APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2008, p. 5366–5372 Vol. 74, No. 17
0099-2240/08/$08.00+0 doi:10.1128/AEM.00783-08

Prevalence and Characterization of Enterotoxin Gene-Carrying *Clostridium perfringens* Isolates from Retail Meat Products in Japan▽

Yasuhiro Miki, Kazuaki Miyamoto,* Ikuko Kaneko-Hirano, Kanako Fujiuchi, and Shigeru Akimoto

Department of Microbiology, School of Medicine, Wakayama Medical University, Wakayama 641-0012, Japan

Received 6 April 2008/Accepted 25 June 2008

Clostridium perfringens **is an important anaerobic pathogen causing food-borne gastrointestinal (GI) diseases in humans and animals. It is thought that *C. perfringens* food poisoning isolates typically carry the enterotoxin gene (*cpe*) on their chromosome, while isolates from other GI diseases, such as antibiotic-associated diarrhea, carry *cpe* on a transferable plasmid. However, food-borne GI disease outbreaks associated with *C. perfringens* isolates carrying plasmid-borne *cpe* (plasmid *cpe* isolates) were recently reported in Japan and Europe. To investigate whether retail food can be a reservoir for food poisoning generally, we evaluated Japanese retail meat products for the presence of two genotypes of enterotoxigenic *C. perfringens*. Our results demonstrated that approximately 70% of the Japanese retail raw meat samples tested were contaminated with low numbers of *C. perfringens* bacteria and 4% were contaminated with *cpe*-positive *C. perfringens*. Most of the *cpe*-positive *C. perfringens* isolates obtained from Japanese retail meat carried *cpe* on a plasmid. The plasmid *cpe* isolates exhibited lower spore heat resistance than did chromosomal *cpe* isolates. Collectively, these plasmid *cpe* isolates might be causative agents of food poisoning when foods are contaminated with these isolates from equipment and/or the environment after cooking, or they may survive in food that has not been cooked at a high enough temperature.**

Clostridium perfringens type A food poisoning is among the most commonly identified food-borne illnesses in Japan, Europe, and the United States (15). The symptoms of diarrhea and abdominal cramping result from *C. perfringens* enterotoxin (CPE) (15). The vehicles of infection are typically meat and poultry. In Japan, approximately 20 to 40 outbreaks of *C. perfringens* food-borne diseases were identified from 2000 to 2005 and approximately 4,000 people became sick each year (http://www.mhlw.go.jp/topics/syokuchu/index.html [text in Japanese]). Only a small fraction (0 to 2%) of *C. perfringens* isolates, mainly type A, from Japanese retail food carry the CPE-encoding gene (*cpe*) (8, 10). It is thought that food poisoning isolates typically carry *cpe* on their chromosome while isolates from other gastrointestinal (GI) diseases, such as antibiotic-associated diarrhea, carry *cpe* on a transferable plasmid (12). A recent report from the United States showed that *C. perfringens* isolates in retail foods such as meat and poultry are chromosomal *cpe* strains (15). However, in Japan and Europe, food-borne GI disease outbreaks caused by plasmid *cpe C. perfringens* were reported recently, while several food-borne isolates carry chromosomal *cpe* (6, 14).

In previous Japanese surveys, the location of *cpe* in enterotoxigenic *C. perfringens* and the heat resistance of spores of these isolates were not investigated, even though these properties are important in establishing a link between plasmid *cpe* isolates and food poisoning (8, 10). Therefore, to investigate whether chromosomal *cpe* strains are the major population in Japanese retail meat, as they are in U.S. retail food, our present study included in-depth molecular genetic and phenotyping analyses of *cpe*-positive *C. perfringens* obtained from retail meat in Japan. We also measured the heat resistance of spores of plasmid *cpe* isolates and compared the results with those of spores of plasmid and chromosomal *cpe* isolates obtained in previous studies (11). Results of the present study indicate that plasmid *cpe* isolates are predominant in Japanese retail meat and that spores of these plasmid *cpe* isolates exhibit lower heat resistance compared to those of chromosomal *cpe* isolates.

MATERIALS AND METHODS

Bacterial strains. A total of eight chromosomal *cpe* isolates were included in this study (9, 12, 15): two food poisoning isolates from Europe (NCTC8239 and NCTC8798), four food poisoning isolates from Japan (W4232, W5603, W6206, and OSAKA2), and two food isolates from the United States (P-1/09/03 and T-1/08/03). Three plasmid *cpe* food poisoning outbreak strains (T1, T16, and T102) belong to the *cpe*-IS*1470*-like genotype, while three *cpe*-positive outbreak strains (no. 2, no. 24, and no. 110) belonging to the *cpe*-IS*1151* genotype were also included (9, 14). Isolates with *cpe* on a plasmid were also included (9, 12): three sporadic diarrhea isolates in Europe (F4969, F4013, and F5603) and a healthy human fecal isolate in Japan (MR2-4). A total of 10 plasmid *cpe* isolates were used in this study.

Collection of food samples. Two hundred samples of retail raw meat were obtained from grocery stores and retail meat shops in Wakayama city between April and September 2006. A breakdown of these meat samples is shown in Table 1.

