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Detection of Enterotoxigenic *Clostridium perfringens* in Meat Samples by Using Molecular Methods▽

Ikuko Kaneko,[1] Kazuaki Miyamoto,[1]* Kanako Mimura,[1] Natsuko Yumine,[1] Hirotoshi Utsunomiya,[2] Shigeru Akimoto,[1] and Bruce A. McClane[3]

Department of Microbiology, Wakayama Medical University School of Medicine, 811-1 Kimiidera, Wakayama 641-0012, Japan[1]; Department of Gastroenterology, Wakayama Medical University School of Medicine, 811-1 Kimiidera, Wakayama 641-0012, Japan[2]; and Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261[3]

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To prevent food-borne bacterial diseases and to trace bacterial contamination events to foods, microbial source tracking (MST) methods provide important epidemiological information. To apply molecular methods to MST, it is necessary not only to amplify bacterial cells to detection limit levels but also to prepare DNA with reduced inhibitory compounds and contamination. Isolates carrying the *Clostridium perfringens* enterotoxin gene (*cpe*) on the chromosome or a plasmid rank among the most important food-borne pathogens. Previous surveys indicated that *cpe*-positive *C. perfringens* isolates are present in only ~5% of nonoutbreak food samples and then only at low numbers, usually less than 3 cells/g. In this study, four molecular assays for the detection of *cpe*-positive *C. perfringens* isolates, i.e., ordinary PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP), were developed and evaluated for their reliability using purified DNA. For use in the artificial contamination of meat samples, DNA templates were prepared by three different commercial DNA preparation kits. The four molecular assays always detected *cpe* when $>10^3$ cells/g of *cpe*-positive *C. perfringens* were present, using any kit. Of three tested commercial DNA preparation kits, the InstaGene matrix kit appeared to be most suitable for the testing of a large number of samples. By using the InstaGene matrix kit, the four molecular assays efficiently detected *cpe* using DNA prepared from enrichment culture specimens of meat samples contaminated with low numbers of *cpe*-positive *C. perfringens* vegetative cells or spores. Overall, the current study developed molecular assay protocols for MST to detect the contamination of foods with low numbers of cells, and at a low frequency, of *cpe*-positive *C. perfringens* isolates.

Clostridium perfringens is an important pathogen of human gastrointestinal (GI) tract diseases such as food poisoning, antibiotic-associated diarrhea, and sporadic diarrhea as well as nosocomial diarrheal disease outbreaks (6, 10). The most important toxin made by this bacterium when it causes human GI tract diseases is *Clostridium perfringens* enterotoxin (CPE) (10, 14). Although *C. perfringens* is a ubiquitous bacterium in the environment, only a small subpopulation of this bacterium, usually less than 5%, harbors the CPE gene (*cpe*) (10). Probably because of this rarity, previous surveys identifying *cpe*-positive *C. perfringens* isolates in food, human feces, and the environment have reported various results (1, 4, 8, 9, 11, 16). Moreover, the number of contaminated *C. perfringens* cells (*cpe*-positive and *cpe*-negative strains) present in most nonoutbreak food samples has been reported to be fewer than 3 using the most-probable-number method (9, 11, 16). Therefore, to prevent food poisoning and nosocomial outbreaks, a method able to detect *cpe*-positive strains with high sensitivity and applicability for the testing of a large number of samples is necessary for use in epidemiological surveys.

Microbial source tracking (MST) methods allow the identification of the types of microbial contaminants, the extent of contamination, and the possible source of contamination (12). In MST, molecular approaches are useful tools for the detection of a low number of bacteria (12). Hence, the molecular methods used for MST should efficiently distinguish *cpe*-positive *C. perfringens* isolates from *cpe*-negative *C. perfringens* isolates in foods and the environment.

