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Abilities of the mCP Agar Method and CRENAME Alpha Toxin-Specific Real-Time PCR Assay To Detect *Clostridium perfringens* Spores in Drinking Water

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We first determined the analytical specificity and ubiquity (i.e., the ability to detect all or most strains) of a *Clostridium perfringens*-specific real-time PCR (rtPCR) assay based on the *cpa* gene (*cpa* rtPCR) by using a bacterial strain panel composed of *C. perfringens* and non-*C. perfringens Clostridium* strains. All non-*C. perfringens Clostridium* strains tested negative, whereas all *C. perfringens* strains tested positive with the *cpa* rtPCR, for an analytical specificity and ubiquity of 100%. The *cpa* rtPCR assay was then used to confirm the identity of 116 putative *C. perfringens* isolates recovered after filtration of water samples and culture on mCP agar. Colonies presenting discordant results between the phenotype on mCP agar and *cpa* rtPCR were identified by sequencing the 16S rRNA and *cpa* genes. Four mCP$^-$/rtPCR$^+$ colonies were identified as *C. perfringens*, whereas 3 mCP$^+$/rtPCR$^-$ colonies were identified as non-*C. perfringens*. The *cpa* rtPCR was negative with all 51 non-*C. perfringens* strains and positive with 64 of 65 *C. perfringens* strains. Finally, we compared mCP agar and a CRENAME (*c*oncentration and *r*ecovery of microbial particles, *e*xtraction of *n*ucleic *a*cids, and *m*olecular *e*nrichment) procedure plus *cpa* rtPCR (CRENAME + *cpa* rtPCR) for their abilities to detect *C. perfringens* spores in drinking water. CRENAME + *cpa* rtPCR detected as few as one *C. perfringens* CFU per 100 ml of drinking water sample in less than 5 h, whereas mCP agar took at least 25 h to deliver results. CRENAME + *cpa* rtPCR also allows the simultaneous and sensitive detection of *Escherichia coli* and *C. perfringens* from the same potable water sample. In itself, it could be used to assess the public health risk posed by drinking water potentially contaminated with pathogens more resistant to disinfection.

The presence of *Escherichia coli* in drinking water indicates a recent fecal contamination, as well as a risk of waterborne gastrointestinal diseases (1). Under some circumstances, however, it has been shown that this microorganism inadequately indicates the presence of viruses and protozoan parasites of human health significance (2). For example, while chlorine in water rapidly inactivates *E. coli*, it leaves most disinfection-resistant pathogens almost unaffected for several hours (3). According to Payment and Franco (3), *Clostridium perfringens* is a suitable indicator of human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts in finished water and can also be used in the assessment of water treatment processes due to the resistance of *Clostridium* spores to chlorine. Furthermore, the presence of *C. perfringens* in water is also associated with fecal contamination, and it has been evaluated and utilized as an alternative indicator of fecal pollution (4–14). Thus, a simple and reliable culture-based method to isolate and enumerate *C. perfringens*, the membrane filtration method on mCP agar, has been elaborated and evaluated to monitor the presence of *C. perfringens* in water (15–20). In the European Union, the mCP agar method is the reference method used to assess the presence of *C. perfringens* in water intended for human consumption (21).

The simultaneous detection of *E. coli* and *C. perfringens* in drinking water could provide a better estimation of the public health risk, since both microorganisms indicate the presence of bacteria and more environmentally resistant or disinfection-resistant pathogens (22, 23). However, this cannot be accomplished by current culture-based techniques, and two independent tests would be required to assess the presence of these indicator microorganisms.

The application of rapid molecular testing to the microbiological quality of water is hampered by the scarcity of simple and robust solutions for concentrating and recovering very low numbers of microbial particles present in a relatively large water sample. In this study, we have determined the analytical specificity and ubiquity (i.e., the ability to detect all or most strains) of a *Clostridium perfringens*-specific real-time PCR (rtPCR) assay based on the *cpa* gene (encoding *C. perfringens* alpha-toxin) (*cpa* rtPCR) (24–26). We then used it to rapidly confirm the identity of putative *C. perfringens* isolates recovered after filtration and culture on mCP agar. Colonies presenting discordant results between the phenotype on mCP agar and *cpa* rtPCR were identified by sequencing the 16S rRNA and *cpa* genes. Finally, we compared mCP agar and

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a CRENAME (*c*oncentration and *r*ecovery of microbial particles, *e*xtraction of *n*ucleic *a*cids, and *m*olecular *e*nrichment) procedure plus *cpa* rtPCR (CRENAME + *cpa* rtPCR) for their abilities to detect *C. perfringens* spores in drinking water (27, 28).

MATERIALS AND METHODS

Analytical performance of *C. perfringens cpa* rtPCR assay. The analytical specificity of the *C. perfringens*-specific (*cpa*-based) rtPCR assay was determined by testing genomic DNAs isolated from 54 clinical and environmental non-*C. perfringens Clostridium* species and other bacterial species phylogenetically related to *C. perfringens* (Table 1). The ubiquity of the *cpa* rtPCR assay (i.e., the ability to detect all or most *C. perfringens* strains) was assessed against 37 *C. perfringens* strains of environmental and nonenvironmental origins. Environmental *C. perfringens* isolates ($n = 33$), recovered from different water sources by the culture-based method on mCP agar, were obtained from the Centre d'Expertise en Analyze Environnementale du Québec (CEAEQ) (Québec City, Québec, Canada). Nonenvironmental *C. perfringens* isolates ($n = 4$) were obtained from the CHU de Québec (Québec City, Canada) ($n = 2$) and the Culture Collection, University of Gothenburg (CCUG) (Gothenburg, Sweden) ($n = 2$). The identification of these isolates was confirmed by 16S rRNA gene nucleotide sequencing analysis.

