a PCR product of the expected size was obtained, the PCR product was purified using a PCR clean-up system (QIAGEN GmbH, Hilden, Germany) for sequencing. The sequencing reactions were performed by the chain termination method with the BigDye® Terminator v1.1 cycle sequencing kit (Thermo Fisher Scientific). Nucleotide sequences were determined using an ABI PRISM® 3130-Avant Genetic analyzer (Thermo Fisher Scientific). The sequence upstream of Ch*cdt-IB* in *C. hyointestinalis* strains 130206DCC11, 130206DCC12, 130325D2aC1, 141007D1C1, S2TDNEFB-1, S3C-1 and S5C-1, and that of Ch*cdt-IIB* in *C. hyointestinalis* strain 141007D2C1, were determined with the primers listed in Table S2 by genome walking as described previously [12].

Undigested PCR products (approximately 500 bp in length) were purified from agarose gel by using the Wizard® SV Gel and PCR clean-up system (Promega Corporation). The nucleotide sequences of undigested PCR products were determined as described above, analyzed using MEGA6, and compared with the sequences of Ch*cdt-IB* and Ch*cdt-IIB*.

Nucleotide sequence accession numbers

The *cdt* gene-sequences determined in this study were deposited to the DNA Data Bank of Japan (DDBJ) with the accession numbers LC175774 to LC175800.

RESULTS

Evaluation of ChcdtB amplification by PCR

By comparing published *cdtB* sequences of *C. hyointestinalis* (GenBank accession nos. AB218983 and AB373951) and those of other *Campylobacter* spp. (AB274783, AB274793, AB274802, AB872889 and AB872911), forward and reverse primers (ChCdt-BF and ChCdt-BR) were designed from the conserved regions of Ch*cdt-IB* and Ch*cdt-IIB*. To evaluate whether these primers can amplify Ch*cdt-IB* and Ch*cdt-IIB*, PCR was carried out using recombinant plasmids, pET28a carrying Ch*cdt-IB* and pET28a carrying Ch*cdt-IIB*, as positive controls. PCR of the positive controls, pET28a carrying Ch*cdt-IB* and pET28a carrying Ch*cdt-IIB*, gave the expected size of the PCR product (507 or 516 bp) in each case (Fig. S1). Sequence analysis further confirmed that these PCR products were amplified from Ch*cdt-IB* and Ch*cdt-IIB* gene-fragments (data not shown).

The sensitivity of the PCR assay was evaluated with the 35 *C. hyointestinalis* strains listed in Table 1. A PCR product of the expected size was obtained from 23 strains, which were previously reported to possess Ch*cdt-I* and/or Ch*cdt-II* (Table 1). Furthermore, a PCR product of the same size was also obtained from 12 newly isolated *C. hyointestinalis* strains in which the presence of Ch*cdt-I* and/or Ch*cdt-II* was confirmed by colony hybridization assay and sequence analysis as described below. Then, the specificity of the PCR assay was evaluated with a total of 42 strains including 17 strains of 11 other *Campylobacter* spp. and 25 strains of 20 non-*Campylobacter* spp. as listed in Table 1. Irrespective of their *cdt* gene-possession, no PCR product was obtained from those 42 strains.

Sequence analysis of cdt gene cluster in 12 C. hyointestinalis strains

To calculate the sensitivity of the PCR-RFLP assay developed in this study, we further evaluated the distribution of *cdt* genes in 12 *C. hyointestinalis* strains, which were newly isolated from pigs and bovines, by colony hybridization assay. The Ch*cdt-IB* gene-probe was reacted with 8 strains (130206DCC11, 130206DCC12, 130325D2aC1, 141007D1C1, 141007D2C1, S2TDNEFB-1, S3C-1 and S5C-1), while the Ch*cdt-IIB* gene-probe was reacted with all the strains (data not shown). Furthermore, the entire nucleotide sequences of these *cdt* gene clusters were determined as described in the Materials and Methods section. The sizes of individual *cdt* genes and the homology of each *cdt* gene are summarized in Table S3. These data indicated that the 12 *C. hyointestinalis* strains possessed Ch*cdt-I* and/or Ch*cdt-II*, except for 1 strain, 141007D2C1, in which *cdtA* was missing.

