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Enumeration and Isolation of *cpe*-Positive *Clostridium perfringens* Spores from Feces

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A hydrophobic grid membrane filter-colony hybridization (HGMF-CH) method for the enumeration and isolation of *cpe* gene-carrying (*cpe*-positive) *Clostridium perfringens* spores from feces was developed. A 425-bp DNA probe specific for the *cpe* gene was sensitive and specific when tested with bacterial DNA and pure cultures. The enumeration of *cpe*-positive *C. perfringens* by the HGMF-CH method proved to be as sensitive as nested PCR combined with the most-probable number technique when tested with fecal samples from healthy individuals. With the aid of the HGMF-CH method, positive hybridization signals were detected from two out of seven fecal samples obtained from healthy individuals. Furthermore, *cpe*-positive *C. perfringens* was successfully isolated from both of these samples. The detection of *cpe*-positive *C. perfringens* by the HGMF-CH method is dependent on the ratio of *cpe*-positive *C. perfringens* colonies to total *C. perfringens* colonies growing on the HGMF–tryptose-sulfite-cycloserine plate. *cpe*-positive *C. perfringens* could be isolated if the ratio of *cpe*-positive *C. perfringens* spores to total *C. perfringens* spores was 6×10^{-5} or higher. The HGMF-CH method provides an aid in the investigation of fecal samples of patients suffering from food poisoning or other diseases caused by *cpe*-positive *C. perfringens*. The method also offers a new approach in the investigation of the epidemiology of *cpe*-positive *C. perfringens* strains.

Clostridium perfringens food poisoning is caused by the ingestion of food that contains large numbers of vegetative cells of *cpe* gene-positive *C. perfringens* strains, usually belonging to type A. These *cpe*-positive cells sporulate in the intestinal tract, producing enterotoxin (CPE), which is responsible for the diarrheal symptoms of the disease (12). When confirming a food poisoning outbreak caused by *C. perfringens*, isolation of the same *cpe*-positive strain from suspect food and from stools of affected individuals is advisable. However, conventional isolation of *cpe*-positive *C. perfringens* from the feces of affected individuals is occasionally complicated by the presence of *cpe*-negative *C. perfringens* as part of the normal fecal microbial population (5, 11, 16, 20, 22, 26). The isolation of the causative agent from fecal samples by direct plating is challenging, particularly if several days have passed since the onset of the illness (16).

The widespread distribution of *C. perfringens* in the environment has been considered to be an important factor in the frequent occurrence of *C. perfringens* type A food poisoning. However, only a small minority of *C. perfringens* strains isolated from humans and animals have been demonstrated to carry the *cpe* gene (1, 4, 8, 10, 24, 27). Studies by Miwa et al. have strengthened the hypothesis that, in fecal samples of animals, a small number of *cpe*-positive *C. perfringens* cells coexist with a large number of *cpe*-negative *C. perfringens* cells (14, 15). It has been shown that the ratio of *cpe*-positive strains to total *C. perfringens* strains in the intestinal contents of cattle, swine, and chickens may be as low as 10^{-4} to 10^{-5} (14, 15).

Due to the low ratio of *cpe*-positive *C. perfringens* strains to total *C. perfringens* strains in clinical samples, the isolation of *cpe*-positive *C. perfringens* strains is difficult and laborious. Thus, due to the lack of a specific method to isolate *cpe*-positive *C. perfringens* from clinical and environmental samples, the present knowledge of the epidemiology of the *cpe*-positive strains is deficient. Several PCR methods for the detection of *cpe*-positive *C. perfringens* from fecal samples have been described (6, 8, 13, 21, 23). The enumeration of *cpe*-positive *C. perfringens* is obtained by nested PCR combined with the most-probable number (MPN) technique (14). Furthermore, there are reports of the use of DNA probe hybridization and PCR–enzyme-linked immunosorbent assay for the detection of the *cpe* gene from food samples (2, 3). Compared to conventional methods, these techniques provide rapid and sensitive detection of the *cpe* gene, but the weakness of the methods is that none of them facilitate the isolation of *cpe*-positive *C. perfringens*. This study describes the design of a hydrophobic grid membrane filter-colony hybridization (HGMF-CH) method for the enumeration and isolation of *cpe*-positive *C. perfringens* spores from fecal samples.