In this study, four kinds of molecular methods, i.e., PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP), were first developed for the detection of *cpe*. Those methods were then applied to artificially contaminated meat samples, since meat samples represent one of the major vehicles of *cpe*-positive *C. perfringens* when causing food poisoning (10). For the detection of the presence of the *cpe* gene in meat samples, three kinds of DNA preparation kits were compared and evaluated with respect to their sensitivity, convenience, and applicability for the testing of large numbers of samples. Moreover, the usefulness of a previously reported enrichment culture method was also evaluated by using meat samples contaminated with a small number of *cpe*-positive *C. perfringens* cells, since bacteria in nonoutbreak foods, water, and the environment are generally present at low numbers (11, 12, 16). For these enrichment culture experiments, vegetative cells and spores of *cpe*-positive *C. perfringens* isolates were used as artificial contaminants.

MATERIALS AND METHODS

Bacterial strains and media. NCTC8239, a *C. perfringens* food poisoning strain with a chromosomal *cpe* gene, and T16, a food-borne outbreak strain with *cpe* carried on a large plasmid, were used (2, 15).

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

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TABLE 1. Primers used in each molecular assay

Assay	Primer	Sequence	Reference
PCR	cpe-F3	5′-ACATCTGCAGATAGCTTAGGAAAT-3′	11
	cpe-B3	5′-CCAGTAGCTGTAATTGTTAAGTGT-3′	11
Nested PCR	cpe	5′-GGAGATGGTTGGATATTAGG-3′[a]	3
	cpe 5R	5′-TCCATCACCTAAGGACTG-3′[a]	14
	cpe-F3	5′-ACATCTGCAGATAGCTTAGGAAAT-3′[b]	11
	cpe-B3	5′-CCAGTAGCTGTAATTGTTAAGTGT-3′[b]	11
Real-time PCR	cpe RT-F	5′-TGGTGTTCGAAAATGCTAAA-3′	This study
	cpe RT-R	5′-TTCCCCTAATATCCAACCA-3′	This study
LAMP	cpe-F3	5′-ACATCTGCAGATAGCTTAGGAAAT-3′	11
	cpe-B3	5′-CCAGTAGCTGTAATTGTTAAGTGT-3′	11
	cpe-FIP	5′-AGCAGCTAAATCAAGGATTTCTTTTT AGGTTCATTAATTGAAACTGGTGA-3′	This study
	cpe-BIP	5′-AACTGATGCATTAAACTCAAATCCAG AGGGTATGAGTTAGAAGAACGC-3′	This study

[a] First primers.
[b] Nested primers.

Both *C. perfringens* strains were grown overnight at 37°C in FTG medium (fluid thioglycolate broth [Difco]) or TGY medium (3% tryptic soy broth [Becton Dickinson], 2% glucose, 1% yeast extract [Difco], 0.1% L-cysteine [Wako]). For spore formation, Duncan-Strong medium was used (11).

Molecular assays for detection of the *cpe* gene. Four representative molecular detection methods were developed: standard PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP). A list of primers used in each assay is shown in Table 1. To investigate the sensitivity of each assay, template DNA was prepared from NCTC8239 according to a method described previously (2). To investigate the specificity of molecular assays, DNA templates from four *Bacillus cereus* food poisoning isolates, six meat isolates (these six meat isolates were identified as *Streptococcus* species and four other *Clostridium* species based on 16S rRNA sequences), and five types of *Clostridium botulinum* strains (types A, B, C, E, and F) were used (11).

For PCR assays and nested PCR assays (first-cycle PCR), each PCR mixture contained 2 μl of template DNA, 0.25 μl of High Fidelity GoTaq polymerase (Promega), 2 μl of 2 mM nucleoside triphosphates (NTPs), 4 μl of 25 mM $MgCl_2$, 10 μl of PCR buffer, and 2 μl of each primer pair (1 μM final concentration). In the second cycle of the PCR for the nested PCR assay, 1 μl of the first-cycle PCR mixture was used as a template.