Isolation and toxin genotype identification of *C. perfringens* strains from meat products. A sample of 100 g or one block mass was aseptically put into a stomacher bag and blended with 100 ml of fluid thioglycolate (FTG) medium II (Eiken) and then incubated at 45°C overnight anaerobically in the bag. One loopful of each thioglycolate medium II culture was streaked onto SFP (Difco) agar containing 50% egg yolk-enriched saline (Kyokuto), 12 mg/ml kanamycin (Wako), and 30 U/ml polymyxin B (MP Biomedicals) and incubated at 37°C overnight anaerobically. From the SFP plates, colonies either showing black in the center or surrounding lecithinase activity were selected and put into 10 ml of TGY medium (3% Trypticase soy broth [Difco], 2% D-glucose [Wako], 1% yeast extract [Difco], 0.1% L-cysteine [Wako]) and incubated at 37°C overnight. Two hundred microliters of each TGY overnight culture was used for the preparation of DNA with InstaGene matrix (Bio-Rad). DNA preparations were used as templates for a *C. perfringens* genotype PCR assay (3). This PCR assay was used to identify *C. perfringens* isolates and also to detect the genes encoding beta-toxin

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-0012, Japan. Phone: 81-73-441-0640. Fax: 81-73-448-1026. E-mail: kazuaki@wakayama-med.ac.jp.

▽ Published ahead of print on 7 July 2008.

TABLE 1. Isolation of *C. perfringens* strains from Japanese retail meats

Food	No. of samples tested	MPN/g range	No. (%) of samples contaminated with *C. perfringens*	No. of samples with		
				Type A	Type A-*cpb2*	Type A-*cpe*
Beef	35	<3	16 (45.7)	19	9	2
Ground beef	22	<3	18 (81.8)	24	11	0
Pork	42	<3	15 (35.7)	22	9	0
Ground pork	21	<3	17 (81.0)	25	16	0
Ground beef-pork mixture	21	<3	20 (95.2)	26	12	0
Chicken	33	3~3.0	32 (97.0)	53	46	1
Ground chicken	22	3~3.6	22 (100.0)	40	38	0
Duck	1	<3	1 (100.0)	2	1	0
Lamb	3	<3	1 (33.3)	1	1	0
Total	200		142 (71.0)	212	143	3

(*cpb*), epsilon-toxin (*etx*), iota-toxin (*ia*), enterotoxin (*cpe*), and beta2-toxin (*cpb2*).

Determination of MPN of *C. perfringens* bacteria per gram in meat samples. When 10 g of ground meat or meat samples chopped into small pieces was available (approximately 140 meat samples were surveyed in this study), a three-tube most-probable-number (MPN) method was used to investigate the *C. perfringens* amounts in the samples (15). Briefly, 10-g aliquots of meat samples were put into a stomacher bag and then 20 ml of 0.1% peptone (Difco) was added. An aliquot was diluted in 10-fold increments (from 10^{-1} to 10^{-3}) in FTG (Difco). Next, 0.2 ml of each dilution from a single sample was inoculated into three tubes containing 5 ml of differential reinforced clostridial broth medium (Merck) and then incubated at 37°C for 16 to 24 h. Cultures testing positive for *C. perfringens* produced a unique black precipitation in this differential reinforced clostridial broth medium. Statistical analyses were performed according to the FDA web page (http://www.cfsan.fda.gov).

PCR detection of *cpa* and *cpe* in enriched cultures. To confirm the presence of *C. perfringens* and enterotoxigenic *C. perfringens*, DNA was prepared from 400-μl enriched-culture samples with InstaGene matrix. For the *cpa* gene assay, the PCR primers used to detect *cpa* (3) and *cpe*, respectively, were cpe-F3 (5′ACA TCTGCAGATAGCTTAGGAAT3′) and cpe-B3 (5′CCAGTAGCTGTAATTG TTAAGTGT3′). The 50-μl PCR volume included 10 μl of GoTaq DNA polymerase buffer (Promega), 4 μl of template DNA preparation, a 1 μM concentration of each primer, 0.2 mM concentrations of deoxynucleoside triphosphates, 2.0 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Promega). Amplification was carried out in a MiniCycler (MJ Research), with 1 cycle of 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 68°C; and a final dwell time of 8 min at 68°C. The PCR-amplified products were analyzed by electrophoresis of 4-μl PCR samples in a 1.5% agarose gel and staining with ethidium bromide. *cpe* and *cpa* PCR products of 248 and 324 bp, respectively, were detected. The specificity of the *cpe* PCR assay was tested with *Clostridium botulinum* types A, B, C, E, and F; four food poisoning isolates of *Bacillus cereus*; and six isolates from meat samples (these six meat isolates were identified as *Streptococcus* species and four *Clostridium* species based on 16S rRNA sequences).

PCR assay for genotyping of *cpe*-positive *C. perfringens* isolates. To investigate whether *cpe* is present on the chromosome or on a large plasmid in food isolates, we performed a *cpe*-genotyping PCR assay as described previously (9). For *cpe*-positive control strains NCTC8239, F4969, and F4013, DNAs prepared with the InstaGene matrix kit were used as the templates. For enriched-culture samples assayed by PCR, DNAs prepared with the InstaGene matrix kit were further purified by phenol-chloroform-isoamyl alcohol extraction before use.

SLST analysis of the superoxide dismutase gene (*sod*) of *C. perfringens* isolates. Sixty-one *C. perfringens* food isolates (both *cpe* positive and *cpe* negative; this survey), eight chromosomal *cpe* strains, and six plasmid *cpe* strains (F4969, F4013, F5603, no. 2, T16, and MR2-4) (9, 14, 15) were used for *sod* gene single-locus sequence typing (SLST) analysis. PCR amplification was performed with primers sodF (5′AGCCTTTAGACTATCCTTATGATGCCC3′) and sodR2 (5′GAACCAAAGGTAGAGATTCCACATTGC3′). The PCR was performed with the following regimen: 1 cycle of 2 min at 94°C; 35 cycles of 30 s at 94°C, 60 s at 55°C, and 60 s at 68°C; and a final dwell time of 8 min at 68°C in a MiniCycler (MJ Research). PCR products were then used as sequence templates. Sequence data were analyzed by ClustalW with LaserGene software.