MATERIALS AND METHODS

Bacterial strains. A total of 11 *cpe*-positive and 19 *cpe*-negative *C. perfringens* strains, 38 strains of other clostridia, and 9 strains of other bacterial species were included in the study (Table 1).

DNA isolation. The DNAs from all clostridial and *Listeria* strains used in the study were isolated as described by Hyytiä et al. (7) and Keto-Timonen et al. (9), respectively. All bacterial DNA was stored at −70°C prior to use.

Preparation of digoxigenin-labeled DNA probe specific for *cpe* gene. In order to generate probes for detection of the *cpe* gene, 425-bp fragments were amplified by PCR (13). Purified DNAs of strains NCTC 8239 and NCTC 10239 were used as templates for PCR. The PCR products obtained with the two strains were combined, purified (High Pure PCR Product Purification kit; Roche, Mannheim, Germany) and labeled (DIG High Prime DNA Labeling and Detection Starter

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TABLE 1. Bacterial strains tested by *cpe* gene-specific PCR and DNA probe

Species	Strain	Source[a]	PCR[b]	CH[c]	DNA-H[d]
Clostridium aerotolerans	108	DFEH	NT	−	−
Clostridium botulinum (group I)	ATCC 25763	ATCC	NT	−	−
	ATCC 3502	ATCC	NT	−	NT
	62A	Riemann-Lindroth[e]	NT	−	NT
	69A	Riemann-Lindroth	NT	−	NT
	Langeland	ATCC	NT	−	NT
	SL-2A	Lindroth	NT	−	−
	SL-3A	Lindroth	NT	−	NT
	SL-4A	Lindroth	NT	−	NT
	SL-6A	Lindroth	NT	−	NT
	SL-1B	Lindroth	NT	−	NT
	RS-3A	Lindroth	NT	−	NT
	RS-4A	Lindroth	NT	−	NT
	NCTC 7272	NCTC	NT	−	NT
	ATCC 17841	ATCC	NT	−	NT
	ATCC 7949	ATCC	NT	−	NT
	ATCC 25764	ATCC	NT	−	NT
	126B	IP	NT	−	NT
	Crab F	Lindroth	NT	−	NT
	133–4803	McClung-Lindroth	NT	−	NT
Clostridium botulinum (group II)	K45E	DFEH	NT	−	NT
	17B	ATCC	NT	−	NT
	ATCC 23387	ATCC	NT	−	NT
	2B	Eklund-Lindroth	NT	NT	−
	250E	Crowther-Lindroth	NT	NT	−
	FT10F	Hobbs-Lindroth	NT	−	−
	K115E	DFEH	NT	−	−
	K44E	DFEH	NT	−	−
	BelugaE	Dolman-Lindroth	NT	−	−
Clostridium chauvoei	41	DFEH	NT	−	−
	103	DFEH	NT	−	−
Clostridium histolyticum	102	DFEH	NT	−	−
Clostridium perfringens	ATCC 3624	ATCC	−	−	−
	ATCC 3626	ATCC	−	−	−
	CCUG 2036	CCUG	−	−	−
	CCUG 2037	CCUG	−	−	−
	NCTC 8239	NCTC	+	+	+
	NCTC 10239	NCTC	+	+	+
	F 3686	Notermans[f]	+	+	+
	4732	DFEH	−	−	NT
	10204	DFEH	−	−	NT
	D9030	DFEH	+	+	+
	D9031	DFEH	+	+	+
	D9032	DFEH	+	+	+
	D9033	DFEH	−	−	−
	D9063	DFEH	−	−	−
	T8	DFEH	−	−	NT
	T9	DFEH	+	+	NT
	T16	DFEH	+	+	NT
	T19	DFEH	−	−	NT
	T20	DFEH	−	−	NT
	T21	DFEH	−	−	NT
	T22	DFEH	−	−	NT
	T25	DFEH	−	−	NT
	T28	DFEH	+	+	NT
	T35	DFEH	+	+	NT
	T37	DFEH	+	+	NT
	T39	DFEH	−	−	NT
	T42	DFEH	−	−	NT
	T43	DFEH	−	−	NT
	T44	DFEH	−	−	NT
	T48	DFEH	−	−	NT
Clostridium putrefaciens	104	DFEH	NT	−	NT
Clostridium septicum	43	DFEH	NT	−	NT
Clostridium sporogenes	ATCC 19404	ATCC	NT	−	−
	Lang	DFEH	NT	−	NT
	472A	DFEH	NT	−	NT
	29A	DFEH	NT	−	NT

