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 IS1470-Like Sequence, or a Plasmid cpe Locus with an IS1151 Sequence

Kazuaki Miyamoto,1 Qi Yi Wen,2,3 and Bruce A. McClane2*

Department of Microbiology, Wakayama Medical College, Wakayama, Japan 641-0021; Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; and School of Veterinary Medicine, University of Yangzhou, Yangzhou, Jiangsu 225009, China

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Clostridium perfringens type A isolates carrying the enterotoxin (cpe) gene are important causes of both food poisoning and non-food-borne diarrheas in humans. In North America and Europe, food poisoning isolates were previously shown to carry a chromosomal cpe gene, while non-food-borne gastrointestinal (GI) disease isolates from those two geographic locations were found to have a plasmid cpe gene. In this report, we describe the development of an economical multiplex PCR cpe genotyping assay that works with culture lysates to distinguish among type A isolates carrying a chromosomal cpe gene, a plasmid cpe gene with a downstream IS1470-like sequence, or a plasmid cpe gene with a downstream IS1151 sequence. The multiplex PCR assay was applied in molecular epidemiologic studies, it was found that (i) all 57 examined type A isolates with a plasmid cpe gene have either IS1470-like or IS1151 sequences downstream of the plasmid cpe gene; (ii) an IS1470-like sequence, rather than an IS1151 sequence, is more commonly present downstream of the plasmid cpe gene (particularly in North American non-food-borne human GI disease isolates); and (iii) as previously shown in the United States and Europe, isolates carrying the chromosomal cpe gene also appear to be the major cause of C. perfringens food poisoning in Japan. The superiority of this new multiplex PCR assay over existing cpe genotyping approaches should facilitate further molecular epidemiologic investigations of C. perfringens enterotoxin-associated GI illnesses and their associated cpe-positive type A isolates.

Clostridium perfringens isolates are classified into five types (A to E), depending upon their expression of alpha-toxin, beta-toxin, epsilon-toxin, and iota toxin (19). About 1 to 5% of type A isolates also produce a medically important toxin (14, 17, 24) named C. perfringens enterotoxin (CPE). CPE-positive type A isolates are responsible for C. perfringens type A food poisoning, which is currently the third most commonly reported food-borne illness in the United States (19). Enterotoxin-producing type A isolates are also an underappreciated cause of non-food-borne human gastrointestinal (GI) diseases (e.g., antibiotic-associated diarrhea and sporadic diarrhea); several epidemiologic surveys have suggested that CPE-positive type A isolates are responsible for up to 5 to 20% of all cases of non-food-borne human GI diseases. CPE-positive type A isolates have also been linked to several veterinary GI diseases (18, 23).

Type A isolates carry their enterotoxin gene (cpe) on either the chromosome or large plasmids (11, 12, 25, 26). Surveys of C. perfringens type A human GI disease isolates from North America and Europe have indicated that food poisoning isolates carry their cpe gene on the chromosome, while non-food-borne GI disease isolates possess a plasmid cpe gene. In North American and European isolates, the chromosomal cpe locus organization is highly conserved (8, 20), but the plasmid cpe locus can have at least two organizational arrangements; i.e., either an IS1470-like or an IS1151 sequence can be immediately downstream of the plasmid cpe gene (20). The small number of isolates with a plasmid cpe gene surveyed to date has not precluded the possibility of additional, unrecognized organizational arrangements in the plasmid cpe locus of other type A isolates.

Interestingly, a type A isolate with a plasmid cpe gene was recently linked to a Japanese food poisoning outbreak (26). It remains unclear whether that outbreak was atypical for Japan or whether the previously noted association between isolates carrying chromosomal cpe and food poisoning holds true only in North America and Europe. The organization of the cpe locus in human GI disease isolates from outside North America and Europe has not yet been examined.