First-cycle PCRs for the nested PCR assay were performed under the following conditions, as described previously (2): 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 60 s, and 68°C for 60 s; and a single extension step at 68°C for 8 min. PCRs and second-cycle reactions of the nested PCR assay were performed under the following conditions, as described previously (11): 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s; and a single extension step at 68°C for 8 min. PCR products were electrophoresed on a 1.5% agarose gel, followed by staining with ethidium bromide and then detection by UV.

For the real-time PCR assay, each reaction mixture contained 0.5 μl of each primer (0.5 μM final concentration), 2× iQ SYBR green Supermix (Bio-Rad), and DNA template (2 μl) to a total volume of 50 μl (Table 1). The reactions were performed and analyzed by using the MyiQ real-time PCR system (Bio-Rad) with a positive control and a "no-template control," with an initial holding temperature of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s and then 81 cycles of 55°C (set point temperature) for 30 s as the melt curve assay. Emission was monitored at the end of every 72°C elongation step on a SYBR green channel. For the reference reaction, a pTrcHis plasmid carrying *cpe* was constructed according to a method described previously (7). Serially diluted aliquots (10^5- to 10^9-fold dilutions) of the *cpe*-carrying plasmid preparation were used as a template for reference reactions. The aliquot of the 10^7-fold reference plasmid dilution, estimated as ~6 copies per reaction by the Reed-Muench formula, was always positive in each independent real-time PCR assay. Therefore, when the C_T (threshold cycle) value of sample specimens was less than that of the 10^7-fold reference dilution, the sample specimen was identified as being positive. The specificity of the positive results was confirmed by the results of the melt curve assay.

For the LAMP assay, four LAMP primers (cpeFIP, cpeBIP, cpeF3, and cpeB3) were designed by using PrimerExplorer LAMP primer software (Table 1). LAMP reactions were carried out at 63°C for 60 min using a Loopamp DNA amplification kit (Eiken) on a block incubator (catalog number BI-516C; Astec). Each reaction mixture contained 2× reaction mixture, 40 pmol FIP and BIP primers, 5 pmol F3 and B3 primers, 1 ml of Bst DNA polymerase, and 2 μl of DNA template to a volume of 25 μl. Positive results were investigated by visualizing the product on a 2% agarose gel stained with ethidium bromide.

Estimation of detectable bacterial cell numbers in meat samples using each *cpe* assay. To estimate the number of bacterial cells required for the detection of the presence of *cpe*-positive strains in meat samples, 10 g of a meat sample was put into a stomacher bag, and 19 ml of 0.1% tryptone (Difco) in distilled water and 1 ml of a serially 10-fold-diluted culture of strain NCTC8239 grown overnight were then added. Also, as a negative control, 20 ml of 0.1% tryptone solution was added. The contents of the bag were then fully mixed. Aliquots of 200 μl of extract were used for DNA preparation. The DNA template for molecular assays was prepared by using three kinds of commercial DNA preparation kits, QIAamp DNA stool minikit (Qiagen), Isogen (Wako), and InstaGene matrix (Bio-Rad), according to the manufacturers' instructions. An aliquot (2 μl) of prepared DNA was used as a template for each assay. Six retail ground meat samples (two beef, two chicken, and two beef and pork mix) were used for six independent experiments.

Detection of enterotoxigenic *C. perfringens* in enrichment culture samples. To investigate the effect of enrichment cultures on the growth of *cpe*-positive *C. perfringens* cells to detectable levels for molecular assays, 12 ground meat samples were independently artificially contaminated and then incubated at 45°C as an enrichment culture step (see Table 4).