Phenotypic and molecular characterization of *C. perfringens* isolates and phylogenetic analyses. CEAEQ provided *C. perfringens*-positive mCP agar plates resulting from the filtration of surface and river water samples (Tables 2 and 3). Only plates with well-isolated colonies were selected, and all colonies were recovered for further analysis; 147 colonies were initially found on mCP agar plates, but 31 did not grow after isolation (Fig. 1). The identities of the remaining 116 colonies isolated on mCP agar were confirmed using the *cpa* rtPCR assay, and the identifications of environmental strains presenting discordant results between culture and *cpa* rtPCR were confirmed by nucleotide sequencing of 16S rRNA and *cpa* genes using amplification and/or sequencing primers listed in Table 4 and experimental conditions described by Isabel et al. (29). Phylogenetic and molecular analyses of sequence contigs were conducted using MEGA version 5 (30). Phylogenetic trees were calculated by using the neighbor-joining method with the maximum composite likelihood substitution model. The topological accuracy of the tree was evaluated using 500 bootstrap replicates.

Components and parameters of the real-time PCR test. (i) Real-time PCR primers and probes. The nucleotide sequences of rtPCR primers and probes used in this study to detect *C. perfringens* DNA and spores, as well as *E. coli* cells and *Bacillus atrophaeus* subsp. *globigii* spores used as a process control, are shown in Table 4. Oligonucleotide primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

(ii) Real-time PCR assay. One microliter of bacterial suspension or of whole-genome amplification (WGA)-amplified products was transferred directly to a 24-μl rtPCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.4 μM primers, 0.2 μM probe, 200 μM each deoxyribonucleoside triphosphate (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, Québec, Canada), 3.3 μg/μl of bovine serum albumin (BSA) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), 0.025 enzyme unit of *Taq* DNA polymerase (Promega, Madison, WI, USA), and TaqStart antibody (Clontech Laboratories, Mountain View, CA, USA). For each experiment, 1 μl of sterile water was added to the rtPCR mixture as a negative control. The *C. perfringens* and *B. atrophaeus* subsp. *globigii* rtPCR mixtures were subjected to thermal cycling in a Rotor Gene 3000 (Corbett Life Science, Sidney, Australia) under the following conditions: 3 min at 95°C and then 45 cycles (35 cycles for specificity and ubiquity analysis) consisting of a denaturation step of 15 s at 95°C and an annealing step of 60 s at 60°C. The *E. coli*/*Shigella* rtPCR mixture was subjected to thermal cycling for 1 min at 95°C and then to 35 cycles of 2 s at 95°C, 10 s at 58°C, and 20 s at 72°C.

TABLE 1 Bacterial panel ($n = 54$ strains) for determining the analytical specificity of the *cpa* rtPCR assay[a]

Bacterial species	Strain	Origin
Anaerococcus lactolyticus	ATCC 51172	Human
Anaerococcus prevotii	ATCC 9321	Human
Anaerococcus tetradius	ATCC 35098	Human
Butyrivibrio fibrisolens	ATCC 19171	Animal
Clostridium acetobutyricum	CCRI-16781	Plant
Clostridium baratii	CCRI-19299	Water
Clostridium beijerinckii	ATCC 8260	NA
Clostridium biofermentans	ATCC 638	NA
Clostridium bolteae	CCRI-16220	NA
Clostridium botulinum	CCRI-25	NA
Clostridium cavendishii	CCRI-19303	Water
Clostridium difficile	ATCC 9689	NA
Clostridium frigidicarnis	CCRI-19297	Water
Clostridium frigidicarnis	CCRI-19301	Water
Clostridium hathewayi	CCRI-16222	NA
Clostridium histolyticum	ATCC 19401	Human
Clostridium innocuum	ATCC 14501	Human
Clostridium novyi	ATCC 19402	NA
Clostridium ramosum	ATCC 25582	NA
Clostridium ruminantium	CCRI-19300	Water
Clostridium saccharoperbutylacetonicum	CCRI-19321	Water
Clostridium septicum	ATCC 12464	NA
Clostridium sphenoides	ATCC 19403	NA
Clostridium sporogenes	CCRI-11132	NA
Clostridium symbosium	ATCC 14940	NA
Clostridium tertium	ATCC 14573	NA
Clostridium tetani	ATCC 19406	NA
Clostridium xylanolyticum	CCRI-16727	Plant
Eubacterium brachy	ATCC 33089	Human
Eubacterium nodatum	ATCC 33099	Human
Fusobacterium mortiferum	ATCC 25557	NA
Fusobacterium neosphorum	ATCC 25286	NA
Fusobacterium gonidiaformans	ATCC 25563	NA
Fusobacterium periodonticum	ATCC 33693	Human
Fusobacterium russi	ATCC 25533	Animal
Fusobacterium ulcerans	CCRI-15825	Human
Fusobacterium varium	ATCC 8501	NA
Helcococcus kunzii	ATCC 51366	Human
Lactobacillus acidophilus	ATCC 4356	Human
Lactobacillus crispatus	ATCC 33820	NA
Lactobacillus gasseri	ATCC 33323	NA
Lactobacillus johnsonii	ATCC 33200	Human
Lactobacillus reuteri	ATCC 23272	Human
Leptotrichia buccalis	ATCC 14201	Human
Peptococcus niger	ATCC 27731	Human
Peptoniphilus asaccharolyticus	CCRI-162	Human
Peptoniphilus indolicus	ATCC 29427	Animal
Peptoniphilus lacrimalis	ATCC 51171	Human
Peptostreptococcus anaerobius	CCRI-16219	NA
Ruminococcus bromii	ATCC 27255	Human
Ruminococcus gnavus	CCRI-16371	Human
Ruminococcus productus	ATCC 27340	Human
Streptococcus mitis	ATCC 49456	NA
Tissierella praeacuta	ATCC 25539	NA