The strong associations reported between certain cpe genotypes and specific CPE-associated human GI diseases in North America and Europe have created clinical and epidemiologic interest in exploiting differences between the chromosomal and plasmid cpe loci to determine the cpe genotypes of type A food or fecal isolates during investigations of CPE-associated human GI disease cases or outbreaks. Early cpe genotyping approaches involved cpe Southern blot analyses of C. perfringens DNA subjected to either pulsed-field gel electrophoresis or restriction length polymorphism (RFLP) analyses (11, 12).
Those Southern blot-based assays were reliable but slow, allowed only small sample numbers to be processed, and involved expensive equipment and technical expertise. In response, we recently developed a duplex PCR cpe genotyping assay that distinguishes type A isolates carrying chromosomal versus plasmid cpe genes (27). That PCR cpe genotyping assay allows large sample numbers to be processed and, when colony lysates can be used, yields results within 1 day (versus 7 to 10 days for Southern blot-based approaches).

Several deficiencies in our initial duplex PCR cpe genotyping technique recently became apparent. First, the assay requires Advantage 2 polymerase mix (Clontech, Palo Alto, Calif.) to amplify its relatively large chromosomal cpe locus (~3.3-kb) or plasmid cpe locus (~2.1-kb) products. That requirement is significant, as Advantage 2 polymerase mix costs about eight times as much per reaction as standard Taq polymerase, and many batches of Advantage 2 polymerase mix do not work in the duplex PCR cpe genotyping assay (unpublished results).

Another drawback of the initial duplex PCR assay is that ~6% of cpe-positive type A isolates yield PCR products when purified DNA is used as the DNA template but not when colony lysates are used as the DNA template. Thus, the initial duplex PCR assay can genotype some cpe-positive isolates only after relatively laborious and time-consuming DNA purification. A final deficiency in the duplex PCR cpe genotyping assay is its inability to distinguish between type A isolates carrying the two different plasmid cpe locus arrangements. For molecular epidemiologic investigations, it could be useful to distinguish type A isolates carrying plasmid cpe loci with downstream IS1470-like sequences versus downstream IS1151 sequences. For example, a hospital experiencing recurrent problems with CPE-induced antibiotic-associated diarrhea might want to determine whether a particular cpe-positive isolate is responsible for all cases. That possibility could be ruled out by demonstrating that feces from some patients contain isolates carrying a cpe plasmid locus with downstream IS1151 sequences, whereas feces from other patients contain isolates carrying a cpe plasmid locus with downstream IS1470-like sequences.

Addressing the limitations of our original duplex PCR cpe genotyping assay, we now report the development of a simple and improved multiplex PCR assay for cpe genotyping. This improved assay requires only standard, economical Taq polymerase, reliably cpe genotypes all type A isolates from colony lysates, and distinguishes type A isolates carrying IS1470-like sequences versus IS1151 sequences downstream of their plasmid cpe genes.

### MATERIALS AND METHODS

**C. perfringens isolates.** In this study, we analyzed 93 cpe-positive isolates previously confirmed to be **C. perfringens** by phenotypic characteristic and to be type A by PCR (24). Isolates were stored frozen in cooked-meat medium or glycerol stocks. Ten years of experience has indicated that the cpe genotype of such frozen stocks is extremely stable; i.e., isolates carrying a chromosomal cpe gene do not become isolates carrying plasmid cpe gene or vice versa. Of the 93 examined isolates, 7 were cpe genotyped by RFLP analysis for the first time in this study (see Results), while the remaining 86 had previously been subjected to Southern blot-based approaches or duplex PCR-based cpe genotyping approaches to determine whether they carried a chromosomal or plasmid cpe gene (20, 27).

Among the 93 examined cpe-positive isolates were 57 type A isolates known to carry a plasmid cpe gene. They were obtained from the following sources: 7 were from 1980s human antibiotic-associated diarrhea cases occurring in the United Kingdom, 10 originated from 1990s human sporadic diarrhea cases in the United Kingdom, 29 were from 1990s human antibiotic-associated diarrhea cases occurring in several hospitals in the United States or Canada, 7 came from 1990s canine diarrhea cases in the United States, 1 originated from a 1990s porcine enteritis case in the United Kingdom, 1 was obtained in the 1990s from canine peritoneal fluid, 1 came from the feces of an asymptomatic dog in the United States in the 1990s, and 1 was collected from the feces of an asymptomatic human in Japan in 2000. A previous study (20) had indicated that five of these type A isolates carried an IS470-like sequence downstream of the plasmid cpe gene, while four of these type A isolates carried an IS1151 sequence downstream of the plasmid cpe gene. The insertion sequence (IS) elements, if any, downstream of the plasmid cpe gene in the remaining 48 type A isolates had not yet been examined.