Detection of *sod* sequence in *cpe*-positive enriched-culture samples. In our first attempt to isolate and detect the chromosomal *cpe*-positive strain, there was no evidence of chromosomal *cpe*-positive strains in retail meat. The low sensitivity of the *cpe*-genotyping assay might have affected the results, because the estimated size of the PCR products in this assay could be more than 1 kb. From the results of *sod* sequence analysis, all of the chromosomal *cpe* isolates belonged to a distinct cluster which could easily differ from other plasmid *cpe*-positive and *cpe*-negative isolates; also, the *sod* sequences of chromosomal *cpe* isolates showed the same sequence variations. To detect chromosomal *cpe* isolates with improved sensitivity, a new PCR assay, detecting ~350 bp, was developed with a newly constructed inner primer pair (sodFPF, 5′-AGCCTTTAGACTATCCTT ATGATGCCC-3′; sodFPR, 5′-CTTTTCTTTAAATTCTTCAAAAGAACC-3′) based on the common nucleotide differences between *sod* sequences from chromosomal *cpe* isolates and other isolates. With this new primer pair, we performed a PCR assay with DNAs from eight *cpe*-positive enriched cultures of food. To detect *sod* on chromosomal *cpe* isolates, a 50-μl PCR solution including 10 μl of GoTaq DNA polymerase buffer (Promega), 4 μl of a template DNA preparation, each primer at 1 mM, deoxynucleoside triphosphates at 0.2 mM each, 2.0 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Promega) was used. The PCR was performed under the following conditions: 1 cycle of 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 68°C; and a final dwell time of 8 min at 68°C in a MiniCycler. When PCR products at the expected band sizes were detected, those PCR products were used as templates for sequencing analysis.

Determination of heat resistance of spores of plasmid *cpe C. perfringens* type A food poisoning outbreak isolates and plasmid *cpe* food isolates. To evaluate the heat resistance of spores from *cpe*-positive *C. perfringens* isolates, the D_{100} value (the time taken to reduce spore survival by a factor of 10 at 100°C) for each isolate's spores was measured as described previously (11). Sporulating cultures of *C. perfringens* were prepared by inoculating a 0.2-ml aliquot of FTG culture into 10 ml of Duncan-Strong (DS) sporulating medium. After 24 h of incubation at 37°C, those DS cultures were heated at 75°C for 20 min to kill any remaining vegetative cells and also to facilitate spore germination (11). Each heat-shocked DS culture (0.2 ml) was serially diluted from 10^{-1} to 10^{-8} with FTG medium. Two 0.2-ml aliquots of each dilution were plated on brain heart infusion (Difco) agar plates to establish the number of viable spores.

The remainder of each heat-shocked DS culture was then heated at 100°C for various times (3 to 10 min for plasmid *cpe* isolates and 15 to 60 min for chromosomal *cpe* strains). At each time point, the boiled DS culture was diluted from 10^{-1} to 10^{-5} with FTG medium. Two 0.2-ml aliquots of each dilution were then plated on brain heart infusion agar plates and incubated anaerobically at 37°C overnight, and then the viable spores were counted. Strains NCTC8239 and F4969 were used as controls for chromosomal and plasmid *cpe* isolates, respectively.

CPE production by *cpe*-positive *C. perfringens* isolates. Isolates confirmed as *cpe*-positive *C. perfringens* were tested for CPE production with a perfringens enterotoxin reverse passive latex agglutination (PET-RPLA) CPE toxin detection kit (Denka Seiken) (10). Briefly, a 0.2-ml aliquot of an FTG culture of each *cpe*-positive type A *C. perfringens* food poisoning isolate and food isolate was inoculated into two tubes of 10 ml of DS sporulating medium. After 24 h of incubation at 37°C, one tube of DS culture was used to determine the heat resistance of the spores. The remaining DS culture tube was incubated at 37°C for another 24 h and then stored at −40°C. Same-lot samples, confirmed by the formation of spores in heat resistance experiments, were used for the CPE production assay. Frozen culture samples were applied to a PET-RPLA kit in accordance with the instructions of the manufacturer.

Nucleotide sequence accession numbers. The sequences determined in this study have been submitted to GenBank and assigned accession numbers AB377403 to AB377501.

RESULTS

Isolation of *C. perfringens* from retail meat products. Our survey was focused on foods most commonly implicated as vehicles for *C. perfringens* type A food poisoning outbreaks, such as pork, beef, and poultry (15). Of the 200 meat samples tested, 142 (71%) were found to be contaminated with *C. perfringens* and a total of 212 strains were isolated (Table 1). All meat products showed *C. perfringens* contamination rates ranging from 33.3% (lamb) to 100% (ground chicken

TABLE 2. Detection of *C. perfringens* in Japanese retail meats by PCR assay

Food	No. of samples tested	No. (%) of samples positive by	
		cpa PCR	*cpe* PCR
Beef	35	19 (54.3)	2 (5.7)
Ground beef	22	19 (86.4)	0 (0.0)
Pork	42	14 (33.3)	1 (2.4)
Ground pork	21	18 (85.7)	0 (0.0)
Ground beef-pork mixture	21	21 (100.0)	1 (4.8)
Chicken	33	32 (97.0)	2 (6.1)
Ground chicken	22	22 (100.0)	2 (9.1)
Duck	1	1 (100.0)	0 (0.0)
Lamb	3	1 (33.3)	0 (0.0)
Total	200	147 (73.5)	8 (4.0)

and duck) (Table 1). Most of the meat samples surveyed in this study had very low MPN-per-gram values of less than 3 (Table 1).