Briefly, 0.2-ml aliquots of the FTG culture were inoculated into 10 ml TGY medium or Duncan-Strong medium and then incubated at 37°C overnight. Those TGY cultures grown overnight were serially diluted to a 10^8-fold dilution, and a 0.2-ml aliquot of each dilution was spread onto brain heart infusion (BHI) agar plates to count viable bacterial cells. Also, 1 ml of a 10^7-fold dilution and 39 ml of fluid thioglycolate II (FTGII) medium (Eiken) were put into a stomacher bag containing a 20-g meat sample; the number of *cpe*-positive bacterial cells added was estimated to be fewer than 3 cells (0.1 to 2.5 cells) per gram (see Table 4). To investigate the capability of detecting a spore in food, samples of Duncan-Strong medium from a culture grown overnight were first heated at 75°C for 20 min to kill vegetative cells. Those cultures were then serially diluted and spread onto BHI agar plates. One milliliter of a 10^5-fold dilution of heated Duncan-Strong medium was added to meat samples. An artificially contaminated meat sample with vegetative cells or spores was mixed well, and a 0.2-ml extract was taken as the 0-h sample. The contaminated meat sample was then anaerobically incubated at 45°C. An extract (an aliquot of 0.2 ml) was recovered from this enrichment culture sample every hour for the first 6 or 7 h and at 24 h. These extracts were subjected to DNA preparation with an InstaGene matrix kit. A meat sample in 40 ml FTGII medium without a bacterial culture was used as a negative control for each experiment.

TABLE 2. Specificity of each molecular assay

Organism	Detection of *cpe* by:			
	PCR	Nested PCR	Real-time PCR	LAMP
Clostridium botulinum				
Strain 98-193 (type A)	−	−	−	−
Strain 98-195 (type B)	−	−	−	−
Strain 98-196 (type C)	−	−	−	−
Strain 98-199 (type E)	−	−	−	−
Strain 98-200 (type F)	−	−	−	−
B. cereus (food poisoning isolates)				
Strain 019	−	−	−	−
Strain 022	−	−	−	−
Strain 025	−	−	−	−
Strain 052	−	−	−	−
Food isolates				
TM011 (*Clostridium sordellii*)	−	−	−	−
TM013 (*Streptococcus* species)	−	−	−	−
TM040 (*Clostridium* species)	−	−	−	−
TM106 (*Clostridium* species)	−	−	−	−
TM147 (*Clostridium* species)	−	−	−	−
TM204 (*Clostridium* species)	−	−	−	−
C. perfringens				
NCTC8533 (type B) (*cpe* negative)	−	−	−	−
NCTC3182 (type C) (*cpe* negative)	−	−	−	−
NCTC8346 (type D) (*cpe* negative)	−	−	−	−
NCTC8084 (type E) (silent *cpe* sequences)	+	+	−	−
NCTC8239	+	+	+	+

RESULTS

Sensitivity and specificity of each molecular assay for *cpe* detection. By using purified DNA from NCTC8239, each molecular assay was performed at least twice to investigate sensitivity. The PCR assay and nested PCR assay required 10 pg and 0.1 pg of bacterial DNA for the detection of the *cpe* gene, respectively. The real-time PCR assay required 0.1 pg, and the loop-mediated isothermal amplification (LAMP) assay required 1 pg.

Four *B. cereus* food poisoning isolates, six meat isolates (these six meat isolates were identified as *Streptococcus* species and four other *Clostridium* species based on 16S rRNA sequences), and five types of *C. botulinum* strains (types A, B, C, E, and F) did not show any products in the PCR, nested PCR, real-time PCR, or LAMP assays (Table 2). Molecular assays did not show a positive reaction with three types of *cpe*-negative *C. perfringens* (types B, C, and D) strains. However, PCR and nested PCR assays, but not real-time PCR or LAMP assays, detected the disrupted *cpe* gene present in a type E *C. perfringens* strain (Table 2).

Estimation of a detectable bacterial cell number with each assay. To estimate a detectable bacterial cell number with each molecular *cpe* assay, *cpe*-positive strain NCTC8239 was artificially inoculated into meat samples, and template DNA was then prepared from extracts of those contaminated samples.

Molecular assays detected *cpe* using DNA from artificially contaminated meat samples when the number of cells added ranged from $\sim10^4$ to $\sim10^8$ cells/g in the PCR assay, from $\sim10^2$ to $\sim10^7$ cells/g in the nested PCR assay, from $\sim10^4$ to $\sim10^7$ cells/g in the real-time PCR assay, and from $\sim10^4$ to more than 10^7 cells/g in the LAMP assay. For the detection of *cpe* in DNA prepared from artificially contaminated meat samples, these molecular assays usually required more than 10^3 bacterial cells per g of sample (Table 3).