In this study, we also analyzed 29 type A isolates known to carry a chromosomal cpe gene. They were obtained from the following sources: 8 were from 1950s human food poisoning cases occurring in the United Kingdom, 2 came from 1960s human food poisoning outbreaks in the United States, 7 were obtained from two food poisoning outbreaks in the United States in the 1980s, 6 originated from two different human food poisoning outbreaks occurring in the United States in the 1990s, 5 came from a human food poisoning outbreak in the United States in 2002, and 1 was from a porcine enteritis case in the United Kingdom in the 1990s. A previous study (27) had demonstrated that 24 of these isolates carried IS470 sequences downstream of the chromosomal cpe gene. The IS elements, if any, associated with the chromosomal cpe gene in the remaining five type A isolates (from the 2002 U.S. outbreak) had not yet been determined.

We also examined seven cpe-positive isolates which came from four different Japanese food poisoning outbreaks (occurring in Tokyo or Osaka between 1995 and 2000) and which had not yet been genotyped. Also examined was ATCC 3624, a *C. perfringens* type A isolate previously shown to be cpe negative (13).

**RFLP Southern blot analyses.** The cpe genotype of the seven isolates obtained from the four Japanese human food poisoning outbreaks was confirmed by our previous duplex PCR cpe genotyping assay (27) and by cpe RFLP Southern blot analyses performed as described previously (11).

**Preparation of C. perfringens culture lysates for PCR assays.** Crude vegetative cell lysates of *C. perfringens* colonies, prepared as described previously (27), were used as a template DNA source for the PCRs described below.

**Duplex PCR for detecting type A isolates carrying a chromosomal cpe locus.** Based upon recent results demonstrating that an IS1470 sequence is present ~1 kb downstream of the chromosomal cpe gene in many, if not all, type A isolates (20), a primer pair was designed to PCR amplify an ~1.3-kb product from the chromosomal cpe locus (Fig. 1): 5'-CTCCTTGATTACAAGCTCCTGGAAGAAGA-3' (primer IS1470R1.3) and 5'-TTAGAACAGTCCTTAGGTGATGGAG-3' (primer cpe4F). In addition, a second primer pair was designed to specifically PCR amplify an ~0.6-kb product from the conserved internal cpe sequences of type A isolates (10): 5'-GATAAGGATGATTGTTATGAT-3' (primer IS1470-likeR1.3 and IS1470-likeR1.6) and 5'-GAGTCCAAGGGTATGAGTTAGAAGG-3' (primer cpe4F). The two primer pairs (final concentrations, 1 µM each IS1470R1.3 and cpe4F and 0.2 µM each 3F and 4R) were added to a 50-µL PCR mixture containing 5 µL of cell lysate (prepared as described above) and 40 µL of Taq complete 1.1 master mix (Gene Choice, Frederick, Md.). The PCR mixture was placed in a thermal cycler (Techne, Cambridge, United Kingdom) and subjected to the following amplification conditions: cycle 1, 94°C for 2 min; cycles 2 through 40, 94°C for 30 s, 61°C for 30 s, and 72°C for 60 s; and extension for 8 min at 68°C.

An aliquot (20 µL) of each PCR sample was electrophoresed on a 1.5% agarose gel and then visualized by staining with ethidium bromide.