Toxin genotype of *C. perfringens* isolates in retail meat products. Multiplex PCR is now routinely used to assign *C. perfringens* isolates to one of five toxinotypes (A to E) based upon whether an isolate carries the gene encoding alpha-, beta-, epsilon-, or iota-toxin (3, 15). Since it has been suggested that beta2-toxin may play a role in enteric disease caused by *cpe*-positive isolates (2), a revised version of the multiplex PCR toxin genotyping assay detecting *cpe* and *cpb2* has been developed (3). All 212 isolates collected in this survey were subjected to a revised multiplex PCR assay to determine their toxin genotypes (A to E) and whether they carry *cpe* and *cpb2*. All of the *C. perfringens* isolates assayed by the multiplex PCR genotyping assay were classified as type A. From the results of this multiplex PCR genotyping assay, three isolates (1.5%) carrying *cpe* were identified and none of these three isolates carried *cpb2*. On the other hand, 143 isolates carried *cpb2*. Approximately half of the isolates from chicken meat products contained *cpb2*, but only one chicken meat product contained *cpe*. Despite the relationship between plasmid *cpe* and *cpb2* for some plasmid *cpe* isolates in a previous report (2), no linkage of these two genes was detected in the present study among isolates from Japanese retail meat.

Detection of enterotoxigenic *C. perfringens* in enriched-culture samples from retail meat products by PCR assay. Because molecular detection methods for many pathogens in food materials have been developed, we investigated the presence of *cpe*-positive *C. perfringens* in DNA preparations of enriched-culture samples from retail meat products with a standard PCR assay. We first tested whether our DNA preparation procedure was sufficient to detect the *cpa* gene encoding alpha-toxin that should be produced by all *C. perfringens* bacteria. In this assay, a primer pair against *cpa* that is routinely used and has known specificity for *C. perfringens* was used in the multiplex PCR genotyping assay (3). Our *cpa* PCR assay, with DNA prepared with a Bio-Rad kit, detected *cpa* in 147 (73.5%) of 200 Japanese retail meat product samples (Table 2). These results indicate that our DNA materials from enriched-culture samples are useful for detecting *C. perfringens*. To directly detect *cpe*-positive *C. perfringens* in enriched-culture samples, we used a *cpe* PCR assay that uses newly constructed primers in this study and confirmed its specificity with several species of *Clostridium* and *Bacillus cereus* isolates (data not shown). Eight (4%) of these enriched-culture meat product samples were positive in the *cpe* PCR assay, and *cpe*-positive *C. perfringens* strains (TM111C1, TM138, and TM178) were isolated from three of them (Table 2).

***cpe*-genotyping PCR assay of *cpe*-positive *C. perfringens* isolates to determine the chromosomal or plasmid location of *cpe*.** Our recent study indicated that a conventional PCR assay detecting the sequence downstream of *cpe* could identify whether the *cpe* gene was on the chromosome or on a plasmid (9). To identify the location of *cpe*, three *cpe*-positive strains obtained in this study and 10 *cpe*-positive food-borne disease isolates from three outbreaks in Japan (11) were assayed by a *cpe*-genotyping PCR assay (9). Three food isolates (TM111C1, TM138, and TM178) and three plasmid *cpe* outbreak strains (T1, T16, and T102) belong to the *cpe*-IS*1470*-like genotype, while the other plasmid *cpe* outbreak strains (no. 2, no. 24, and no. 110) belong to the *cpe*-IS*1151* genotype and are *cpb2* positive, as previously reported (Fig. 1A) (14). However, four strains (W4232, W5837, W6205, and OSAKA2) from a food poisoning outbreak in Japan belonged to the chromosomal *cpe* genotype, as described previously (Fig. 1A) (9).

Interestingly, of eight DNA preparations from enriched-culture samples which showed *cpe* positivity by the *cpe*-specific PCR assay, four also showed a positive reaction in the *cpe*-genotyping PCR assay. Enriched-culture sample no. 149 was contaminated with *cpe*-IS*1470*-like genotype *C. perfringens*, although no plasmid *cpe* strain was isolated. *cpe*-IS*1470*-like genotype *C. perfringens* strains (TM111C1, TM138, and TM178) were isolated from three of those four positive samples (no. 088, no. 113, and no. 153, respectively) (Fig. 1B). However, four enriched-culture samples were not found to be contaminated with known *cpe* genotype *C. perfringens* strains.

Detection of *sod* from possible chromosomal *cpe* isolates in *cpe*-positive enriched-culture samples. In the *cpe*-genotyping PCR assay, four of eight enriched-culture samples from retail meat products did not show any PCR products. These samples might have been contaminated with small populations of a chromosomal *cpe* strain(s) because the *cpe*-genotyping assay is less sensitive than the *cpe*-detecting PCR assay. In the *cpe*-genotyping assay, the size of PCR products from chromosomal *cpe* strains is more than 1 kb, compared to the ~200-bp size of PCR products in the *cpe*-detecting PCR assay. To further investigate the presence or absence of chromosomal *cpe* strains in all eight *cpe* assay-positive enriched samples including four *cpe*-genotyping assay-negative samples, we performed a *sod* sequence analysis. In the *sod* sequence phylogenetic assay, chromosomal *cpe* isolates belonged to one definitive cluster, which was easily differentiated from other isolates such as plasmid *cpe* strains (Fig. 2).