[a] None of these strains tested positive with the *cpa* rtPCR assay. ATCC, American Type Culture Collection; CCRI, culture collection of Centre de Recherche en Infectiologie de l'Université Laval; NA, not available.

Process control. A process control consisting of approximately 60 spores/100 ml of *B. atrophaeus* subsp. *globigii* CCRI-9827 (equivalent to strain NRS1221 A) was added to all water samples prior to filtration. Spores were prepared as described by Picard et al. (31). The detection of *B.*

TABLE 2 Phenotypes and *cpa* rtPCR profiles of putative *C. perfringens* isolates found on mCP agar from surface water samples[a]

Sample	Reference strain	Phenotype on mCP agar	*C. perfringens cpa* rtPCR result	16S rRNA gene-based identification
1	CCRI-19605	Pink	+	NA
	CCRI-19606	Pink	+	NA
	DNG	Colorless	ND	NA
	CCRI-19608	Pink	+	NA
	CCRI-19609	Pink	−	*Clostridium baratii*
	CCRI-19610	Pink	+	NA
	CCRI-19607	Colorless	−	NA
	DNG	Colorless	ND	NA
2	CCRI-19611	Pink	+	NA
	CCRI-19612	Pink	+	NA
	CCRI-19613	Pink	+	NA
	CCRI-19614	Pink	+	NA
	CCRI-19615	Pink	+	NA
	CCRI-19710	Colorless	−	NA
	DNG	Colorless	ND	NA
	CCRI-19711	Colorless	−	NA
	CCRI-19616	Colorless		NA
	CCRI-19617	Colorless	+	*C. perfringens*
	CCRI-19618	Pink	+	NA
3	CCRI-19619	Pink	+	NA
	CCRI-19620	Pink	+	NA
	CCRI-19654	Pink	+	NA
	CCRI-19621	Pink	+	NA
	CCRI-19622	Pink	−	*Clostridium sardiniense*
	CCRI-19623	Pink	+	NA
	DNG	Colorless	ND	NA
	CCRI-19655	Colorless	−	NA
	CCRI-19656	Colorless	−	NA
4	CCRI-19624	Pink	+	NA
	CCRI-19625	Pink	+	NA
	CCRI-19626	Pink	+	NA
	CCRI-19627	Pink	+	NA
	CCRI-19628	Pink	+	NA
	CCRI-19630	Pink	+	NA
	CCRI-19631	Pink	+	NA
	CCRI-19632	Pink	+	NA
	CCRI-19633	Pink	+	NA
	CCRI-19634	Pink	+	NA
	CCRI-19635	Pink	+	NA
	CCRI-19636	Pink	+	NA
	CCRI-19637	Pink	+	NA
	CCRI-19638	Pink	+	NA
	DNG	Pink	ND	NA
	CCRI-19639	Pink	+	NA
	CCRI-19640	Pink	+	NA
	CCRI-19657	Pink	+	NA
	CCRI-19641	Pink	+	NA
	CCRI-19658	Pink	+	NA
	CCRI-19665	Pink	+	NA
	CCRI-19642	Pink	+	NA
	CCRI-19643	Pink	+	NA
	DNG	Pink	ND	NA
	DNG	Pink	ND	NA
	CCRI-19666	Pink	+	NA
	CCRI-19660	Pink	+	NA
	CCRI-19644	Pink	+	NA
	CCRI-19645	Pink	+	NA
	CCRI-19646	Pink	+	NA
	CCRI-19667	Pink	+	NA
	CCRI-19647	Pink	+	NA
	CCRI-19662	Pink	+	NA
	CCRI-19720	Pink	+	NA
	CCRI-19650	Pink	+	NA
	CCRI-19723	Pink	+	NA
	CCRI-19651	Pink	+	NA
	CCRI-19652	Colorless	−	NA
	DNG	Colorless	ND	NA
	CCRI-19653	Colorless	+	*C. perfringens*

[a] The identification of discordant strains ($mCP^+/rtPCR^-$ or $mCP^-/rtPCR^+$) was done by 16S rRNA gene sequence analysis. CCRI, culture collection of Centre de Recherche en Infectiologie de l'Université Laval; NA, not applicable; DNG, did not grow upon isolation attempt; ND, not done.

atrophaeus subsp. *globigii* serves to monitor for the integrity of the sample preparation method and nucleic acid extraction and for the absence of inhibition of the molecular enrichment process by WGA, as well as target amplification by rtPCR.