**Duplex PCR for detecting type A isolates carrying a plasmid cpe locus with an IS1470-like sequence.** Based upon the results of a recent study (20), a primer pair was designed to PCR amplify an ~1.6-kb product (Fig. 1) from type A isolates carrying an IS1470-like sequence downstream of the plasmid cpe locus: 5'-CTTGGTATTACAAGCTCCTGGAAGAAGA-3' (primer IS1470-likeR1.3) and primer cpe4F (used in the duplex PCR amplifying the chromosomal cpe locus). In addition, this duplex PCR was done with primer pair 3F-4R (described above) to amplify an ~0.6-kb product from the conserved internal cpe sequences of type A isolates. The two primer pairs (final concentrations, 1 µM each IS1470-likeR1.6 and cpe4F and 0.2 µM each 3F and 4F) were added to a 50-µL PCR mixture containing the same volumes of template and reagents as those described above for the chromosomal cpe duplex PCR. The reaction mixtures were subjected to the same PCR amplification conditions and electrophoretic analyses as those described above for the chromosomal cpe duplex PCR.

**Duplex PCR for detecting type A isolates carrying a plasmid cpe locus with an IS1151-like sequence.** Based upon recent results demonstrating that an IS1151 sequence is found in many, if not all, type A isolates (20), a primer pair was designed to PCR amplify an ~1-kb product from the plasmid cpe locus: 5'-CTCCTTGATTACAAGCTCCTGGAAGAAGA-3' (primer IS1470R1.3 and cpe4F) and 5'-GAGTCCAAGGGTATGAGTTAGAAGG-3' (primer cpe4F). The two primer pairs (final concentrations, 1 µM each IS1470R1.3 and cpe4F and 0.2 µM each 3F and 4R) were added to a 50-µL PCR mixture containing 5 µL of cell lysate (prepared as described above) and 40 µL of Taq complete 1.1 master mix (Gene Choice, Frederick, Md.). The PCR mixture was placed in a thermal cycler (Techne, Cambridge, United Kingdom) and subjected to the following amplification conditions: cycle 1, 94°C for 2 min; cycles 2 through 40, 94°C for 30 s, 61°C for 30 s, and 72°C for 60 s; and extension for 8 min at 68°C. An aliquot (20 µL) of each PCR sample was electrophoresed on a 1.5% agarose gel and then visualized by staining with ethidium bromide.
RESULTS

Development of a duplex PCR assay to specifically identify C. perfringens type A isolates carrying a plasmid cpe locus with a downstream IS1470-like sequence. To substitute Taq polymerase for the more expensive and variable Advantage 2 polymerase mix used in our initial PCR cpe genotyping assay, to develop a PCR genotyping assay capable of distinguishing type A isolates carrying plasmid cpe genes with downstream IS1470-like versus IS1151 sequences, and to investigate whether some type A isolates have as-yet-unrecognized sequences immediately downstream of their plasmid cpe genes, several new primer pairs were designed from recently discovered (20) variations in sequences downstream of the plasmid cpe genes of type A isolates.

The first such primer pair (cpe4F and IS1470-likeR1.6) specifically amplifies a shorter (~1.6-kb) PCR product from type A isolates carrying a plasmid cpe locus with a downstream IS1470-like sequence. The second primer pair (3F and 4R) included in this duplex PCR specifically amplifies an ~0.6-kb product from internal cpe sequences; this amplification serves as an internal control to ensure the presence of template-quality DNA in all samples (even those not supporting amplification of the ~1.6-kb product). The cpe–IS1470-like duplex PCR assay was first tested with culture lysates from 14 type A isolates previously characterized for IS elements present immediately downstream of their cpe genes. As shown in Fig. 2A, this duplex PCR correctly amplified the expected ~0.6-kb internal cpe product from culture lysates of all 14 cpe-positive type A isolates. However, an ~1.6-kb product was amplified only from culture lysates of the five isolates (e.g., F4969) known to have an IS1470-like sequence downstream of the plasmid cpe gene. No ~1.6-kb PCR product was amplified from culture lysates of the five isolates (e.g., NCTC8239) carrying a chromosomal cpe gene or the four isolates (e.g., F4013) carrying an IS1151 sequence downstream of the plasmid cpe gene. Neither ~0.6-kb nor ~1.6-kb PCR products were amplified from culture lysates of cpe-negative type A isolate ATCC 3624 (data not shown).