Continued on following page

TABLE 1—*Continued*

Species	Strain	Source[a]	PCR[b]	CH[c]	DNA-H[d]
Listeria monocytogenes	TT7E	DFEH	NT	NT	−
	HT47E	DFEH	NT	NT	−
	AT12E	DFEH	NT	NT	−
	HT33E	DFEH	NT	NT	−
	LT15E	DFEH	NT	NT	−
	RT2E	DFEH	NT	NT	−
Proteus vulgaris	33	DFEH	NT	−	NT
Serratia marcescens	37	DFEH	NT	−	NT
Streptococcus lactis	12	DFEH	NT	−	NT

[a] ATCC, American Type Culture Collection, Manossas, Va.; IP, Institute Pasteur, Paris, France; DFEH, Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland; CCUG, Culture Collection, University of Gothenburg, Gothenburg, Sweden; NCTC, National Collection of Type Cultures, London, United Kingdom.

[b] PCR, detection of the *cpe* gene by PCR (20) from the bacterial cell lysate. +, positive PCR result; −, negative PCR result; NT, not tested.

[c] CH, colony hybridization with *cpe* gene-specific DNA probe from pure cultures growing on HGMF- TSC plates. +, positive signal obtained by hybridization; −, no signal obtained by hybridization; NT, not tested.

[d] DNA-H, hybridization with *cpe* gene-specific DNA probe. +, positive signal obtained by hybridization; −, no signal obtained by hybridization; NT, not tested.

[e] Collected from various sources by the late Seppo Lindroth (University of California, Davis). The first name in each pair is the original source.

[f] Obtained from Serve Notermans (Laboratory for Water and Food Microbiology, Bilthoven, The Netherlands).

kit I; Roche). The efficiency of the labeling reaction was determined as recommended by the manufacturer.

Detection of the *cpe* gene from bacterial DNA with the DNA probe. In order to determine the specificity of the probe, bacterial DNA was denatured at 95°C for 5 min and spotted on a positively charged nylon membrane (Roche). The bacterial DNA was fixed to the membrane with a UV cross-linker (Spectroline; Spectronics Corp., Westbury, N.Y.). Subsequently, the membrane was placed in a roller bottle containing hybridization solution (DIG Easy Hyb; Roche) and prehybridized at 41°C for 1 h. The hybridization solution was discarded and replaced with fresh solution containing 25 ng of denatured probe/ml, and incubation was continued at 41°C overnight. Thereafter, the hybridization solution was discarded and the membrane was washed twice (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] plus 0.1% sodium dodecyl sulfate) in roller bottles at room temperature for 5 min each time, followed by two 15-min washes in the same buffer at 65°C. The hybrids were detected with a chromogenic assay using the protocol provided by the DIG Application Manual (Roche).