Determination of the analytical limit of detection (LOD) of CRENAME + *cpa* rtPCR method. (i) Preparation of *Clostridium perfringens* spores. A *C. perfringens* cell suspension was prepared by inoculating liquid thioglycolate medium with *C. perfringens* CCRI-16107 and incubating the culture under aerobic conditions for 24 h at 37°C, as described by Tórtora (32). In our hands, *C. perfringens* CCRI-16107 had better spore generation yields than the type strain (ATCC 13124^T). Sporulating cultures of *C. perfringens* were prepared by inoculating 0.5 ml of an overnight culture into 10 ml of Duncan-Strong sporulation medium at pH 8.5 (33, 34) and by incubating the culture under aerobic conditions for 24 h at 37°C to induce the formation of spores. Finally, spore preparations were heated at 75°C for 20 min, rapidly cooled on ice, concentrated by low-speed centrifugation, purified by repeated washing with sterile distilled water until they were >99% free of sporulating cells, cell debris, and germinated spores (based on microscopic analysis), and stored at 4°C (35, 36).

(ii) Preparation of water samples spiked with *C. perfringens* spores. To determine the analytical limit of detection (LOD) of the mCP agar and CRENAME + *cpa* rtPCR methods, spiked samples were made by inoculating water with predetermined numbers of *C. perfringens* spores, on the basis of a count obtained with a Petroff-Hauser chamber, to produce suspensions having approximately 300, 150, 75, 25, 12, 5, 2, 1, 0.5, and 0.1 CFU/100 ml (Table 5). The drinking water used for the concentration and recovery method experiments was ozonated spring water from Sainte-Marie-de-Blandford (Comté de Bécancour, Québec, Canada) (total dissolved mineral salt content, 60 ppm [40 mg/liter HCO_3^-, 11 mg/liter Ca^{2+}, 1 mg/liter Cl^-, 0.1 mg/liter F^-, 2.7 mg/liter Mg^{2+}, 1 mg/liter K^+, 3 mg/liter Na^+, and 8 mg/liter SO_4^-]). The bacterial spore count was verified by filtering 100 ml of each spiked water sample through a GN-6 membrane filter (47-mm diameter, 0.45-μm pore size; Pall Corporation, Mississauga, Ontario, Canada) with a standard platform manifold (Millipore Corporation, Billerica, MA, USA). The filter was then incubated on sheep blood agar plates for 24 ± 2 h at 35.0 ± 0.5°C in an anaerobic chamber prior to the determination of colony counts. Spiked samples were tested on mCP agar and by CRENAME + *cpa* rtPCR.

Membrane filtration. Membrane filtration is the water sample concentration step used for both classical (mCP agar) and molecular

TABLE 3 Phenotypes and *cpa* rtPCR profiles of putative *C. perfringens* isolates found on mCP agar from river water samples[a]

Sample	Reference strain	Phenotype on mCP agar	*C. perfringens cpa* rtPCR result	16S rRNA gene-based identification
5	CCRI-20251	Pink	−	*C. perfringens*
	CCRI-20253	Pink	+	NA
	CCRI-20304	Pink	+	NA
	CCRI-20305	Pink	−	*C. sardiniense*
	CCRI-20306	Pink	+	NA
	CCRI-20307	Colorless	−	NA
	CCRI-20308	Colorless	+	*C. perfringens*
	CCRI-20309	Colorless	−	NA
	CCRI-20310	Colorless	+	*C. perfringens*
	CCRI-20328	Colorless	−	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	CCRI-20313	Pink	+	NA
	CCRI-20314	Pink	+	NA
	CCRI-20315	Pink	+	NA
	CCRI-20316	Pink	+	NA
	CCRI-20317	Pink	+	NA
	CCRI-20318	Pink	+	NA
	DNG	Pink	ND	NA
	DNG	Pink	ND	NA
	DNG	Colorless	ND	NA
	CCRI-20339	Colorless	−	NA
	DNG	Colorless	ND	NA
	CCRI-20324	Colorless	−	NA
	CCRI-20325	Colorless	−	NA
	CCRI-20326	Colorless	−	NA
	CCRI-20327	Colorless	−	NA
	DNG	Colorless	ND	NA
	CCRI-20329	Colorless	−	NA
	CCRI-20330	Colorless	−	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	CCRI-20335	Colorless	−	NA
	CCRI-20336	Colorless	−	NA
	CCRI-20337	Colorless	−	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	CCRI-20329	Colorless	−	NA
	CCRI-20342	Colorless	−	NA
	CCRI-20343	Colorless	−	NA
	CCRI-20344	Colorless	−	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	CCRI-20349	Colorless	−	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	CCRI-20353	Colorless	−	NA
	DNG	Colorless	ND	NA
	DNG	Colorless	ND	NA
	CCRI-20356	Colorless	−	NA
	DNG	Colorless	ND	NA
	CCRI-20358	Pink	+	NA
	CCRI-20359	Pink	+	NA
	CCRI-20360	Colorless	−	NA
	CCRI-20361	Colorless	−	NA
	CCRI-20362	Colorless	−	NA
	CCRI-20363	Colorless	−	NA
	CCRI-20364	Colorless	−	NA
	CCRI-20365	Colorless	−	NA
	CCRI-20366	Colorless	−	NA
	CCRI-20367	Colorless	−	NA
	CCRI-20368	Colorless	−	NA
	CCRI-20369	Colorless	−	NA
	CCRI-20370	Colorless	−	NA
	CCRI-20371	Colorless	−	NA
	CCRI-20372	Colorless	−	NA
	CCRI-20373	Colorless	−	NA
	CCRI-20374	Colorless	−	NA
	CCRI-20375	Colorless	−	NA
	CCRI-20376	Colorless	−	NA
	CCRI-20377	Colorless	−	NA
	CCRI-20378	Colorless	−	NA
	CCRI-20379	Colorless	−	NA
	CCRI-20380	Colorless	−	NA