Development of a duplex PCR to specifically identify C. perfringens type A isolates carrying a plasmid cpe locus with a downstream IS1151 sequence. Another primer pair (cpe4F and IS1150.8R) was designed to obtain a Taq-amplifiable, ~0.8-kb PCR product from type A isolates carrying an IS1151 sequence downstream of the plasmid cpe gene. To test their reliability, the primers amplifying cpe-IS1151 sequences were first tested in a duplex PCR that also contained the same primer pair as that used in the experiment shown in Fig. 2A to specifically amplify an ~0.6-kb product from internal cpe sequences. When culture lysates from the same 14 cpe-positive type A isolates as those tested in the experiment shown in Fig. 2A were used, the cpe-IS1151–cpe duplex PCR amplified the expected ~0.6-kb product from all of those cpe-positive type A isolates (Fig. 2B). However, the cpe-IS1151–cpe duplex PCR amplified only an ~0.8-kb product when culture lysates from the four isolates (e.g., F4013) known to carry an IS1151 sequence downstream of the plasmid cpe gene were used. No ~0.8-kb product was amplified from culture lysates of the five isolates (e.g., NCTC8239) carrying a chromosomal cpe gene or the five isolates (e.g., F4969) carrying an IS1470-like sequence.
downstream of the plasmid cpe gene. Neither ∼0.6-kb nor ∼0.8-kb products were amplified by the cpe-IS1151-cpe duplex PCR when culture lysates from cpe-negative type A isolate ATCC 3624 were used (data not shown).

Development of a duplex PCR to specifically identify C. perfringens type A isolates carrying a chromosomal cpe locus. A recent study (20) found that an IS1470 sequence is typically present ∼1.2 kb downstream of the chromosomal cpe gene in type A isolates (note that in Fig. 1, this IS1470 sequence is oriented in the opposite direction from the IS1470-like sequence present downstream of the plasmid cpe gene in some type A isolates). To obtain an easily Taq-amplifiable PCR product, a primer pair (cpe4F and IS14701.3R) was designed to amplify an ∼1.3-kb PCR product from the chromosomal cpe locus. To test their reliability, the new primers amplifying cpe-IS1470 sequences were first tested in a duplex PCR that also contained the primer pair specifically amplifying an ∼0.6-kb product from internal cpe sequences. When culture lysates from the same cpe-positive type A isolates as those tested in the experiments shown in Fig. 2A and B were used, the cpe-IS1470-cpe duplex PCR amplified the expected ∼0.6-kb product from those 14 cpe-positive type A isolates (Fig. 2C). However, the cpe-IS1470 primers amplified an ∼1.3-kb product only when culture lysates from the five isolates (e.g., NCTC8239) carrying an IS1470 sequence downstream of the chromosomal cpe gene were used. No ∼1.3-kb product was amplified from culture lysates of the nine type A isolates carrying a plasmid cpe gene, regardless of whether an IS1470-like or IS1151 sequence was present downstream of the plasmid cpe gene. Neither ∼0.6-kb nor ∼1.3-kb products were amplified by this duplex PCR when culture lysates from cpe-negative type A isolate ATCC 3624 were used (data not shown).

Development and testing of a multiplex PCR assay for comprehensive cpe locus genotyping. The success of the individual duplex PCR assays in the experiments shown in Fig. 2 suggested that the primers for all three duplex PCRs can be combined in a single multiplex PCR cpe genotyping assay that can use Taq polymerase to distinguish among type A isolates carrying a chromosomal cpe locus, a plasmid cpe locus with a downstream IS1470-like sequence, or a plasmid cpe locus with a downstream IS1151 locus. That hypothesis was first tested with culture lysates from the same 14 well-characterized cpe-positive type A isolates as those examined in the experiments shown in Fig. 2. As shown in Fig. 3, the multiplex PCR assay amplified the expected ∼0.6-kb internal cpe product when culture lysates from those isolates were used. Furthermore, the multiplex PCR correctly amplified an ∼0.8-kb band only from culture lysates of the four isolates known to carry an IS1151 sequence downstream of the plasmid cpe gene, an ∼1.3-kb product only from culture lysates of the five isolates known to carry a chromosomal cpe gene, and an ∼1.6-kb product only from culture lysates of the five isolates known to carry an IS1470-like sequence downstream of the plasmid cpe gene. No product was amplified from culture lysates of cpe-negative strain ATCC 3624 in the multiplex PCR assay (data not shown).