From *sod* sequence alignment analyses, we could identify nucleotide differences relatively specific for chromosomal *cpe* isolates. To sensitively detect *sod* in chromosomal *cpe* isolates, a primer pair was constructed based on the alignment analysis results that could produce ~350-bp PCR products, smaller than those of the *cpe*-genotyping assay. In the PCR for detecting *sod* in chromosomal *cpe* strains, two of eight food samples were positive and yielded PCR products of the expected size

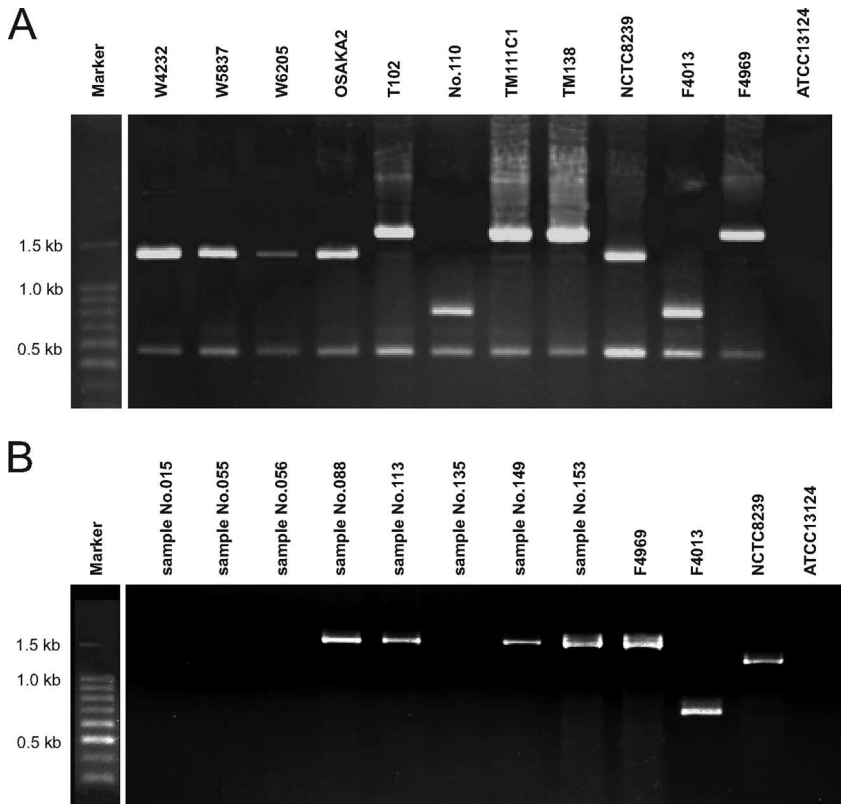


FIG. 1. PCR *cpe*-genotyping assay of food poisoning isolates and food isolates and *cpe*-positive enriched-culture samples. A PCR *cpe*-genotyping assay of isolates is shown in panel A, and the same assay with DNA preparations of enrichment culture samples is shown in panel B. In panel A, food poisoning chromosomal *cpe* isolates in Japan (W4232, W5837, W6205, and OSAKA2) and food poisoning plasmid *cpe* isolates in Japan (T102 and no. 110) are shown. Food isolates with *cpe* on a plasmid (TM111C1 and TM138) are also shown. Strains NCTC8239 (chromosomal *cpe* isolate), F4969 (plasmid *cpe* isolate with a downstream IS*1470*-like sequence), and F4013 (plasmid *cpe* isolate with a downstream IS*1151* sequence) were used as controls. ATCC 13124 was a *cpe*-negative type strain. In panel B, DNA preparations from enriched-culture samples (no. 015, no. 055, no. 056, no. 088, no. 113, no. 135, no. 149, and no. 153) were positive by *cpe* PCR assay. Plasmid *cpe* strains TM111C1, TM138, and TM178 were isolated from sample no. 088, no. 113, and no. 153, respectively.

(Fig. 3). These two *sod* PCR products (sample no. 055 and sample no. 153) might have come from *sod* of chromosomal *cpe* strains. To investigate whether these PCR products were from *sod* of a chromosomal *cpe* strain(s), we performed a sequence analysis. Based on the sequence information and subsequent phylogenetic analysis of the sequences of these *sod* PCR products, these two samples did not contain chromosomal *cpe* strains but might have been contaminated with *cpe*-positive strains of an unknown genotype, as has been reported elsewhere (4) (indicated by the arrow in Fig. 2).