TABLE 3. Estimation of numbers of bacterial cells required for detection in meat samples

Assay	Estimated no. of bacterial cells (10^3)/g required for detection		
	QIAamp DNA stool minikit	InstaGene matrix	Isogen
PCR	1300–280,000	13–1,000	13–2,000
Nested PCR	20–28,000	0.2–1,000	8.5–100
Real-time PCR	1,000–85,000	20.0–2,000	13.0–2,800
LAMP	100–28,000	20.0–>85,000	8.5–280

Comparing the three investigated commercial DNA preparation kits, the highest number of bacterial cells ($\sim10^4$ to $\sim10^8$ cells/g) was required for the detection of *cpe* using DNA prepared with the column-based QIAamp DNA stool minikit (Table 3). One reason for this may be that only a portion of the DNA-containing supernatant was applied onto the column device used in this kit for the removal of residual sediments before proceeding to further DNA purification steps. The protocol for the QIAamp DNA stool minikit is also complicated, containing 18 or 19 steps and using 6 supplied regents, which makes it less applicable to the screening of large numbers of samples.

The Isogen DNA extraction kit includes phenol-chloroform extraction and ethanol precipitation steps. As expected, by using DNA prepared with this kit, molecular assays required the lowest number of contaminated cells ($\sim10^3$ to $\sim10^5$ cells) for the detection of the presence of the *cpe* gene (Table 3). Compared with the other two DNA preparation kits, the range of cell numbers required for detection was narrower when *cpe* was detected with four molecular assays (Table 3). This is probably because of low levels of contaminating protein. However, this kit has seven independent steps, and both ethanol and an in-house-prepared reagent are necessary, in addition to the supplied reagents. When applied to a large number of samples, the use of these many reagents might induce cross-contamination.

For the InstaGene matrix kit, template DNA is extracted by heating, and some PCR inhibitors are then adsorbed by a matrix. PCR-based assays detected *cpe* when *cpe*-positive cells were added to meat samples at levels of between $\sim10^2$ and $\sim10^6$ cells. This number of cells is closely correlated with those needed for PCR-based assays using DNA prepared by the Isogen kit (Table 3). However, the LAMP assay did not show a positive reaction using DNA prepared from four of six investigated meat samples contaminated by the inoculation of more than 10^7 cells (Table 3). However, using the same DNA specimens after a 10-fold dilution with distilled water, or the same DNA specimens with additional phenol-chloroform extraction, the LAMP assay detected *cpe* more efficiently (dropping from $>10^7$ to 10^4 or 10^5 cells [data not shown]). The LAMP assay could detect *cpe* by using DNA from other two meat samples contaminated with 10^4 or 10^5 cells (Table 3).

From these findings, DNA prepared by the InstaGene matrix kit might still contain a LAMP-specific inhibitor(s) in some meat samples, which did not act as an inhibitor in PCR-based assays. Another possibility is that the inhibitor(s) in the DNA preparation has a higher level of activity in the LAMP reaction than in PCR-based reactions. According to the manufacturer's instructions for the InstaGene matrix kit, DNA templates are prepared after only four independent steps, which is able to reduce cross-contamination compared to the other two kits; therefore, this kit might be suitable for the testing of a large number of samples.

Effect of enrichment culture on detection of a low number of contaminated *cpe*-positive *C. perfringens* vegetative cells and spores. From the results of experiments estimating the numbers of detectable *cpe*-positive bacterial cells with molecular assays, more than 10^3 cells per g were required to detect *cpe* in all tested samples (Table 3). This finding indicated that to consistently detect low numbers of *cpe*-positive *C. perfringens* bacteria in meat samples, an enrichment culture for *C. perfringens* growth should be crucial. However, it is still unclear whether an enrichment culture could make growth to sufficient levels for detection possible and how long of an incubation time is required to grow sufficient numbers of cells for detection with molecular assays.