Detection of *cpe* gene from pure bacterial cultures with DNA probe. Tryptose-sulfite-cycloserine (TSC) agar was prepared by adding 1% D-cycloserine (Sigma-Aldrich, St. Louis, Mo.) to Shahidi-Ferguson Perfringens agar (Difco Laboratories, Detroit, Mich.). HGMFs (Iso-Grid; Neogen, Baltimore, Md.) were placed on freshly prepared TSC agar plates, and the bacterial strains were streaked onto HGMF-TSC plates with a sterile loop. After the plates were incubated under anaerobic conditions at 37°C for 24 h, colonies on the HGMF-TSC plates were replicated by placing a nylon membrane aseptically on top of the HGMF. The membranes carrying the colony lifts were laid colony side up on Whatman (Kent, United Kingdom) 3MM blotting paper soaked in the denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 min, in neutralization solution (1.0 M Tris-HCl, 1.5 M NaCl, pH 7.4) for 15 min, and in 2× SSC for 10 min. The membranes were briefly air dried after each step and then were thoroughly dried after the 2× SSC treatment. Volumes of 1 μl of positive and negative control DNAs were spotted on dry membranes before the DNA was fixed to the membranes as described above. To remove cell debris, the membranes were treated at 37°C for 1 h with 0.5 ml of proteinase K (Finnzymes, Espoo, Finland) diluted 1:9 in 2× SSC. The debris was removed from the membranes by tightly pressing a sheet of blotting paper soaked with sterile distilled water onto the membranes. This procedure was repeated until all visible debris was removed. Subsequently, the membranes were prehybridized and hybridized as described above. Washing procedures and the chromogenic detection of the hybrids were carried out as described above.

Detection and enumeration of *cpe*-positive *C. perfringens* spores from fecal samples using the HGMF-CH method. In order to test the applicability of the HGMF-CH method in the investigation of fecal specimens, seven fecal samples from healthy individuals were included in the study. Each fecal sample was diluted 10-fold in 0.1% peptone water and heated at 75°C for 20 min in order to kill vegetative cells and to promote spore germination. A total of 0.1 g per sample was filtered using 10 HGMFs in a membrane filtration system (Iso-Grid). After filtration, the HGMFs were placed on TSC plates and incubated for 36 to 42 h at 37°C. Bacterial colonies grown on HGMF-TSC agar plates were replicated by placing a nylon membrane disk aseptically onto the HGMF. The disk was marked in order to orient the replica in relation to the HGMF. The nylon replicas were transferred to a dry sheet of blotting paper and prepared for hybridization as described above. After chromogenic detection, the nylon membranes were examined carefully in order to enumerate the positive hybridization signals corresponding to *cpe*-positive colonies on the original HGMF-TSC plate. As each HGMF allows the growth of 1,600 bacterial colonies, the theoretical detection limit of the method is 1 *cpe*-positive *C. perfringens* colony per 1,600 total *C. perfringens* colonies, provided that no more than one filter is investigated. When investigating 10 HGMFs, it is theoretically possible to detect and isolate 1 *cpe*-positive colony among 1.6×10^4 *cpe*-negative colonies.

As a reference to the quantification obtained by the HGMF-CH method, the number of *cpe*-positive *C. perfringens* spores in the fecal samples was estimated using nested PCR combined with the MPN technique (MPN-PCR) (13, 18), and the total number of *C. perfringens* spores was determined by plating fecal dilutions onto TSC agar (17). A total of three or four colonies from each sample containing typical colonies on TSC agar were confirmed by culture methods to be *C. perfringens* (17).

Isolation of *cpe*-positive *C. perfringens* spores from fecal samples using HGMF-CH method. During the hybridization procedure of the nylon membranes, the HGMF-TSC plates were kept under anaerobic conditions at room temperature in order to maintain the bacterial growth on the plates. The colonies yielding a positive signal on the nylon membranes were localized on the original HGMF-TSC plates. Several probe-positive colonies were picked from the original plates, streaked onto blood agar, and incubated for 24 h at 37°C. These isolates were further analyzed for the presence of the *cpe* gene by PCR (20).