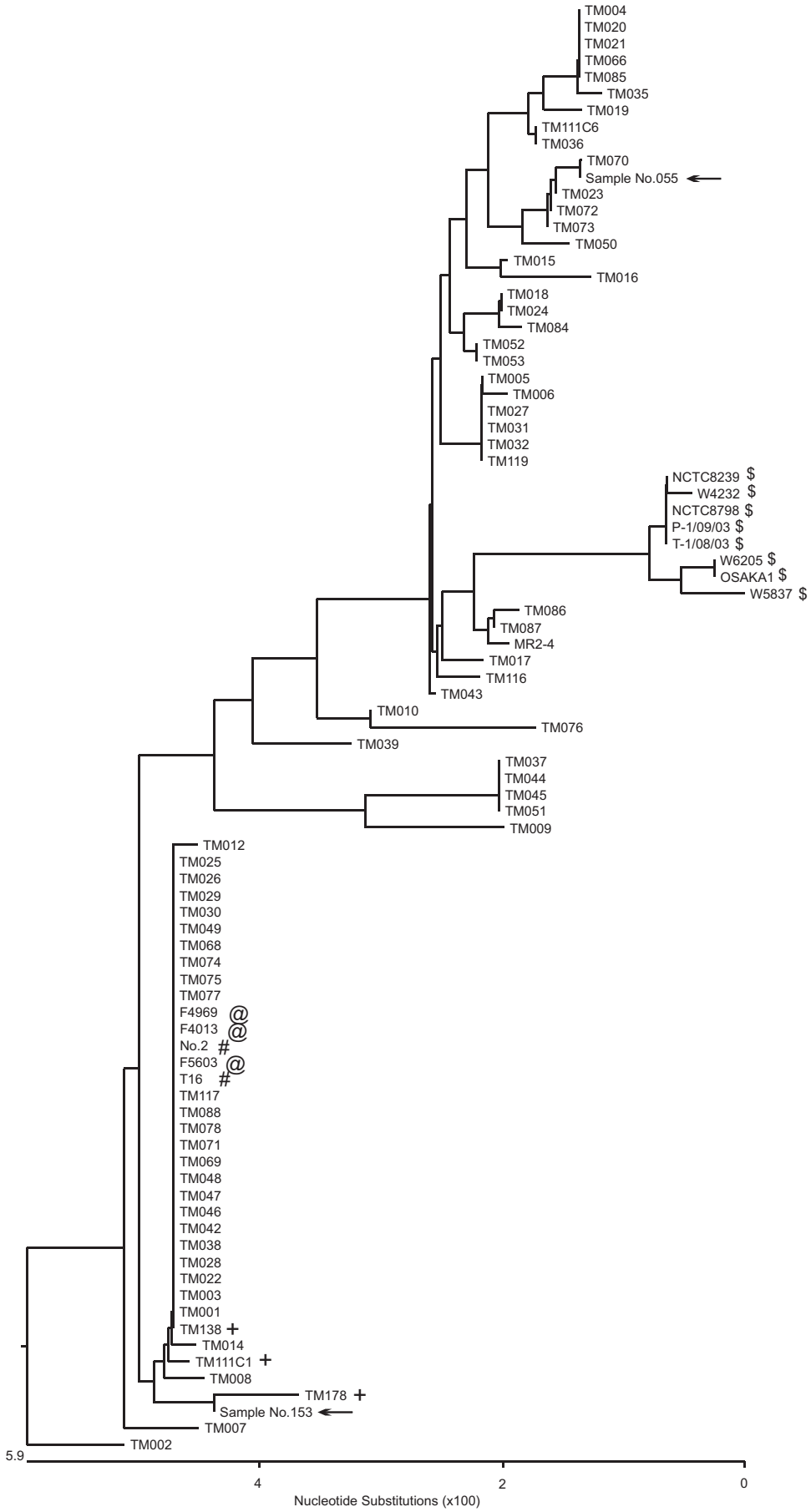
Heat resistance properties of spores of plasmid *cpe* food isolates and plasmid *cpe* food poisoning strains. Type A food poisoning isolates carrying *cpe* on the chromosome typically produce more heat-resistant spores than type A isolates carrying *cpe* on a plasmid (11). Possession of this heat resistance phenotype should be favorable for causing typical *C. perfringens* type A food poisoning, since this disease usually results from improper temperatures during the cooking or storage of foods (15).

To investigate the heat-resistant spore formation by the three *cpe*-positive type A food isolates and two plasmid *cpe* type A food poisoning outbreak isolates in Japan (T16 and no. 2), *D* values at 100°C were determined for sporulating cultures of these isolates. As expected from previously reported results that spores of strain NCTC8239 (a control chromosomal *cpe* isolate) exhibited higher heat resistance than spores of strain F4969 (a control plasmid *cpe* isolate) (Table 3) (11), spores of our surveyed isolates exhibited heat resistance lower than that of NCTC8239 spores (average D_{100} value of 2.0 versus 16.5) but similar to that of F4969 spores (average D_{100} value of 2.0 versus 2.2) (Table 3).

CPE production. All of our *cpe*-positive isolates, including plasmid *cpe* food poisoning isolates (no. 2 and T16), plasmid *cpe* food isolates (TM111C1, TM138, and TM178), and control strains (NCTC8239 and F4969), produced CPE, but a *cpe*-negative strain (ATCC 3624) did not show any positive reaction (Table 3).

DISCUSSION

C. perfringens is one of the most important food-borne GI pathogens and is also ubiquitous in nature. CPE is known to be the most important of the toxins of this bacterium for food poisoning. In a survey of retail food in the United States, ~1.4% of the food samples tested were contaminated with enterotoxigenic *C. perfringens* and all of those isolates carried



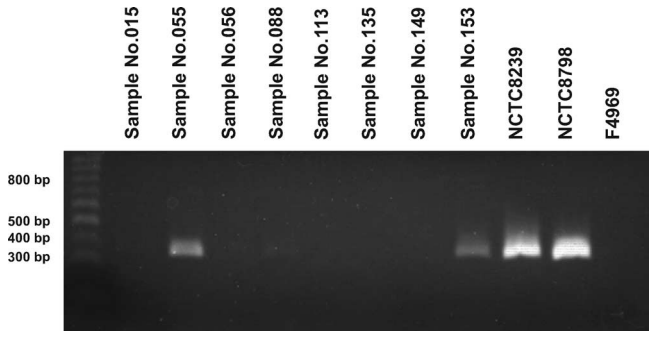


FIG. 3. Sequence-based PCR assay of *sod* from chromosomal and plasmid *cpe* isolates. PCR assay with a primer pair based on the *sod* sequence for detecting chromosomal *cpe* strains. Of eight *cpe* PCR assay-positive enriched-culture samples, two (no. 055 and no. 153) were positive by the *sod*-based assay.

cpe on their chromosome and exhibited extremely high spore heat resistance (15). However, recent studies suggest that *C. perfringens* strains from several food poisoning outbreaks carry *cpe* on a plasmid (6, 14).

