

Humans as Reservoir for Enterotoxin Gene-carrying *Clostridium perfringens* Type A

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We found a prevalence of 18% for enterotoxin gene-carrying (*cpe*+) *Clostridium perfringens* in the feces of healthy food handlers by PCR and isolated the organism from 11 of 23 PCR-positive persons by using hydrophobic grid membrane filter-colony hybridization. Several different *cpe* genotypes were recovered. The prevalence was 3.7% for plasmidial IS1151-*cpe*, 2.9% for plasmidial IS1470-like-*cpe*, 0.7% for chromosomal IS1470-*cpe*, and 1.5% for unknown *cpe* genotype. Lateral spread of *cpe* between *C. perfringens* strains was evident because strains from the same person carried IS1470-like *cpe* but shared no genetic relatedness according to pulsed-field gel electrophoresis analysis. Our findings suggest that healthy humans serve as a rich reservoir for *cpe*+ *C. perfringens* type A and may play a role in the etiology of gastrointestinal diseases caused by this organism. The results also indicate that humans should be considered a risk factor for spread of *C. perfringens* type A food poisoning and that they are a possible source of contamination for *C. perfringens* type A food poisoning.

Clostridium perfringens is classified into 5 types (A–E) on the basis of its ability to produce ≥ 1 of the major lethal toxins α , β , ϵ , and ι (1). Enterotoxin (CPE)-producing (*cpe*+) *C. perfringens* type A is reported continuously as 1 of the most common food poisoning agents worldwide (2–4). An increasing number of reports also implicate the organism in 5%–15% of antimicrobial drug-associated diarrhea (AAD) and sporadic diarrhea (SD) cases in humans as well as diarrhea cases in animals (1,5–9). Most food poisoning strains studied to date carry *cpe* in their chromosomes; isolates from AAD and SD cases bear *cpe* in a plasmid (10,11). Furthermore, genetic studies have

shown that in *C. perfringens* strains with the chromosomally located *cpe*, IS1470 sequences are found upstream and downstream of *cpe* (12,13). However, in strains with *cpe* in the plasmid, 2 different genetic arrangements (either IS1151 or IS1470-like sequences) have been recognized downstream of *cpe* (11,14).

Why *C. perfringens* strains with *cpe* located on chromosomes or plasmids cause different diseases has not been satisfactorily explained. However, the relatively greater heat resistance of the strains with chromosomally located *cpe* is a plausible explanation for these strains' survival in cooked food, thus causing instances of food poisonings (15). The presence of *C. perfringens* strains with chromosomally located *cpe* in 1.4% of American retail food indicates that these strains have an access to the food chain (16). The sources and routes of contamination are unclear.

An explanation for the strong association between *C. perfringens* strains with plasmidially located *cpe* and cases of AAD and SD disease may be in vivo transfer of the *cpe* plasmid to *C. perfringens* strains of the normal intestinal microbiota (17). Thus, a small amount of ingested *cpe*+ *C. perfringens* would act as an infectious agent and transfer the *cpe* plasmid to *cpe*– *C. perfringens* strains of the normal microbiota. This process would result in the persistence of *cpe*+ *C. perfringens* in the intestines. Chronic exposure to CPE would explain the severity and long duration of symptoms (17). Conjugative transfer of the *cpe* plasmid has been demonstrated in vitro (18), but currently no data exist on lateral transfer of *cpe* in vivo, and whether *cpe*+ strains that cause AAD and SD are resident in the gastrointestinal tract or acquired before onset of the disease is unknown.

Although the ubiquitous distribution of *C. perfringens* in nature is well documented, the epidemiology of *cpe*+ strains has not yet been established. Less than 5% of global

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C. perfringens isolates are estimated to carry *cpe* (1), and the prevalence of different *cpe* genotypes in specific ecological niches is not known. In this study, healthy persons were screened for fecal carriage of *cpe*, and *cpe*⁺ strains were further isolated by using hydrophobic grid membrane filter-colony hybridization. The *cpe* genotype and location of *cpe* were determined from strains by detecting different insertion sequence (IS) elements attached to *cpe* (plasmid types IS1151-*cpe* and IS1470-like-*cpe* and chromosomal type IS1470-*cpe*). The genetic relationship between *cpe*⁺ and *cpe*[−] *C. perfringens* isolates obtained from *cpe* carriers was assessed with pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Fecal Samples

A total of 136 fecal samples, 102 (75%) from female food handlers and 34 (25%) from male food handlers, were collected from food handlers in southern Finland during summer 2003. These persons reported no gastrointestinal symptoms at the time of sampling. Their ages ranged from 15 to 65 years (mean 30 years, median 24 years). The samples were kept at −70°C until investigated.