Detection limit of the PCR-RFLP assay

The detection limit of this assay was evaluated using *C. hyointestinalis* ATCC35217^T(Ch*cdt-II*), 022 (Ch*cdt-I* and Ch*cdt-II*) and 3197 (Ch*cdt-I*, Ch*cdt-II* and Ch*cdt* homologue gene) strains. The detection limits of this PCR assay were determined to be $4.9 \pm 1.8 \times 10^2$, $4.4 \pm 0.47 \times 10^2$ and $4.9 \pm 3.2 \times 10^2$ colony-forming units (CFU)/20 μl of reaction mixture for strains ATCC35217^T, 022 and 3197, respectively.

A ChcdtB-based PCR-RFLP assay

EcoT14-I restriction enzyme was selected for the PCR-RFLP assay by comparing the sequences of Ch*cdt-IB* and Ch*cdt-IIB*. When the PCR products obtained from the positive controls were digested with EcoT14-I, the Ch*cdt-IB* DNA fragment yielded two bands of 363 and 144 bp (Fig. 1A, lane 1), and in the case of Ch*cdt-IIB*, the fragment sizes were 261 and 255 bp (Fig. 1A, lane 2). We then evaluated the utility of the PCR-RFLP assay with the PCR products obtained from 35 *C. hyointestinalis* strains (Table 1). Among the 35 strains, the *cdt* gene-possession profile of 23 strains was previously analyzed by colony hybridization assay and the same types of *cdt* were successfully identified by their RFLP patterns, even though both Ch*cdt-I* and Ch*cdt-II* were present (Fig. 1A, lane 4). When the PCR products obtained from 12 other strains were analyzed by the RFLP assay, their RFLP patterns indicated that all *C. hyointestinalis* strains contained Ch*cdt-IIB* and 7 strains additionally possessed Ch*cdt-IB* (Table 1). These data indicated that the PCR-RFLP assay could identify the *cdt* gene-profile. However, undigested PCR products were obtained from 7 strains (3197, 3535, 3839, 3857, 87–4, 130206DCC11 and 141007D2C1) among 35 strains when digested with EcoT14-I (Fig. 1A, lane 5).

The undigested PCR products obtained from 7 strains were purified and sequenced. This analysis revealed that the undigested PCR products carried sequences of *cdt* genes with only 45–46% and 64–66% homologies to the corresponding regions of Ch*cdt-IB* and Ch*cdt-IIB*, respectively. These data indicated that the *C. hyointestinalis* strains might possess new variants of *cdt* genes.

To avoid the possibility of obtaining ambiguous results from the PCR-RFLP assay, we further digested the PCR products with DdeI in addition to EcoT14-I. When the PCR products obtained from positive controls were digested with these enzymes, the Ch*cdt-IB* gene-fragment and Ch*cdt-IIB* gene-fragment were theoretically digested to yield fragments of 363, 101 and 43 bp, and 261, 113, 70, 41 and 31 bp, respectively. The agarose gel separation result is shown in Fig. 1B. Furthermore, the PCR products of