RESULTS

Detection of the *cpe* gene from bacterial DNA and pure bacterial cultures with the DNA probe. DNA and pure cultures of all *cpe*-positive *C. perfringens* strains tested were positive as determined by the hybridization method (Table 1). The positive hybridization signals were seen as clearly visible purple spots on the nylon membrane after chromogenic detection. All *cpe*-negative *C. perfringens* strains and the other bacterial strains tested yielded no hybridization signals (Table 1).

Enumeration and isolation of *cpe*-positive *C. perfringens* spores from fecal samples using the HGMF-CH method. The HGMF-CH method proved to be as sensitive as MPN-PCR in detecting *cpe*-positive *C. perfringens* spores in fecal samples (Table 2). Positive hybridization signals were detected from two fecal samples, N1 and N4 (Table 2). These signals were strong, purple, round or square spots on the nylon membrane (Fig. 1). The numbers of *cpe*-positive *C. perfringens* spores in the samples were 60 and 1,500 CFU/g. The other five samples

TABLE 2. Enumeration and isolation of *cpe*-positive *C. perfringens* from fecal samples[a]

Sample no.	Age[b] (yr)	No. of *cpe*-positive *C. perfringens* spores/g[c]	Total no. of *C. perfringens* spores/g[d]	No. of *cpe*-positive *C. perfringens* spores/ total no. of *C. perfringens* spores[e]	HGMF-CH	
					No. of *cpe*-positive *C. perfringens* spores/g[f]	Isolation of *cpe*-positive *C. perfringens*[g]
N1	30	74	5.8×10^5	1×10^{-4}	60	+
N2	57	<3	2.1×10^6	ND	<10	−
N3	17	<3	1.5×10^4	ND	<10	−
N4	55	7,500	1.3×10^5	6×10^{-2}	1,500	+
N5	24	3.6	3.2×10^3	1×10^{-3}	<10	−
N6	51	<3	2.3×10^2	ND	<10	−
N7	24	9.2	2.5×10^5	4×10^{-5}	<10	−

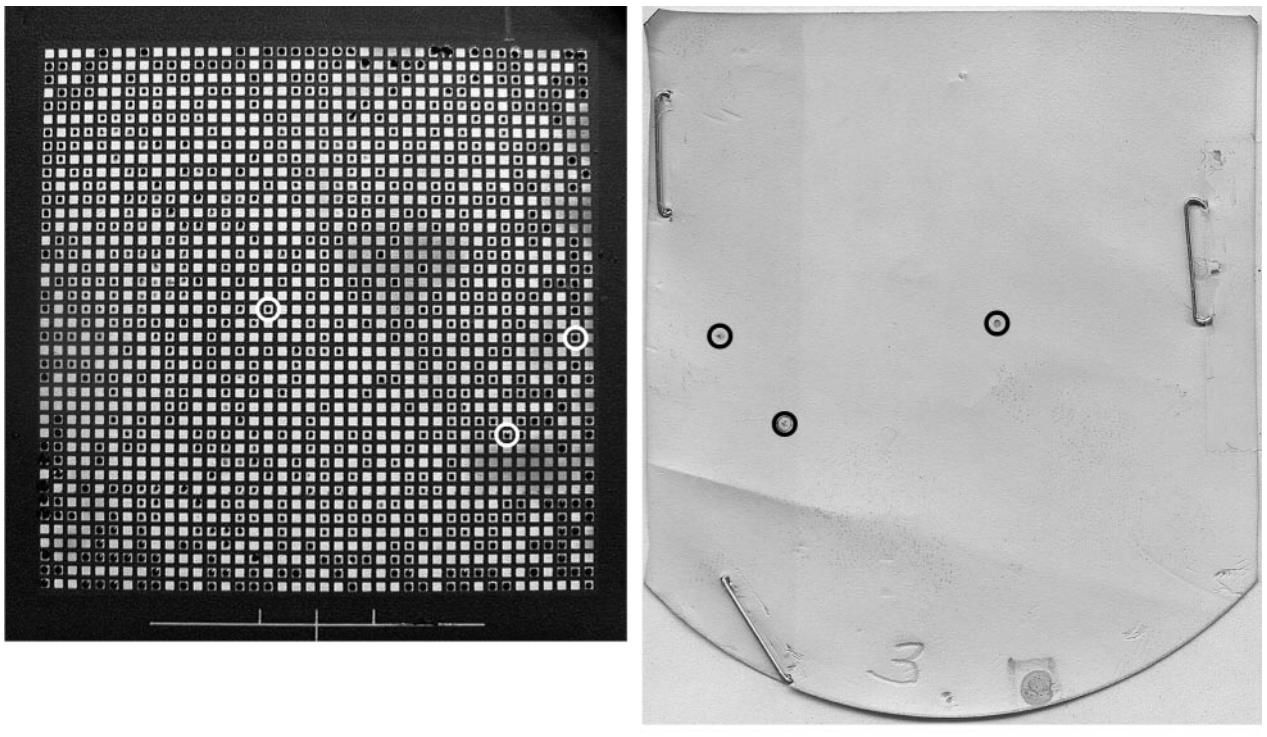
[a] Sample heated at 75°C for 20 min.
[b] Age of donor.
[c] Number of *cpe*-positive *C. perfringens* spores in the sample determined by nested PCR combined with MPN technique. A value of <3/g was assigned when no positive PCR results were obtained.
[d] Determined by plate count on TSC agar and confirming three or four counts of typical colonies by culture methods.
[e] Ratio of number of *cpe*-positive *C. perfringens* spores determined by nested PCR combined with MPN technique to total number of *C. perfringens* spores obtained by plate count on TSC agar. ND, no ratio for *cpe*-positive *C. perfringens* and total *C. perfringens* spore numbers could be determined.
[f] Number of *cpe*-positive *C. perfringens* spores determined by HGMF-CH method. A value of <10/g was assigned when no hybridization signals were obtained.
[g] +, *cpe*-positive *C. perfringens* isolates obtained; −, no *cpe*-positive *C. perfringens* isolates obtained.