Detection and Isolation

Each fecal sample (1 g) was divided into 2 tubes that contained freshly prepared thioglycollate (40 mL) (Oxoid, Basingstoke, UK). One tube was heated at 75°C for 20 min; the other was left unheated. After anaerobic incubation at 37°C for 20–22 h, the presence of *cpe* in each tube was determined by nested PCR (19). Persons with a *cpe*-positive fecal sample are hereafter referred to as *cpe* carriers.

Hydrophobic grid membrane filter-colony hybridization (HGMF-CH) (20) was used to isolate *cpe*⁺ *C. perfringens* from *cpe* carriers. In addition to probe-positive colonies, those showing no hybridization signals but having a typical color for *C. perfringens* colonies (usually black, occasionally gray or grayish yellow) were isolated from each sample to obtain a collection of both *cpe*⁺ and *cpe*[−] *C. perfringens* from a single sample and to further study the genetic relatedness of these isolates by PFGE (see below).

The isolates were subjected to PCR to determine the toxinotype (A–E) and the presence of *cpe* (21). *C. perfringens* strains NCTC 8239, ATCC 3626, CCUG 2036, CCUG 2037, and CCUG 44727 were used as positive controls.

Molecular Typing

C. perfringens isolates that possessed *cpe* were further studied by PCR to determine the *cpe* genotype of the strain. Total DNA was isolated by using Advamax beads (Edge Biosystems, Gaithersburg, MD, USA) according to the manufacturer's instructions. IS elements downstream

of *cpe* that determine the *cpe* genotype (IS1151-*cpe*, IS1470-like-*cpe*, or IS1470-*cpe*) of each isolate were characterized by using primers and protocols described in Table 1. The location of *cpe* (plasmid or chromosome) was concluded according to the genotyping results (Table 1).

C. perfringens isolates were then typed by PFGE with *Apa*I and *Sma*I (New England Biolabs, Beverly, MA, USA) to study the genetic relationships between isolates (25). PFGE profiles were analyzed visually and with a computer software program (Bionumerics, version 4.5; Applied Maths, Kortrijk, Belgium). The similarities between macrorestriction patterns (MRP) were expressed by Dice coefficient correlation, and clustering by the unweighted pair-group method with arithmetic averages was used to construct a dendrogram.

Cytotoxicity Test on Vero Cells

To test the capability of *cpe*⁺ strains to produce CPE, 1–3 *cpe*⁺ isolates representing each MRP were sporulated (26). The final culture in modified Duncan-Strong medium (Sigma-Aldrich Chemie, Steinheim, Switzerland) was examined by phase-contrast microscopy to confirm the sporulation of the strain. The culture was then sonicated until >95% of the spores were free, as determined by phase-contrast microscopy. The culture was centrifuged at 1,500× *g* for 25 min at 4°C, and cytotoxicity of the supernatant was tested with a Vero cell assay according to Sandvig and Olsnes (27). The assay monitors the inhibition of protein synthesis in Vero cells after addition of toxic proteins, including CPE. The Vero cells were grown in a minimal essential medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum.

The supernatant was precipitated in 80% saturated ammonium sulfate and kept at 4°C before the Vero cell assay. The strain was defined as producing CPE when the inhibition of protein synthesis of Vero cells was >20%.

Results

Detection and Isolation

The gene encoding for *cpe* was detected in the feces of 25 food handlers (18%). For 23 samples (92%), the heated tube showed the positive result, and for 3 of them the unheated tube also yielded a positive PCR result. For 2 samples, only the unheated tube showed a positive PCR result. No association between gender or age and carrier status of *cpe* was found (Table 2).