To answer these questions, the effect of an enrichment culture was investigated by using meat samples artificially contaminated by low numbers of bacterial cells, <3 cells (0.1 to 2.5 cells) per g, of two types of *cpe*-positive strains, i.e., *C. perfringens* food-borne outbreak isolates harboring *cpe* either on the chromosome or on a large plasmid (2, 10, 15). For enrichment cultures, the incubation temperature was 45°C, because *C. perfringens* grew better than other contaminated bacteria at 45°C in our previous study (11). Also, DNA templates were prepared by the InstaGene matrix kit, because the protocol of this kit has the least number of steps for the preparation of DNA templates, making it most suitable to investigate a large number of samples and least at risk for cross-contamination events. In addition, in our previous study using this kit, most *C. perfringens* cells in meat samples were detected within 24 h after the enrichment culture (11).

After artificial contamination with vegetative cells of the chromosomal *cpe* strain or the plasmid *cpe* strain, all four investigated molecular assays detected *cpe* by using DNA prepared from >6-h enrichment culture samples but not by using DNA prepared from <1-h enrichment cultures (Table 4). In samples contaminated with spores, all four molecular assays also detected *cpe* by using DNA from enrichment cultures grown for >7 h (Table 4). By using DNA from extracts of the same enrichment culture experiment, nested PCR and real-time PCR assays required shorter incubation times, usually 1 h less, than the other two assays for the detection of the *cpe* gene, indicating that nested PCR and real-time PCR assays might have higher sensitivities than the other two assays. However, the required incubation time for the detection of *cpe* among all experiments for the detection of *cpe* showed more diversity: 4 to 7 h for PCR, 2 to 7 h for nested PCR, 3 to 6 h for real-time PCR, and 3 to 6 h for the LAMP assay. These findings revealed that an enrichment culture step is beneficial for the detection of *cpe*-positive strains in meat samples. The LAMP assay could detect *cpe* in all investigated samples with a sensitivity similar to those of other assays: the effect of the LAMP inhibitor(s) found for DNA from non-culture-contaminated samples was not detected with the DNA specimens from enrichment culture samples prepared with the InstaGene kit. No apparent correlation between the number of cells added and the incubation time of the enrichment culture was found. Also, the type of meat sample did not consistently affect the time needed for enrichment cultures, which the investigated molecular assays required. Overall, the four investigated molecular assays were able to detect *cpe*-positive *C. perfringens* by using DNA specimens from enrichment cultures incubated for more than 7 h, while DNA from noncontaminated samples always showed a negative reaction (Table 4).

DISCUSSION

Generally, *Clostridium perfringens* is a difficult target bacterium for microbial source tracking (MST) because it is so broadly distributed in the environment (12). However, only a small proportion of isolates of this bacterium can produce toxins specific for human and animal gastrointestinal diseases. One group of these toxin-producing strains, i.e., CPE-producing type A strains, has been found in <5% of human fecal samples or environmental sources such as nonoutbreak retail foods (1, 4, 8, 9, 11, 16). Moreover, the open reading frame (ORF) of the *cpe* gene in human isolates has a highly conserved sequence (10). Hence, *C. perfringens* strains carrying the *cpe* gene should be an adequate candidate for MST (13).

In Japan, the number of patients suffering from *C. perfringens* food poisoning ranks this as the third or fourth most common cause of bacterial food poisoning in recent years. For the prevention of food poisoning caused by *cpe*-positive *C. perfringens* strains, epidemiological surveys using MST methods should elucidate the specific locations or reservoirs and food chain of *cpe*-positive *C. perfringens* strains. In cases of food poisoning, protocols for the identification of *C. perfringens* as the causative bacterium have been developed. These protocols can identify contaminated food containing a large number, usually more than 10^5, of CPE-producing *C. perfringens* cells and can identify ill patients by use of fecal samples that contain CPE and a large number of *cpe*-positive *C. perfringens* bacteria. However, these protocols cannot detect a small number of *cpe*-positive *C. perfringens* cells present in foods prior to temperature abuse. Like other bacteria, *cpe*-positive and/or *cpe*-negative *C. perfringens* strains are usually present in low numbers in food ingredients or contaminated environments prior to temperature abuse (9, 11, 12, 16).