With those promising initial results, the specificity of the multiplex PCR cpe genotyping assay was assessed with culture lysates or extracted DNA from two other gram-positive foodborne pathogens, i.e., Bacillus cereus and Clostridium botuli-
a chromosomal assay had shown that those 72 isolates included 24 isolates with cpe-positive type A isolates. Previous genotyping by either Southern blot techniques or our original duplex PCR cpe genotyping assay had shown that those 72 isolates included 24 isolates with a chromosomal cpe gene and 48 isolates with a plasmid cpe gene (note that the IS elements associated with the plasmid cpe gene of those isolates had not yet been examined). In this additional testing, the multiplex PCR assay amplified the expected ~0.6-kb internal cpe product from all 72 isolates. Furthermore, an ~1.3-kb product was amplified from culture lysates from all 24 additionally tested type A isolates carrying a chromosomal cpe gene. Culture lysates from all 48 additionally tested isolates carrying a plasmid cpe gene showed amplification of products of a plasmid cpe genotype, with culture lysates from 36 of the 48 isolates showing amplification of the ~1.6-kb product indicative of an IS1470-like sequence downstream of the cpe gene. Culture lysates from the remaining 12 additionally tested isolates showed amplification of the ~0.8-kb product indicative of an IS1151 sequence downstream of the plasmid cpe gene.

Use of the multiplex PCR cpe genotyping assay to investigate previously nongenotyped cpe-positive type A food poisoning isolates in Japan. To date there has been very limited genotyping of cpe-positive human GI disease isolates from outside the United States or the United Kingdom. This issue gained particular relevance from a recent report (26) identifying type A isolates carrying a plasmid cpe gene as the cause of a food poisoning outbreak in Japan. To evaluate whether that was an atypical Japanese outbreak or whether food poisoning outbreaks in Japan differ from those in the United States or the United Kingdom by mainly involving type A isolates with a plasmid cpe gene instead of a chromosomal cpe gene, the new multiplex PCR cpe genotyping assay was applied to seven cpe-positive isolates from four randomly chosen Japanese food poisoning outbreaks occurring from the mid-1990s to 2000. Multiplex PCR analysis indicated (Fig. 4) that all seven of those food poisoning isolates carried a chromosomal cpe gene; i.e., culture lysates from those isolates showed amplification of the ~0.6-kb product from internal cpe sequences and the ~1.3-kb product from cpe-IS1470 sequences. This genotyping conclusion was confirmed (data not shown) by our previous duplex PCR cpe genotyping assay and by classical cpe RFLP Southern blot analyses.

DISCUSSION

The new multiplex PCR cpe genotyping assay reported in this study retains all the advantages of our duplex PCR cpe genotyping assay (27) but addresses the limitations of that previous assay. First, the new multiplex PCR assay reduces costs by ~8-fold per reaction by using standard Taq polymerase instead of Advantage 2 polymerase mix; this cost reduction is even greater because the new multiplex PCR assay works consistently with all Taq polymerase batches, thus avoiding the lot-to-lot variability noted with Advantage 2 polymerase mix in the duplex PCR assay. Second, the new assay uses colony lysates from all cpe-positive type A isolates as a DNA template source, eliminating the need of the duplex PCR to isolate and purify DNA for the genotyping of ~6% of all type A isolates. Third, the new assay specifically differentiates among type A isolates carrying a chromosomal cpe locus, an IS1470-like sequence downstream of the plasmid cpe gene, or an IS1151 sequence downstream of the plasmid cpe gene. This new assay is currently intended for the genotyping of known cpe-positive C. perfringens type A isolates obtained from disease-associated food or feces; whether the assay may also be useful for the direct genotyping of cpe-positive isolates present in disease-associated food or fecal samples will be evaluated when such specimens become available for testing.