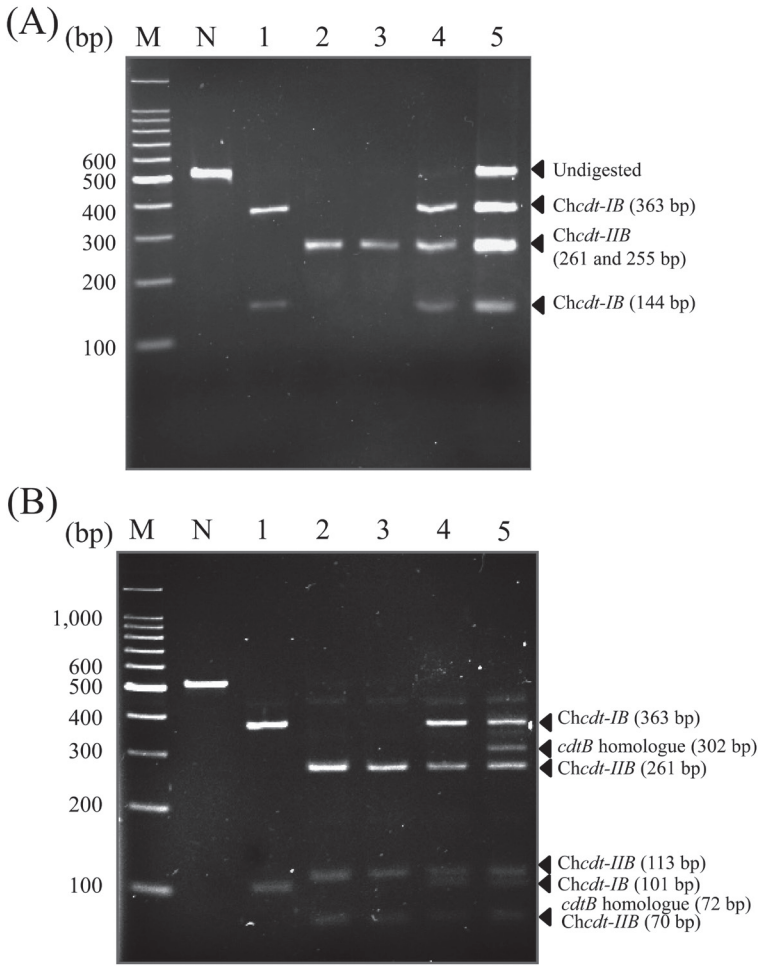

Fig. 1. (A) PCR-RFLP with EcoT14-I to evaluate the presence of the Ch*cdtB* gene in *C. hyointestinalis*. PCR products digested with EcoT14-I were analyzed by electrophoresis on a 3% agarose gel. Undigested PCR product in lane 5 was extracted from the gel, purified, and subjected to a sequencing analysis. Undigested PCR product was identified as a possible Ch*cdtB* variant gene. Lanes: M, 100-bp DNA ladder (Takara Bio Inc.); N, PCR product of *C. hyointestinalis* 3197 strain; 1, pET28a Ch*cdt-IB*; 2, pET28a Ch*cdt-IIB*; 3, *C. hyointestinalis* ATCC35217^T strain; 4, *C. hyointestinalis* 022 strain; 5, *C. hyointestinalis* 3197 strain. (B) PCR-RFLP with EcoT14-I and DdeI to evaluate the presence of the Ch*cdtB* gene in *C. hyointestinalis*. Lanes: M, 100-bp DNA ladder (Takara Bio Inc.); N, PCR product of *C. hyointestinalis* 3197 strain; 1, pET28a Ch*cdt-IB*; 2, pET28a Ch*cdt-IIB*; 3, *C. hyointestinalis* ATCC35217^T strain; 4, *C. hyointestinalis* 022 strain; 5, *C. hyointestinalis* 3197 strain.

the *cdtB* gene-homologue were theoretically digested to yield fragments of 302, 72, 51, 27 and 24 bp (Fig. 1B). Then, when we evaluated the utility of this modified PCR-RFLP assay with the PCR products obtained from 35 *C. hyointestinalis* strains, Ch*cdt-IB,* Ch*cdt-IIB* and *cdtB* gene-homologue could be clearly distinguished from one another.

DISCUSSION

Although the clinical significance of *C. hyointestinalis* infection has not yet been clearly established, *C. hyointestinalis* is implicated as a pathogen of both humans and animals. We have previously reported that two types of the *cdt* genes, namely Ch*cdt-I* and Ch*cdt-II*, are present in *C. hyointestinalis* and encode biologically active CDTs [12, 17]. Therefore, CDT is a candidate virulence factor in *C. hyointestinalis.* To understand the importance of CDT in the pathogenesis of *C. hyointestinalis*, we attempted to establish a PCR-RFLP assay for the detection and differentiation of Ch*cdt-I* and Ch*cdt-II* in *C. hyointestinalis*. Since *cdtB* was previously demonstrated to be more conserved than *cdtA* and *cdtC* in *C. jejuni*, *C. coli* and *C. fetus* [2], various *cdtB* gene-based multiplex PCR and PCR-RFLP assays have been developed to detect and differentiate *Campylobacter* species [1, 11, 13]. In this study, we have developed a Ch*cdtB*-based PCR-RFLP assay that can detect and differentiate between Ch*cdt-I* and Ch*cdt-II*.