revealed no positive hybridization signals (Table 2). The total number of *C. perfringens* spores on the HGMFs was similar to the results obtained by the plate count on TSC agar.

The *cpe*-positive *C. perfringens* isolates were obtained from both of the samples revealing positive hybridization signals. The isolates were confirmed to be *cpe* positive by PCR.

DISCUSSION

An HGMF-CH method for the enumeration and isolation of *cpe*-positive *C. perfringens* spores from feces was developed. The method provides a marked improvement in the investigation of *cpe*-positive *C. perfringens* from feces, since none of the



A B

FIG. 1. (A) HGMF containing *C. perfringens* growth after incubation on TSC agar plate. *cpe*-positive colonies detected later with hybridization are circled. (B) Nylon membrane replica of growth on HGMF-TSC plate, hybridized with *cpe* gene-specific DNA probe. The circles indicate positive hybridization signals corresponding to *cpe*-positive colonies on the original HGMF membrane.

previously reported detection methods facilitates the isolation of *cpe*-positive *C. perfringens* (2, 3, 6, 8, 13, 14, 21, 23). With the HGMF-CH method, up to 1,600 separate colonies on a single membrane can be simultaneously screened for the presence of the *cpe* gene. The method thus provides the possibility of enumerating and isolating *cpe*-positive colonies.

When tested with bacterial DNA and pure cultures, the DNA probe proved to be specific and sensitive, revealing strong hybridization signals with all *cpe*-positive *C. perfringens* strains. Stringent hybridization and washing conditions ensured high specificity, and none of the *cpe*-negative bacterial strains revealed positive signals. Even a 2-h hybridization period was sufficient to provide a strong hybridization signal, but for practical purposes the hybridization was continued overnight.