The ability of the multiplex PCR cpe genotyping assay to distinguish between type A isolates carrying IS1470-like versus IS1151 sequences downstream of the plasmid cpe gene is al-
ready providing useful molecular epidemiology insights. A limited initial survey of three European isolates had suggested that most type A isolates carry an IS1151 sequence downstream of the plasmid \( cpe \) gene (12), but later studies questioned that hypothesis (20). Our current survey of 57 isolates carrying a plasmid \( cpe \) gene (48 of which had unexamined sequences downstream of the plasmid \( cpe \) gene) now confirms that IS1470-like sequences, rather than IS1151 sequences, are found downstream of the plasmid \( cpe \) gene in ~70% of the 57 type A isolates. The 16 remaining isolates all carry IS1151 sequences downstream of the plasmid \( cpe \) gene, indicating that either IS1470-like or IS1151 sequences are present downstream of the \( cpe \) gene in virtually all type A isolates carrying a plasmid \( cpe \) gene. Our multiplex PCR survey results further suggest an interesting geographic pattern with respect to plasmid \( cpe \) locus arrangements. Surveyed United Kingdom isolates that had a plasmid \( cpe \) gene and that were obtained from non-food-borne GI disease cases had nearly equal representations of downstream IS1470-like and IS1151 sequences (8 and 10 isolates, respectively), but 24 (83%) of 29 surveyed North American non-food-borne GI disease isolates had an IS1470-like sequence downstream of the plasmid \( cpe \) gene. These preliminary results could indicate that isolates that have a plasmid \( cpe \) gene and that cause non-food-borne human GI diseases vary by geographic location.

The multiplex PCR assay also revealed that type A isolates carrying a chromosomal \( cpe \) gene were responsible for all four Japanese food poisoning outbreaks examined in this study. These results, confirmed by duplex PCR \( cpe \) genotyping and \( cpe \) Southern blot-based RFLP genotyping assays, provide the first evidence that isolates with a chromosomal \( cpe \) gene cause food poisoning outbreaks in Japan. As an ~1.3-kb product was amplified from the chromosomal \( cpe \) locus of all seven tested Japanese food poisoning isolates, it now becomes apparent that, regardless of geographic origin, the chromosomal \( cpe \) locus of virtually all type A food poisoning isolates is highly conserved; i.e., all 36 isolates with a chromosomal \( cpe \) gene examined to date carry an IS1470 sequence downstream of the \( cpe \) gene. Our identification of isolates with a chromosomal \( cpe \) gene as the cause of all four randomly chosen Japanese food poisoning outbreaks strongly suggests that, as in the United States and the United Kingdom, isolates with a chromosomal \( cpe \) gene cause most \( C. \) perfringens type A food poisoning outbreaks in Japan. However, even if atypical, the recently identified (26) Japanese food poisoning outbreak involving isolates with a plasmid \( cpe \) gene remains interesting.

A previous study (22) suggested that isolates with a chromosomal \( cpe \) gene are predominantly associated with food poisoning outbreaks (at least in part) because of greater heat resistance, which probably facilitates their survival in improperly cooked or stored foods (19). Therefore, it is notable that the Japanese food poisoning outbreak caused by heat-sensitive isolates with a plasmid \( cpe \) gene involved boiled beans (26), an unusual \( C. \) perfringens food poisoning vehicle prepared by brief immersion in boiling water. In contrast, a Japanese food poisoning outbreak shown in the present study to be caused by isolates with a chromosomal \( cpe \) gene involved a typical \( C. \) perfringens type A food poisoning vehicle, i.e., contaminated meat stew (food poisoning vehicles for the other three Japanese outbreaks examined in our study were not identified).

Collectively, these observations suggest that isolates with a chromosomal \( cpe \) gene are the predominant global cause of \( C. \) perfringens type A food poisoning outbreaks but that isolates with a plasmid \( cpe \) gene occasionally cause outbreaks when the food vehicle is atypical or is not prepared by substantial heating.

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