The sensitivity and specificity of the PCR-RFLP assay were shown to be 100%. Furthermore, the PCR-RFLP assay could clearly identify the *cdt* gene-profile, even though *C. hyointestinalis* contained both Ch*cdt-I* and Ch*cdt-II* (Fig. 1A, lane 4). Kamei *et al*. have reported that Ch*cdt-II* may be ubiquitously conserved in *C. hyointestinalis* [12]. In this study, we evaluated the presence of

cdt genes using 12 *C. hyointestinalis* strains that were newly isolated from pigs and bovines. As expected, Ch*cdt-IIB* was detected in all the *C. hyointestinalis* strains, while Ch*cdt-IB* was also detected in 7 strains (Table 1). Furthermore, the presence of Ch*cdt-IIA* and Ch*cdt-IIC* in these 12 strains was analyzed by colony hybridization assay, and their nucleotide sequences were determined by sequence analysis (Table S3). Although Ch*cdt-IIA* was not detected in *C. hyointestinalis* strain 141007D2C1, 11 other strains were demonstrated to possess Ch*cdt-IIA*, Ch*cdt-IIB* and Ch*cdt-IIC*. It appears that Ch*cdt-IIB* and Ch*cdt-IIC* are ubiquitously present in *C. hyointestinalis* and may be suitable target genes for the detection and identification of this species.

The isolation of *C. hyointestinalis* is difficult if *C. jejuni* and *C. coli* are targeted for isolation because of its susceptibility to cephem antibiotics, which are included in the modified charcoal-cefoperazone-deoxycholate agar and Bolton broth that are normally used for the isolation of *C. jejuni* and *C. coli*. Therefore, a PCR-based method is required to detect this bacterium before initiating cultivation. The *cdtB* gene-based PCR assay developed in this study could detect *C. hyointestinalis* with 100% sensitivity and specificity (Table 1). The detection limit of the assay was determined to be approximately 10^2 CFU/20 μl of reaction mixture. These data indicate that the PCR assay developed in this study is useful for the detection of *C. hyointestinalis*.

However, PCR products obtained from some template DNAs of *C. hyointestinalis* strains remained undigested by EcoT14-I (Fig. 1A, lane 5). A sequence analysis of these PCR fragments showed that these strains carry the homologous *cdtB* gene sequence, indicating the detection of a new *cdt* gene-variant of *C. hyointestinalis*. Therefore, we further included DdeI digestion in the *cdtB* gene-based PCR-RFLP assay to distinguish not only Ch*cdt-IB* and Ch*cdt-IIB* but also a possible new *cdtB* gene-variant. The *cdtB* gene-based PCR-RFLP assay with EcoT14-I and DdeI could clearly distinguish three different *cdtB* genes as PCR products including Ch*cdt-IB* and Ch*cdt-IIB* and a possible new *cdtB* gene-variant in the 35 *C. hyointestinalis* strains listed in Table 1. Further analysis to characterize the *cdt* gene-variant is currently ongoing in our laboratory.

In conclusion, the Ch*cdtB* gene-based PCR-RFLP assay developed in this study is useful to detect and differentiate not only Ch*cdt-I* and Ch*cdt-II* but also a possible new *cdt* gene-variant in *C. hyointestinalis*. The ubiquitous presence of Ch*cdt-IIB* among the tested *C. hyointestinalis* strains also confirmed it to be an appropriate target gene for the identification of *C. hyointestinalis*. Further studies are required for the evaluation of the *cdtB* gene-based PCR-RFLP assay for the detection and differentiation of *cdt* genes present in *C. hyointestinalis*.

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