When the HGMF-CH method was applied to the investigation of feces from healthy individuals, the method was shown to be as sensitive as the MPN-PCR employed for detecting *cpe*-positive *C. perfringens* spores in fecal samples. The detection limit of the method was dependent on at least two factors. First, the size of the sample filtered defines the lowest level of *cpe*-positive *C. perfringens* spores detected. Thus, when a total of 0.1 g of a sample was filtered, the limit of detection was 10 CFU/g. However, a lower detection limit would be achieved by studying a larger amount of sample. Secondly, the detection limit of the HGMF-CH method is dependent on the ratio of *cpe*-positive *C. perfringens* strains to total *C. perfringens* strains growing on the HGMF-TSC. By studying 10 HGMFs, the lowest detectable ratio is 6×10^{-5}. When the HGMF-CH method was applied in the investigation of feces from healthy individuals, positive hybridization signals were detected from two samples, N1 and N4 (Table 2). When 0.1 g of sample was filtered, totals of 6 and 150 *cpe*-positive colonies were detected from samples N1 and N4, respectively. *cpe*-positive *C. perfringens* was successfully isolated from both of these samples. However, *cpe*-positive colonies could not be detected or isolated from samples N5 and N7, which contained *cpe*-positive *C. perfringens* according to the results obtained by MPN-PCR. This is explained by the fact that the numbers of *cpe*-positive *C. perfringens* spores in these two samples were below the detection limit of the HGMF-CH method (Table 2). In sample N7, the number of *cpe*-positive *C. perfringens* spores was 9.2/g, which is close to the 10 CFU/g detection limit. The ratio of *cpe*-positive *C. perfringens* strains to total *C. perfringens* strains in this sample was 4×10^{-5}, which is also close to the detection limit of the method. Thus, detection of *cpe*-positive *C. perfringens* spores from sample N7 should have been possible by examining a greater number of membranes and/or filtering a larger amount of feces.

As the number of *cpe*-positive *C. perfringens* spores and the ratio of *cpe*-positive spores to total *C. perfringens* spores in the intestinal contents of humans and animals seem to be extremely low, the HGMF-CH method offers a powerful tool in the investigation of the reservoirs for *cpe*-positive *C. perfringens*. By initial screening of samples for the presence of the *cpe* gene by PCR and choosing only the PCR-positive samples for HGMF-CH, the diagnostics cost would be reduced and studying of reservoirs for the *cpe*-positive strains would be more effective. As indicated by the results of this study, a larger sample size and a higher number of membranes hybridized are recommended when positive hybridization signals are not found from PCR-positive samples.

The total time required to detect the *cpe*-positive *C. perfringens* spores in a sample was 3 to 4 days. The filtration of a fecal sample was laborious due to the occasional obstruction of the filtration apparatus and the membrane. Sample enrichment has been used in previous studies of the HGMF technique combined with DNA hybridization (19, 25). Enriching the sample would probably prevent the filtration apparatus and the membrane from becoming obstructed and improve the filtration rate. However, enrichment procedures would naturally hamper the quantitative analysis and therefore other sample preparation methods should be considered in order to improve the efficacy of the method.

The samples were heated in order to kill the vegetative cells and to increase spore germination. However, without heating the sample, the vegetative cells could be detected and enumerated. Thus, in *C. perfringens* food poisoning outbreaks, the HGMF-CH method could also be applied to the investigation of the suspected food, since the disease is caused by food containing a large number of vegetative cells of *cpe*-positive *C. perfringens*.

The HGMF-CH method provides a new approach in the diagnostics of food poisoning caused by *cpe*-positive *C. perfringens*, since the conventional isolation of *cpe*-positive *C. perfringens* from the feces of affected individuals is occasionally complicated by the presence of *cpe*-negative *C. perfringens* (5, 11, 16, 20, 22, 26). As the HGMF-CH method provides a means to determine the presence of the *cpe* gene in a large number of isolates simultaneously and to enumerate and further isolate the *cpe*-positive strain, the method markedly improves the investigation of the epidemiology of *cpe*-positive *C. perfringens* strains. The method may also help in the diagnostics of food poisoning and other gastrointestinal diseases caused by *cpe*-positive *C. perfringens*, especially if the samples are collected several days after the onset of the disease (16).

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