The HGMF-CH method was used to determine whether *cpe*⁺ *C. perfringens* was present in 23 persons; isolation was successful from 11 persons. The average number of *cpe*⁺ *C. perfringens* isolates carried by these persons was 2.5×10^2 CFU/g. In 10 of these persons, *cpe*[−] *C. perfringens* was also recovered; the average number was $1.8 \times$

Table 1. Primers and PCR protocols for determining the genotype and location of *cpe**

Primer	Sequence (5' to 3')	Primer target	Sequence ref, GenBank accession no.	Ref	Size	<i>cpe</i> genotype	<i>cpe</i> location
CPEmmF 1151 R	CAAGTCAAATTCTTAATCCT CATGGCCGTCAACCCTAAGAAG	<i>cpe</i> IS1151	(22), Y16009 (23), X60694	(12)	1.2 kb	IS1151- <i>cpe</i>	Plasmid
CPEmmF 1470mR	CAAGTCAAATTCTTAATCCT TGAAAACCGTGAAGAATTTGG	<i>cpe</i> IS1470	(22), Y16009 (12), X71844	(12)	2.4 kb	IS1470- <i>cpe</i>	Chromosome
<i>cpe</i> 4F	TTAGAACAGTCCTTAGGTGATGG AG	<i>cpe</i>	(14), AF511071	(24)	1.6 kb	IS1470-like- <i>cpe</i>	Plasmid
IS1470- likeR1.6	CTTTGTGTACACAGCTTCGCCAA TGTC	IS1470-like	(24), AF416450				

*Ref, reference.

10⁴ CFU/g. In each of these 10 case-patients, *cpe*– *C. perfringens* formed a 10- to 1,000-fold majority of the *C. perfringens* population. In 1 person, only *cpe*+ *C. perfringens* isolates were recovered. A total of 77 *C. perfringens* isolates (average of 7 isolates per person) were recovered; all possessed the gene encoding for the α toxin only, which signified that they belonged to type A (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12/no11/06-0478_appT.htm). Of these, 36 isolates were positive for *cpe* (Appendix Table).

Molecular Typing

When the relative prevalence of different *cpe* genotypes was determined with PCR, strains representing the IS1151-*cpe* type were found in 5 persons (3.7%) and strains representing the IS1470-like-*cpe* type were found in 4 persons (2.9%), findings that indicated that all of these persons carried *C. perfringens* strains with plasmidially located *cpe*. A strain with chromosomally located *cpe*, representing the IS1470-*cpe* type, was detected in 1 person (0.7%). Furthermore, *cpe*+ *C. perfringens* strains representing none of the aforementioned types, referred to as *cpe*+ strains with an unknown genetic arrangement downstream of *cpe*, were observed in 2 persons (1.5%) (Appendix Table) (Figure 1).

In PFGE analysis, all isolates were typeable with *Sma*I restriction enzyme, and all but 2 isolates were typeable with *Apa*I. The discriminatory power was equal with both enzymes used, which showed a high genetic diversity

among *C. perfringens*. PFGE analysis showed 1–5 different MRPs in each person, with *cpe*+ and *cpe*– isolates generally being unrelated to each other. However, in 1 case, 2 isolates (CPI 57–1 and CPI 57–2) from the same person showed identical MRPs with both restriction enzymes, the former being *cpe*+ and the latter *cpe*– (Appendix Table; Figure 2). In 8 of 11 persons, *cpe*+ isolates with similar MRPs were identified, whereas 3 persons carried *cpe*+ isolates with 2 different MRPs. In 2 of these persons (numbers 18 and 57), both MRPs represented the same *cpe* genotype (IS1470-like-*cpe*); the third person (number 75) had MRPs with different *cpe* genotypes (IS1470-like-*cpe* and IS1151-*cpe*) (Appendix Table).

Cytotoxicity Test on Vero Cells

Successful sporulation was achieved with isolates representing 9 different MRPs. In 8 (89%) of these MRPs, $\geq 20\%$ inhibition of protein synthesis was detected in Vero cells, which suggested CPE production of the strain (Table 3).

Discussion

We report the first in-depth study of the fecal carriage of *cpe*+ *C. perfringens* by healthy humans, which showed that the organism is widely distributed in this ecologic niche. By using the novel HGMF-CH method, we demonstrated that low numbers of *cpe*+ *C. perfringens* strains are frequently present among the dominant *cpe*– *C. perfringens*. HGMF-CH proved invaluable in the isolation of *cpe*+ *C. perfringens*; in all instances, when both *cpe*+ and *cpe*– *C. perfringens* were isolated, the former existed as a minority and thus would have been missed by conventional isolation methods.