In this survey, approximately 70% of retail meat products were positive for *C. perfringens* by our bacterial isolation procedures. Previous surveys showed incidences of 8 to 37% in retail meat in Japan (8, 10) and 30 to 80% in American retail meat (7, 15). In the present study, *cpe*-positive *C. perfringens* strains were isolated from 3 out of 200 meat product samples. This rate is similar to the contamination rates from two previous surveys in Japan (8, 10). In those surveys, 0 and 2% of the tested meat samples were contaminated with CPE-positive *C. perfringens* by a PET-RPLA assay (8, 10). Since insufficient heating is thought to be a cause of *C. perfringens* food poisoning, the heat resistance of spores of CPE-positive isolates is likewise important. From this viewpoint, the significance of those previous Japanese surveys was limited because neither the location of *cpe* nor the heat resistance of spores was examined.

This study, for the first time, demonstrated the presence of plasmid *cpe* isolates in Japanese retail meat products, while all of the isolates in retail food in the United States carry *cpe* on their chromosome (15). Interestingly, these food isolates (TM111C1, TM138, and TM178) belong to the *cpe*-IS*1470*-like genotype, as observed with plasmid *cpe* outbreak isolates (T1, T16, and T102) (Fig. 1A). However, other plasmid *cpe* outbreak strains (no. 2, no. 24, and no. 110) belong to the *cpe*-IS*1151* genotype, as previously reported (Fig. 1A) (14). Spores of chromosomal *cpe* isolates (previously reported control strain NCTC8239 [11] and Japanese food poisoning chromosomal *cpe* strains W4232 and W5837 [data not shown]) showed extremely high heat resistance, with a *D* value of more than 15 min at 100°C, as expected. In contrast, spores of two different food poisoning strains with *cpe* on a plasmid (no. 2 and T16) were relatively heat sensitive, with a *D* value of less than 2 min at 100°C. The food isolates obtained in this survey (TM111C1, TM138, and TM178) also produced relatively heat-sensitive spores, similar to plasmid *cpe* food poisoning strains and previously reported plasmid *cpe* strain F4969 (Table 3). Food isolates with *cpe* on a plasmid could produce CPE. Collectively, these three CPE-producing food isolates have the potential to cause food poisoning, as some previously isolated food poisoning strains carry *cpe* on a plasmid (14).

TABLE 3. Heat resistance and CPE production of enterotoxigenic *C. perfringens* isolates from food poisoning outbreaks and Japanese retail meats

Strain	Origin	*cpe* genotype	*D* value at 100°C (min)	CPE production
No. 2	Food poisoning	Plasmid (IS*1151*)	2.4	+
T16	Food poisoning	Plasmid (IS*1470* like)	2.5	+
TM111C1	Food	Plasmid (IS*1470* like)	1.9	+
TM138	Food	Plasmid (IS*1470* like)	1.3	+
TM178	Food	Plasmid (IS*1470* like)	1.9	+
NCTC8239	Food poisoning	Chromosome	16.5	+
F4969	Sporadic diarrhea	Plasmid (IS*1470* like)	2.2	+
ATCC 3624		*cpe* negative	NT[a]	−

[a] NT, not tested.

To further investigate the presence of chromosomal *cpe* strains in Japanese retail meat products, we applied sequence-based molecular epidemiology by *sod* sequence analysis. We used *sod* for sequence analysis because (i) *sod* is a representative housekeeping gene; (ii) *sod* is not a very large gene, and we can analyze ~80% of the entire open reading frame; (iii) *sod* is a pathogenic gene and is thought to be important in retail food (5); and finally, (iv) *sod* is located far away from *cpe* and thus might not be affected by so-called *cpe* movement (1). Polymorphological analyses with the ClustalW program showed that chromosomal *cpe* food poisoning and retail food isolates in the United States comprised one distinct cluster, and this cluster is distant from plasmid *cpe*-positive and *cpe*-negative isolates (Fig. 2). By our newly constructed molecular method, our *sod* sequence analyses could not identify chromosomal *cpe* isolates in two *cpe* PCR-positive enriched samples (no. 055 and no. 153) that showed positive reactions in a chromosomal *cpe* strain-detecting *sod*-based PCR assay (Fig. 3). Collectively, our present study could not isolate any *C. perfringens* strains with *cpe* on their chromosome from Japanese retail meat by three different approaches (bacterial iso-

FIG. 2. Phylogenetic tree analysis of the superoxide gene (*sod*) of chromosomal *cpe* strains, plasmid *cpe* strains, and *cpe*-negative food isolates. The phylogenetic tree was constructed with 505 bp of sequence information from the *sod* open reading frame (full length, 760 bp) from food and food poisoning isolates and with 347 bp of sequence information from *sod*-based assay-positive samples. Symbols: $, chromosomal *cpe* strains recovered from food poisoning outbreaks in Japan and Europe and chromosomal *cpe* strains recovered from food isolates in the United States; @, plasmid *cpe*-positive European diarrhea isolates; #, plasmid *cpe*-positive strains recovered from food poisoning outbreaks in Japan; +, plasmid *cpe*-positive food isolates (strain MR2-4 is from a healthy human fecal isolate with *cpe* on a plasmid; the other strains are *cpe*-negative food isolates); arrows, *sod* PCR-positive enriched-culture samples. Chromosomal *cpe* strains (indicated by the symbol $) formed a distinct cluster which could be easily differentiated from other strains such as plasmid *cpe* strains. From the sequence information of PCR-positive products from a *sod*-based assay (indicated with arrows), the PCR products could not have come from a chromosomal *cpe* strain.

lation, direct PCR *cpe* genotyping, and indirect *sod* SLST analysis). In our study, in-depth molecular analyses to detect chromosomal *cpe* isolates, in addition to bacterial isolation procedures, were performed. However, no Japanese retail meat products were contaminated with a chromosomal *cpe* strain. While chromosomal *cpe* strains were isolated in food poisoning outbreaks in Japan, the rate of presence of chromosomal *cpe* strains in Japanese retail meat products was lower, less than 0.5%, than in U.S. foods (15).