[a] The identification of discordant strains ($mCP^+/rtPCR^-$ or $mCP^-/rtPCR^+$) was done by 16S rRNA gene sequence analysis. CCRI, culture collection of Centre de Recherche en Infectiologie de l'Université Laval; NA, not applicable; DNG, did not grow upon isolation attempt; ND, not done.

(CRENAME + *cpa* rtPCR) microbiology methods. For the detection limit determination study, for example, each 400-ml sample spiked with *C. perfringens* spores was subdivided into four subsamples of 100 ml separately filtered on GN-6 membranes with a standard platform manifold. Tests to confirm the sterility of filter membranes and buffer used for rinsing the filtration apparatus were also performed. One filter was used for mCP agar method, while 3 filters (triplicates) were processed by the CRENAME water sample preparation procedure before subjecting the WGA-amplified nucleic acid sample to specific detection of *C. perfringens* by rtPCR. Since DNA is nonspecifically amplified by WGA, real-time PCR is used for amplicon detection, not for quantification. It is well known that the closed-tube format of rtPCR may significantly minimize cross-contamination.

To perform the mCP agar method, a membrane filter is placed onto mCP solid medium and incubated under anaerobic conditions at 44.5 ± 0.2°C for 24 ± 2 h (37). After incubation, filtration membranes containing straw yellow-colored colonies are transferred to pads saturated with ammonium hydroxide in a chemical fume hood. Following a 15-s exposure, colonies that turn dark pink to magenta are counted as *C. perfringens*. Quality control for each batch of mCP agar was performed, and filter, buffer, and rinse water blanks were included as sterility controls. For performing CRENAME on concentrated water samples, GN-6 filters were aseptically transferred to 3 individual 15-ml polypropylene tubes (Sarstedt, Newton, NC, USA) and processed as described by Maheux et al. (27, 28).

Simultaneous detection of *C. perfringens* and *E. coli* in drinking water. To determine if CRENAME coupled to rtPCR can simultaneously detect *C. perfringens* spores and *E. coli* cells from the same drinking water sample, 30 different well water samples were collected in the Québec City area and blindly spiked by the CEAEQ with diluted sewage (10^{-1} in phosphate-buffered saline [PBS]) to produce 10 well water samples containing approximately 40 CFU/100 ml and 10 well water samples containing approximately 80 CFU/100 ml of *C. perfringens*. *E. coli* counts exceeded 200 CFU/100 ml. Ten well water samples were not spiked. These samples were filtered on GN-6 membranes and prepared by CRENAME before aliquots

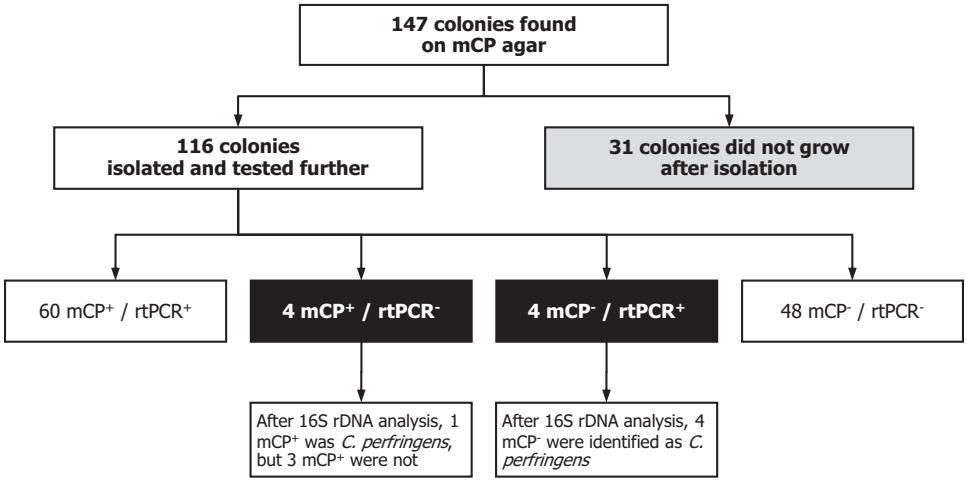

FIG 1 Schematic overview of the comparative study. The identities of discordant strains ($mCP^+/rtPCR^-$ and $mCP^-/rtPCR^+$) were determined by 16S rRNA gene (rDNA) nucleotide sequence analysis.

of WGA-amplified DNA were used as input in independent rtPCRs for the detection of *cpa* and *tuf* gene targets.