Healthy persons are a rich reservoir for *cpe*+ *C. perfringens*; type A strains representing several different *cpe* genotypes (strains with plasmidially located IS1470-like-*cpe* or IS1151-*cpe* and chromosomally located IS1470-*cpe*) as well as type A strains with unrecognized genetic arrangement attached to *cpe* were present. That IS1151-*cpe* was the most prevalent and IS1470-*cpe* represented the minority of the *cpe* genotypes are findings in

Table 2. Association of sex and age with *cpe* in feces of healthy humans

Characteristic	<i>cpe</i> detected by PCR/ total no. fecal samples (%)
Sex	
Female	19/102 (18.6)
Male	6/34 (17.6)
Total	25/136 (18.4)
Age, y	
<20	6/40 (15.0)
20–50	16/79 (20.3)
>50	3/17 (17.6)
Total	25/136 (18.4)

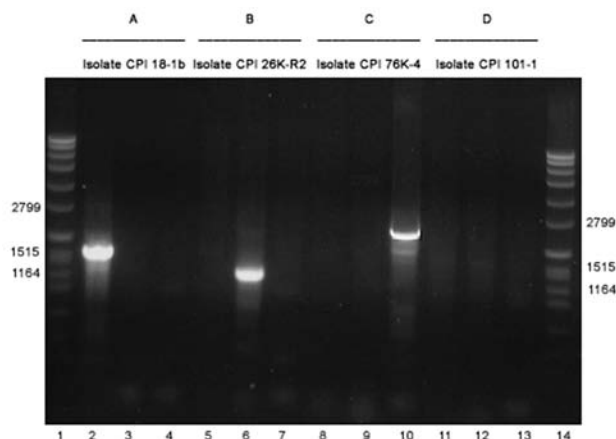


Figure 1. PCR analysis determining the genotype of enterotoxin gene-carrying *Clostridium perfringens* type A isolates obtained from healthy persons. All 4 strains are studied with 3 different primer sets, described in Table 1. For A, B, C and D, the isolate represents genotype IS1470-like-*cpe*, IS1151-*cpe*, IS1470-*cpe*, or unknown genotype, respectively.

line with a previous study that suggested that *cpe*⁺ *C. perfringens* strains with plasmidially located *cpe* are more common in nature than *cpe*⁺ *C. perfringens* strains with chromosomally located *cpe* (28). The presence of strains with unrecognized genetic arrangement attached to *cpe* reflects the wide genetic variety of *cpe*⁺ strains. Because production of CPE was demonstrated in 1 of these *cpe*⁺ strains with an unknown *cpe* genotype, the gene was apparently intact and functional.

The presence of *cpe*⁺ *C. perfringens* type A strain with chromosomally located *cpe* and a full capacity to produce CPE in the feces of healthy food handlers indicates that human handling of food should be considered a risk factor for contamination. *C. perfringens* type A food poisoning typically follows from the ingestion of *cpe*⁺ *C. perfringens* vegetative cells formed in food during storage and serving (29). The low numbers of *cpe*⁺ *C. perfringens* spores present in the feces of the person handling the food may be transferred to the food. Under favorable conditions, *cpe*⁺ strains with chromosomally located *cpe* will easily survive and multiply during food processing and

cause food poisoning because they survive broader temperature ranges during growth and maintenance phases than other *C. perfringens* strains (15,30).

Evidence of in vivo horizontal transfer of *cpe* between *C. perfringens* strains was obtained in the PFGE analysis. First, in 2 persons (numbers 18 and 57), IS1470-like-*cpe* was observed in strains with no genetic relatedness, which indicated lateral spread of IS1470-like-*cpe* (Appendix Table). In vitro conjugative transfer of the *cpe* plasmid has been demonstrated with strain F4969 carrying IS1470-like-*cpe* (18,24), which supports our findings. Second, the evidence of in vivo horizontal transfer of *cpe* was further strengthened by observing a loss or acquisition of IS1470-like-*cpe* in 1 strain (MRP 15) (Appendix Table). In this case, the isolate CPI 57-1 carried IS1470-like-*cpe*, whereas CPI 57-2 was lacking in the same element; these strains were nevertheless isolated from the same person and shared an identical MRP. A potential donor or recipient strain (MRP 13 and 13b) was demonstrated from the same sample, which carried IS1470-like-*cpe* but shared no genetic relatedness to CPI 57-1 (Appendix Table) (Figure 2). All IS1151-*cpe* isolates from the same persons shared identical MRPs; thus, no evidence for in vivo lateral transfer of IS1151-*cpe* was observed.