Therefore, in this study, we first developed four sensitive and specific representative molecular assays. However, even though molecular methods are often touted as being highly sensitive (detection limit of 1 to 10 gene targets), they are generally not of value if employed directly for the detection of organisms in food or environmental samples (12). In fact, for the detection of *cpe*-positive strains from artificially contaminated meat samples without preincubation, molecular assays required more than 10^3 cells per g and showed a wide range of detection diversity among the investigated samples. These results suggest that the efficiency of DNA extraction and the reduction of presumable inhibitors were highly variable among the investigated food samples, even when the same kit was

TABLE 4. Detection of *cpe*-positive cells in enrichment culture samples[a]

Expt	Molecular method	Detection of *cpe*-positive cells in: Contaminated samples at incubation time (h) of: 0	1	2	3	4	5	6	7	24	Noncontaminated samples at incubation time (h) of: 0	24	No. of cells added/amt of sample (g)	Type of meat sample
1	PCR	−	−	−	−	−	+	+	NT	+	−	−	1.5/20	Ground beef
	Nested PCR	−	−	−	−	+	+	+	NT	+	−	−		
	Real-time PCR	−	−	−	−	+	+	+	NT	+	−	−		
	LAMP	−	−	−	−	+	+	+	NT	+	−	−		
2	PCR	−	−	−	−	+	+	+	NT	+	−	−	20/20	Ground pork
	Nested PCR	−	−	−	+	+	+	+	NT	+	−	−		
	Real-time PCR	−	−	−	−	+	+	+	NT	+	−	−		
	LAMP	−	−	−	−	−	+	+	NT	+				
3	PCR	−	−	−	−	−	−	+	NT	+	−	−	38/20	Ground chicken
	Nested PCR	−	−	−	−	−	−	+	NT	+	−	−		
	Real-time PCR	−	−	−	−	−	+	+	NT	+	−	−		
	LAMP	−	−	−	−	−	−	+	NT	+	−	−		
4	PCR	−	−	−	−	+	+	+	NT	+	−	−	26/20	Ground beef
	Nested PCR	−	−	−	+	+	+	+	NT	+	−	−		
	Real-time PCR	−	−	−	−	+	+	+	NT	+	−	−		
	LAMP	−	−	−	+	+	+	+	NT	+	−	−		
5	PCR	−	−	−	−	+	+	+	NT	+	−	−	35/20	Ground pork
	Nested PCR	−	−	−	+	+	+	+	NT	+	−	−		
	Real-time PCR	−	−	−	+	+	+	+	NT	+	−	−		
	LAMP	−	−	−	−	−	+	+	NT	+	−	−		
6	PCR	−	−	−	−	−	+	+	NT	+	−	−	50/20	Ground chicken
	Nested PCR	−	−	−	−	+	+	+	NT	+	−	−		
	Real-time PCR	−	−	−	−	+	+	+	NT	+	−	−		
	LAMP	−	−	−	−	+	+	+	NT	+	−	−		
7	PCR	−	−	−	−	−	+	+	+	+	−	−	6.5/20	Ground beef
	Nested PCR	+[b]	−	−	−	+	+	+	+	+	−	−		
	Real-time PCR	−	−	−	−	+	+	+	+	+	−	−		
	LAMP	+[b]	−	−	−	+	+	+	+	+	−	−		
8	PCR	−	−	−	−	−	+	+	+	+	−	−	1.8/20	Ground pork
	Nested PCR	−	−	−	−	−	+	+	+	+	−	−		
	Real-time PCR	−	−	−	−	−	+	+	+	+	−	−		
	LAMP	−	−	−	−	−	+	+	+	+	−	−		
9	PCR	−	−	−	−	−	−	−	+	+	−	−	2.4/20	Ground chicken
	Nested PCR	−	−	−	−	−	−	−	+	+	−	−		
	Real-time PCR	−	−	−	−	−	−	+	+	+	−	−		
	LAMP	−	−	−	−	−	+	+	+	+	−	−		