Enterotoxigenic *C. perfringens* strains with chromosomal *cpe* are still considered one of the important pathogens of food poisoning outbreaks, because those strains were almost always isolated from patients suffering from diarrhea secondary to *C. perfringens* in Europe, the United States, and Japan (9); were present in retail foods; and could produce highly heat-resistant spores (15). Recently, it was reported that plasmid *cpe* strains were isolated from several food poisoning outbreaks in Japan and Europe (6, 14). However, plasmid *cpe* food poisoning strains, at least in Japan, produced relatively heat-labile spores, similar to sporadic and antibiotic-associated diarrheal strains. These findings suggest that plasmid *cpe* isolates in food products could be a causative agent of *C. perfringens* food poisoning.

Because enterotoxigenic *C. perfringens* in retail foods in the United States usually carry *cpe* on their chromosome and produce heat-resistant spores, the contaminated food material itself could be a cause of food poisoning (15). However, plasmid *cpe* food poisoning strains and strains carrying *cpe* on a plasmid found in food could produce relatively heat-labile spores, as is the case with sporadic and antibiotic-associated diarrheal strains. Thus, these plasmid *cpe* strains in meat products could be easily killed during cooking. In food poisoning outbreaks, strains with *cpe* on a plasmid might be a causative agent when heating during cooking is inadequate (13) or when food might be contaminated during processing. From this point of view, the well-known food poisoning with heat-resistant-spore-forming strains carrying *cpe* on their chromosome could be called a classical type and food poisoning by plasmid *cpe* strains might be called an emerging type.

ACKNOWLEDGMENTS

We thank Mahfuzur R. Sarker for helpful discussion. We thank Daisuke Tanaka, Teizo Tsukamoto, and Akemi Kai for providing Japanese food poisoning strains.

REFERENCES

1. **Brynestad, S., B. Synstad, and P. E. Granum.** 1997. The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. Microbiology **143:**2109–2115.
2. **Fisher, D. J., K. Miyamoto, B. Harrison, S. Akimoto, M. R. Sarker, and B. A. McClane.** 2005. Association of β2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Mol. Microbiol. **56:**747–762.
3. **Garmory, H. S., N. Chanter, N. P. French, D. Bueschel., J. G. Songer, and R. W. Titball.** 2000. Occurrence of *Clostridium perfringens* β2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiol. Infect. **124:**61–67.
4. **Heikinheimo, A., M. Lindstrom, P. E. Granum, and H. Korkeala.** 2006. Humans as reservoir for enterotoxin gene-carrying *Clostridium perfringens* type A. Emerg. Infect. Dis. **12:**1724–1729.
5. **Jean, D., V. Briolat, and G. Reysset.** 2004. Oxidative stress response in *Clostridium perfringens*. Microbiology **150:**1649–1659.
6. **Lahti, P., A. Heikinheimo, T. Johansson, and H. Korkeala.** 2008. *Clostridium perfringens* type A isolates carrying the plasmid-borne enterotoxin gene (genotype IS*1151-cpe* or IS*1470*-like-*cpe*) are a common cause of food poisonings. J. Clin. Microbiol. **46:**371–373.
7. **Lin, Y., and R. Labbe.** 2003. Enterotoxigenicity and genetic relatedness of *Clostridium perfringens* isolates from retail foods in the United States. Appl. Environ. Microbiol. **69:**1642–1646.
8. **Miwa, N., T. Nishina, S. Kubo, M. Atsumi, and H. Honda.** 1998. Amount of enterotoxigenic *Clostridium perfringens* in meat detected by nested PCR. Int. J. Food Microbiol. **42:**195–200.
9. **Miyamoto, K., Q. Wen, and B. A. McClane.** 2004. Multiplex PCR genotyping assay that distinguishes between isolates of *Clostridium perfringens* type A carrying a chromosomal enterotoxin gene (*cpe*) locus, a plasmid *cpe* locus with an IS*1470*-like sequence, or a plasmid *cpe* locus with an IS*1151* sequence. J. Clin. Microbiol. **42:**1552–1558.
10. **Saito, M.** 1990. Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, foods, and the natural environment in Japan. J. Food Prot. **53:**115–118.
11. **Sarker, M. R., R. P. Shivers, S. G. Sparks, V. K. Juneja, and B. A. McClane.** 2000. Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid versus chromosomal enterotoxin genes. Appl. Environ. Microbiol. **66:**3234–3240.
12. **Sparks, S. G., R. J. Carman, M. R. Sarker, and B. A. McClane.** 2001. Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. J. Clin. Microbiol. **39:**883–888.
13. **Sutton, R. G. A., and B. C. Hobbs.** 1968. Food poisoning caused by heat-sensitive *Clostridium welchii*. A report of five recent outbreaks. J. Hyg. **66:**135–146.
14. **Tanaka, D., K. Kimata, M. Shimizu, J. Isobe, M. Watahiki, T. Karasawa, T. Yamagishi, S. Kuramoto, T. Serikawa, F. Ishiguro, M. Yamada, K. Yamaoka, M. Tokoro, T. Fukao, M. Matsumoot, R. Hiramatsu, C. Monma, and Y. Nagai.** 2007. Genotyping of *Clostridium perfringens* isolates collected from food poisoning outbreaks and healthy individuals in Japan based on the *cpe* locus. Jpn. J. Infect. Dis. **60:**68–69.
15. **Wen, Q., and B. A. McClane.** 2004. Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. Appl. Environ. Microbiol. **70:**2685–2691.