Statistical analysis. Statistical analysis by logistic regression was done using the JMP v8.0 software (38).

RESULTS AND DISCUSSION

Analytical performance of the *C. perfringens cpa* rtPCR assay. The *C. perfringens*-specific rtPCR assay used in this study, targeting the *C. perfringens* alpha-toxin gene (*cpa*) (25), was previously validated by Grant et al. (39) against a panel composed of 253 *C. perfringens* isolates, 19 isolates selected from 14 other *Clostridium* species, and *Listeria monocytogenes* and *Bacillus* sp. isolates. The *cpa* gene was (i) detected by rtPCR in all 253 *C. perfringens* cultures that had been identified as *C. perfringens* by standard microbiological tests and (ii) not amplified from the 14 *Clostridium* species other than *C. perfringens*; that work reported specificity and sensitivity rates of 100% for the *cpa* rtPCR assay.

In our study, the analytical specificity and the ubiquity of the *cpa* rtPCR assay were verified using a bacterial panel of clinical and environmental strains consisting of 54 non-*C. perfringens* species of the *Clostridium* genus and 37 *C. perfringens* strains (Table 1). None of the 54 non-*C. perfringens* strains tested positive with the *cpa* rtPCR assay, for an analytical specificity of 100%, whereas the *cpa* rtPCR assay tested positive with 37 of 37 *C. perfringens* strains, for an ubiquity of 100%.

Phenotypic and molecular characterization of *C. perfringens* isolates. Of the 116 colonies recovered from mCP agar, 64 were positive (yellow initially but pink after exposure to ammonium

TABLE 4 Primers and probes used in this study

rtPCR assay (target)	Primers and probe	Sequence (5′ → 3′)[a]	Reference
cpa (*C. perfringens*)	CpaTQ1	CTAGATATGAATGGAAGAGGAAACTA	25
	CpaTQ2	TTAGCAGGATGATATGGAGTATCTATATCTC	
	CpaTQP	FAM-AAACAAGCTACATTCTATCTTGGAGAGGCTATGCACTATT-BHQ-1	
tuf (*E. coli*/*Shigella*)	TEcol553	TGGGAAGCGAAAATCCTG	28
	TEcol754	CAGTACAGGTAGACTTCTG	
	TEcol573-T1-B1	TET-AACTGGCTGGCTTCCTGG-BHQ-1	
atpD (*B. atrophaeus* subsp. *globigii*)	ABgl158	CACTTCATTTAGGCGACGATACT	31
	ABgl345a	TTGTCTGTGAATCGGATCTTTCTC	
	ABgl-T1-A1	FAM-CGTCCCAATGTTACATTACCAA-CCGGCACT-(BHQ-1)-GAAATAGG	
cpa (*C. perfringens*)	cpaCperseq298	CCTGATACARATMATAATTTYWCAAAGG	This study
	cpaCperseq961	CATAAARTCATTTCCWGGGTTRTC	
Small-subunit (16S) rRNA gene (bacteria)	SSU27	AGAGTTTGATCMTGGCTCAG	41
	SSU1492	TACGGYTACCTTGTTACGACTT	
	SSU536[b]	GTGCCAGCMGCCGCGGTAATAC	
	SSU685[b]	TCTACGCATTTCACYGCTAC	
	SSU926[b]	AAACTYAAAKGAATTGACGG	

[a] FAM, 6-carboxyfluorescein (fluorescent reporter dye); BHQ-1, Black Hole Quencher-1 (fluorescence quencher dye); TET, tetrachlorofluorescein (fluorescent reporter dye); R, A/G; M, A/C; W, A/T; Y, C/T; K, G/T.
[b] Internal sequencing primer.

TABLE 5 Determination of the analytical limit of detection of *C. perfringens* on mCP agar and by the CRENAME + *cpa* rtPCR procedure

C. perfringens count (CFU/100 ml)		WGA rtPCR (presence/absence for each replicate)		
Estimated	On mCP agar			
300	329	+	+	+
150	154	+	+	+
75	74	+	+	+
25	24	+	+	+
12	12	+	+	+
5	4	+	+	+
2	2	+	+	−
1	1	+	−	−
0.5	0	+	−	−
0.1	0	−	−	−
Negative control	0	−	−	−

hydroxide) and 52 were negative (colorless) (Tables 2 and 3 and Fig. 1). Both mCP agar and the *cpa* rtPCR assay were negative for 48 of 52 colorless colonies and positive for 60 of 64 pink colonies. After the identification of the colonies presenting discordant results between culture and molecular assay by 16S rRNA gene sequencing, 3 pink colonies were identified as *Clostridium baratii*, whereas 4 colorless colonies were identified as *C. perfringens* (Fig. 1 and 2). Thus, mCP agar was positive with 61 of 65 *C. perfringens* strains as well as 3 of 51 non-*C. perfringens* strains, and with these 116 colonies on mCP agar, the *cpa* rtPCR assay was negative with all 51 non-*C. perfringens* strains and positive with 64 of 65 *C. perfringens* strains.