10	PCR	−	−	−	+	+	+	+	+	−	5.0/20	Ground pork						
	Nested PCR	−	−	+	+	+	+	+	+	−								
	Real-time PCR	−	−	−	+	+	+	+	+	−								
	LAMP	−	−	−	+	+	+	+	+	−								
11	PCR	−	−	−	+	+	+	+	+	−	4.0/20	Ground chicken						
	Nested PCR	−	−	+	+	+	+	+	+	−								
	Real-time PCR	−	−	−	+	−	+	+	+	−								
	LAMP	−	−	−	+	+	+	+	+	−								
12	PCR	−	−	−	+	−	−	+	+	−	8.5/20	Ground beef						
	Nested PCR	−	−	−	+	−	+	+	+	−								
	Real-time PCR	−	−	−	+	+	+	+	+	−								
	LAMP	−	−	−	+	+	+	+	+	−								

[a] For experiments 1 to 6, a 10^7-fold dilution of a culture of chromosomal *cpe* strain NCTC8239 grown overnight in TGY medium was used; for experiments 7 to 9, a 10^5-fold dilution of chromosomal *cpe* strain NCTC8239 grown overnight in Duncan-Strong medium was used after heat inactivation at 75°C for 20 min for the selection of spores; for experiments 10 to 12, a 10^7-fold dilution of plasmid *cpe* strain T16 grown overnight in TGY medium was used. NT, not tested.

[b] This specimen might contain a sufficient number of heat-killed *cpe*-positive cells for the detection of *cpe* with molecular assays.

used. Generally, current molecular methods designed for the detection of common pathogens in foods include an enrichment step that serves two purposes: to dilute out the interfering substances in the food matrix and to provide some assurance that the DNA sequences being detected are viable cells (12). In addition to these purposes, an enrichment culture step for the detection of *C. perfringens* strains induces the production of several enzymes that might reduce known inhibitors (e.g., collagen molecules) of PCR (5). An enrichment culture step also enables molecular assays to detect spores (16). In fact, the molecular assays used in this study efficiently and accurately detected low numbers of vegetative cells (fewer than 3 cells per g of sample) of chromosomal and plasmid *cpe* strains, as well as spores, using an enrichment procedure. Concerning the incubation time for the enrichment culture, a 24-h or overnight incubation may not be necessary when time is an issue; i.e., a culture incubated for 7 h appears to be sufficient for the detection of *cpe*-positive *C. perfringens* isolates in food.

The inclusion of enrichment steps in a detection protocol may still have limitations. For example, food samples stored in a refrigerator might be contaminated with only low numbers of bacteria. The use of enrichment steps in this scenario could give false-negative results if the bacteria were killed by low-temperature storage, since enrichment cultures detect only viable cells. In addition, these assays are qualitative in nature, and heavy contamination with *cpe*-negative *C. perfringens* bacteria in meat samples might affect the detection of *cpe*-positive strains. Despite these limitations, molecular assays will be useful for the detection of *cpe*-positive *C. perfringens* in foods when combined with our enrichment procedures and DNA preparation system. Therefore, these molecular assays might facilitate the identification of food poisoning outbreaks, putative reservoirs, and vector foods for *cpe*-positive *C. perfringens* strains. The detection of *cpe*-positive *C. perfringens* by molecular assays might provide a rather comprehensive understanding of the ecology of *cpe*-positive *C. perfringens* when combined with traditional, but more complicated, bacterial isolation procedures.

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