Finally, our study provides a new insight into the pathogenesis of AAD and SD caused by *cpe*⁺ *C. perfringens* type A. These diseases have been speculated to result from the ingestion of small numbers of *cpe*⁺ strains, which transfer the *cpe* plasmid to *cpe*⁻ *C. perfringens* strains present in the normal intestinal microbiota (17). Our results support this theory because we found small numbers of *cpe*⁺ *C. perfringens* type A with plasmidially located *cpe* in the human gastrointestinal tract as well as evidence for the lateral spread of *cpe*. Our findings therefore indicate that AAD or SD caused by *cpe*⁺ *C. perfringens* type A may occur as an endogenous infection, present in the gastrointestinal tract and causing the disease after exposure to antimicrobial drugs or other predisposing factors. However, the capacity of these *cpe*⁺ strains to persist in the gastrointestinal tract remains unknown; as does how these strains find their way to the gastrointestinal tract. Nevertheless, because they are apparently common in human feces, *cpe*⁺ strains are presumably acquired from

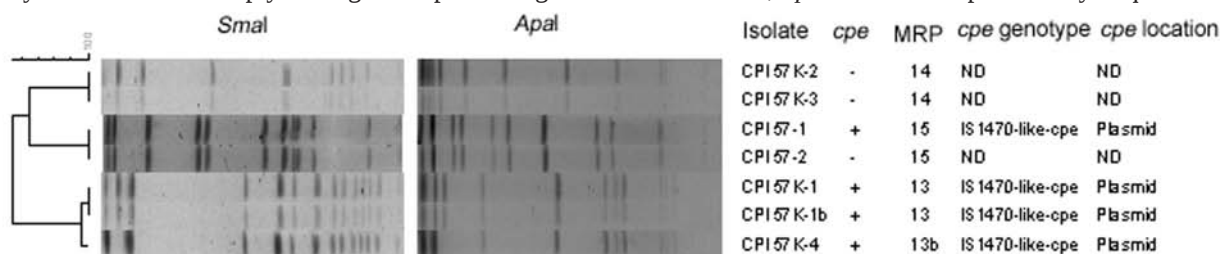


Figure 2. Pulsed-field gel electrophoresis analysis and determination of the *cpe* genotype of *Clostridium perfringens* isolates obtained from a healthy person. ND, not determined.

Table 3. Cytotoxicity test on Vero cells to determine the production of CPE by *cpe*+ *C. perfringens* strains obtained from healthy persons

Isolate	MRP*	CPE†	<i>cpe</i> genotype	<i>cpe</i> location
CPI 18–3	1	+	IS1470-like- <i>cpe</i>	Plasmid
CPI 18–2	2	NS	IS1470-like- <i>cpe</i>	Plasmid
CPI 26K-R3p	5	+	IS1151- <i>cpe</i>	Plasmid
CPI 39–1b, CPI 39K-7	6	+	IS1470-like- <i>cpe</i>	Plasmid
CPI 44K-R3, CPI 44K-R7	10	NS	Untypeable	Not known
CPI 53K-R3	11	NS	IS1151- <i>cpe</i>	Plasmid
CPI 57K-1	13	+	IS1470-like- <i>cpe</i>	Plasmid
CPI 57–1	15	NS	IS1470-like- <i>cpe</i>	Plasmid
CPI 63K-R5	19	NS	IS1151- <i>cpe</i>	Plasmid
CPI 75–3a, CPI 75–3b, CPI 75–5b	21	–	IS1151- <i>cpe</i>	Plasmid
CPI 75–4	22	+	IS1470-like- <i>cpe</i>	Plasmid
CPI 76K-4, CPI 76K-5	24	+	IS1470- <i>cpe</i>	Chromosome
CPI 101–4	25	+	Untypeable	Not known
CPI 103K-3, CPI 103K-5, CPI 103K-6	29	+	IS1151- <i>cpe</i>	Plasmid

*MRP, macro restriction pattern.

†+, present; –, absent, NS, not sporulated and thus cytotoxicity test on Vero cells not performed.

the environment. The question then arises whether these *cpe*+ strains are ingested with food, which would indicate that AAD and SD caused by *cpe*+ *C. perfringens* type A are foodborne.

In conclusion, healthy humans serve as a rich reservoir for *cpe*+ *C. perfringens* type A strains and may play an important role in gastrointestinal diseases caused by this pathogen. Humans should therefore be considered a risk factor for spread of *C. perfringens* type A food poisoning. Future studies to determine the presence of different *cpe* genotypes in other ecologic niches are warranted to elucidate the epidemiology of this major pathogen.

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