The nucleotide sequence of the 16S rRNA gene of the only *C. perfringens* strain (CCRI-20251) that was not detected by the *cpa* rtPCR assay was 99.4% identical to that of the type strain (Fig. 2). No phenotypic test was performed on this discordant strain. However, we sequenced the *cpa* gene, and using a 635-bp region, we found that this gene is 96.4% identical to that of *C. perfringens* SWCP, a strain isolated from an avian source (40). The *cpa* gene of *C. perfringens* SWCP is 85.5% identical to its homolog of the *C. perfringens* type strain (Fig. 3). When we aligned the primers and probe designed by Amar et al. (25) to the *cpa* sequence of strain CCRI-20251, 6 mismatches were observed with forward and reverse primers. Mismatches were also observed with the internal TaqMan detection probe, thereby providing an explanation for the negative *cpa* rtPCR result with *C. perfringens* CCRI-20251. To date, it has been difficult to establish the impact of this strain on

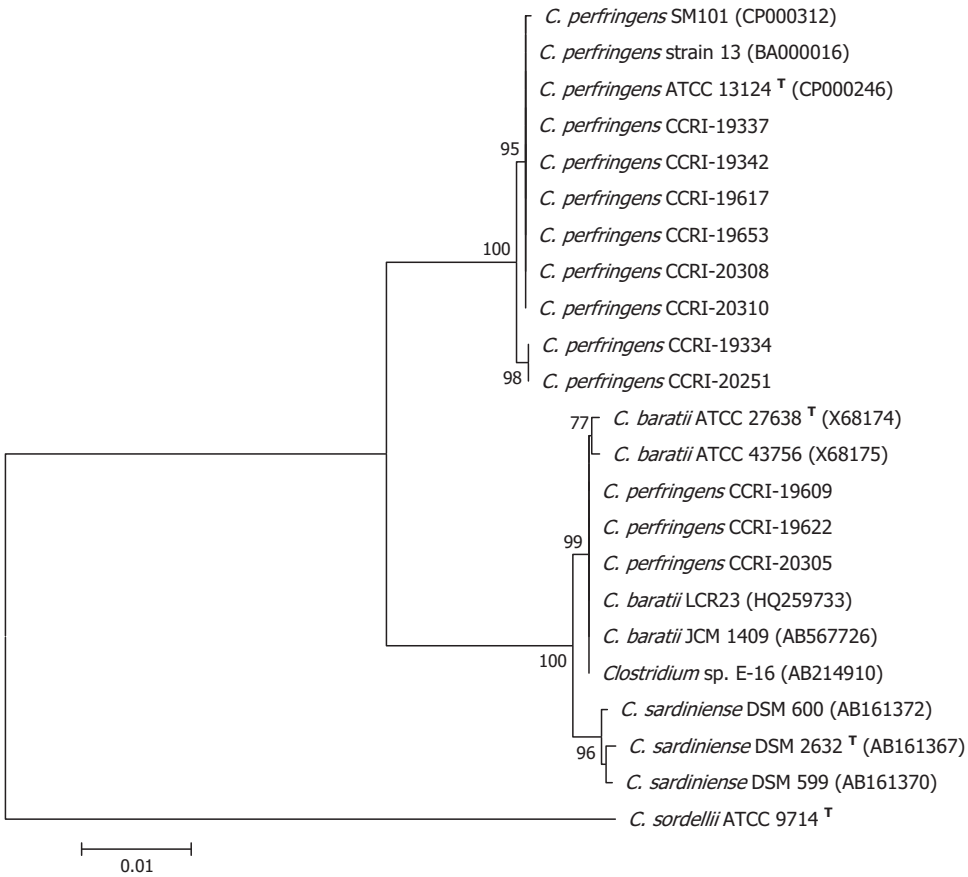

FIG 2 Phylogenetic tree based on a 1,384-bp portion of the 16S rRNA genes of discordant or atypical *C. perfringens* strains isolated during this study. Strains CCRI-19609, CCRI-19622, CCRI-20305, CCRI-19334, and CCRI-20251 were $mCP^+/rtPCR^-$. Strains CCRI-19617, CCRI-19653, CCRI-20308, and CCRI-20310 were $mCP^-/rtPCR^+$. For clarity, the original species identification of discordant strains has been conserved in the phylogenetic tree. The phylogenetic analysis was performed with the neighbor-joining method, and evolutionary distances were computed using the maximum composite likelihood model of the MEGA version 5.01 software. The topological accuracy of the tree was evaluated using 500 bootstrap replicates. Bootstrap values lower than 70% are not shown. GenBank accession numbers are given in parentheses.

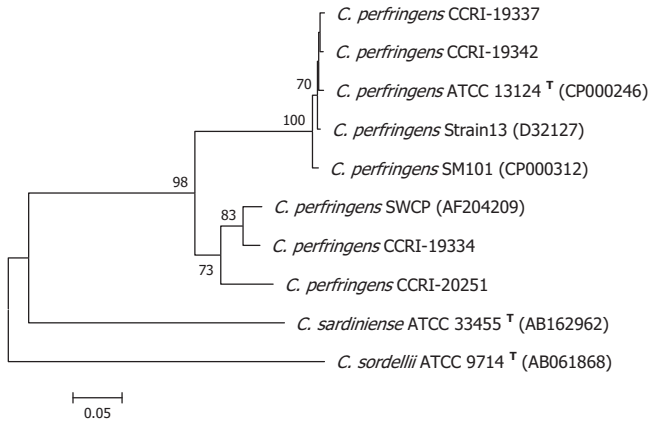

FIG 3 Phylogenetic tree based on a 632-bp portion of the *cpa* genes from *C. perfringens* strains. Atypical strain CCRI-20251 was identified as *C. perfringens* on mCP agar and by 16S rRNA gene analysis but was not detected by the *cpa* rtPCR assay. *C. sardiniensis* and *C. sordellii* alpha-toxin genes are represented as outgroups. The phylogenetic analysis was performed with the neighbor-joining method, and evolutionary distances were computed using the maximum composite likelihood model of the MEGA version 5.01 software. The topological accuracy of the tree was evaluated using 500 bootstrap replicates. Bootstrap values lower than 70% are not shown. GenBank accession numbers are given in parentheses.

the sensitivity of the *cpa* rtPCR assay, since its prevalence in water remains unknown.

Determination of the analytical LOD of the CRENAME + *cpa* rtPCR method. The limit of detection (LOD) of the CRENAME-*cpa* rtPCR was evaluated by testing 100-ml well water samples spiked with different concentrations of *C. perfringens* CCRI-16107 spores. *C. perfringens* spores were always detected down to concentrations as low as 4 spores per 100 ml. Compared to colony count on mCP agar plates, the complete molecular microbiology method was able to detect as few as 1 spore per 100 ml (Table 5), which corresponds to the microbiological criterion for this indicator microorganism in Europe (21). No signal was observed with negative controls, and the process control tested positive for every sample. The LOD at 95% confidence for *C. perfringens* spores, as determined by logistic regression, was estimated to be 3.57 spores/100 ml.

Simultaneous detection of *C. perfringens* and *E. coli* in drinking water. Enteric viruses, *Cryptosporidium parvum* oocysts, and *Giardia intestinalis* cysts can survive for several months in water. On one hand, the high persistence of *Cryptosporidium* and *Giardia* (oo)cysts limits the usefulness of the fecal contamination indicator *E. coli*, traditionally used to determine the microbial safety of water, while on the other hand, the rapid die-off of *E. coli* and fecal enterococci makes these parameters less suitable as indicators of the presence of waterborne pathogens such as enteric viruses, *C. parvum* oocysts, and *G. intestinalis* cysts (3, 14, 23). Since *C. perfringens* (spores) can survive in water for periods of time similar to those for the latter pathogens, their detection could provide a better indication of their presence in water than current fecal contamination indicators and thus a more adequate estimation of the public health risk (3, 14), but this issue is still controversial (13).

In consideration of these facts and taking into account that the determination of a single indicator is unlikely to be strategically appropriate in all instances, the recognition of *C. perfringens* as an indicator microorganism to predict the presence of waterborne pathogens that are more persistent and disinfection resistant than *E. coli* should stimulate the development and implementation of methods enabling multiparametric detection of fecal contamination indicators and microbial pathogens from a single water sample, to avoid equivocal results due to the stochastic distribution of microbial particles at very low concentrations (~1 CFU/100 ml). Multiple analysis is not compatible with current classical microbiology approaches for testing drinking water, since a 100-ml sample cannot be tested on many different media, but we believe that molecular microbiology tests, in the form of multiplex rtPCRs or microarrays, could be tailored to detect many indicators and pathogens in a time- and cost-effective manner.

The ability of the CRENAME + rtPCR to simultaneously detect *C. perfringens cpa* and *E. coli tuf* genes in drinking water was verified by testing 30 drinking well water samples blindly spiked by CEAEQ with diluted sewage to produce suspensions having approximately 80, 40, and 0 CFU/100 ml of *C. perfringens* (10 samples with each concentration; *E. coli* counts exceeded 200 CFU/100 ml when sewage was added to drinking water samples). Before spiking, all well water samples tested negative for the presence of *C. perfringens* and *E. coli*, whereas 20 water samples tested positive after spiking with diluted sewage, corresponding to the 20 well water samples spiked by the CEAEQ. The process control, *B. atrophaeus* subsp. *globigii*, was detected in all instances, and the cycle thresholds (C_Ts) obtained with *C. perfringens*-positive samples were similar (data not shown) indicating that inhibitory substances present in well water were either (bio)chemically equivalent or not in sufficiently high concentrations to inhibit the WGA or rtPCR process.

Conclusions. As CRENAME provides a nucleic acid sample (WGA-amplified DNA) that can be interrogated repetitively, technologies such as multiplex rtPCR or microarrays could be used for the simultaneous detection of many fecal contamination indicators, including *C. perfringens*, and pathogens from the same water sample, thereby providing a novel, time-efficient solution to better assess the microbial quality of drinking water. As *C. perfringens* is recognized to provide an indication of more persistent and disinfection-resistant waterborne pathogens such as enteric viruses and protozoan (oo)cysts, a CRENAME multiplex rtPCR test detecting *E. coli* and *C. perfringens* could be developed to yield a better assessment of the public health risk posed by drinking water potentially contaminated with pathogens.

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