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Food Analytical Methods

ISSN 1936-9751
Volume 10
Number 5

Food Anal. Methods (2017)
10:1139-1147
DOI 10.1007/s12161-017-0821-6

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Quantitative Detection of *Clostridium perfringens* by Real-Time PCR in Raw Milk

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Received: 7 January 2017 / Accepted: 7 January 2017 / Published online: 29 January 2017
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Abstract *Clostridium perfringens* causes a broad spectrum of diseases in both humans and animals and is an important cause of foodborne illness. We developed and tested a real-time (Q-)PCR assay for the species-specific detection of *C. perfringens* that targets the phospholipase C (*plc*) gene and includes an internal amplification control (IAC), making it possible to identify false-negative results, which are common due to the high level of PCR inhibition by food compounds. The *CPplc*-IAC real-time PCR (RTi-PCR) assay was 100% selective, as shows with 36 *Clostridium* strains and 85 non-*Clostridium* strains, with an analytical sensitivity of 1 genome equivalent (GE) in 23% of the reactions and 10 GE in 100% of the reactions. The quantification was linear (R^2 = 0.9990) over a 7-log dynamic range, down to 10 GE, with a PCR efficiency E = 0.841. The applicability of this RTi-PCR assay was assessed in milk samples. The assay detected as few as 300 spores in 25 mL of artificially contaminated raw sheep milk with 78% probability and 30 spores in 25 mL with 50% probability. It also has accuracy of 83.03 to 151.18%, as shown by an evaluation of the correspondence between RTi-PCR assay results and the number of spores per milliliter determinated by standard plating. This RTi-PCR method was effective for the detection and quantification of *C. perfringens* in milk having an important applicability in the control of this pathogen in the dairy food industry.

Keywords *Clostridium perfringens* · RTi-PCR · *plc* gene · Milk

Electronic supplementary material The online version of this article (doi:10.1007/s12161-017-0821-6) contains supplementary material, which is available to authorized users.

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Introduction

Clostridium perfringens is a Gram-positive spore-forming anaerobic rod microorganism which can be found in an ample range of food-related environments as soil, water, milk, dust, or sewage, but also, it is commonly isolated as commensal bacteria from the intestinal tracts of animals and humans (Hatheway 1990). *C. perfringens* is considered as one of the main anaerobic bacterial pathogen for humans and domestic animals (Czeczulin et al. 1993). It is the most important cause of clostridial disease in domestic animals (Songer 2006a, b), where it can produce gas gangrene (clostridial myonecrosis) (Mainil 2006); clostridial abomasitis in domestic ruminants (Songer 2006a); enteritis and enterotoxaemia in bovine (Songer 2006b), small ruminants (lamb dysentery, struck, and pulpy kidney disease; Bourtzi-Hatzopoulou et al. 2006), pigs (Taylor 2006), and poultry (Engström et al. 2006); and enterocolitis in horses (Baverud 2006). It also can produce enteritis and enterotoxaemia in humans, including food poisoning (Delmeé 2006). *C. perfringens* diseases are mainly related with the production of extracellular enzymes or toxins like alpha, beta, epsilon, iota, and beta2 toxins, with the

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exception of human food poisoning, which involves an enterotoxin. This clostridial species produces the highest number of toxins, although each bacterium only produces a subset of these. Depending on the ability to produce any of the four major toxins (alpha, beta, epsilon, and iota), *C. perfringens* strains are classified into five toxigenic types, from A to E (Petit et al. 1999).

The identification of *C. perfringens* from food or environmental samples is usually performed by conventional microbiological procedures (FDA 2001). They rely on the isolation of the microorganism on selective media in anaerobic conditions, and the presumptive anaerobe sulfite-reducing colonies are confirmed by biochemical tests. Consequently, the identification of this agent is a time-consuming procedure that does not meet the needs of the diagnostic laboratories especially in the diagnosis of food-borne outbreaks. Reliable, specific, and quick identification and enumeration methods are the goal in food safety and in clinical diagnosis of human and animal diseases (Wang et al. 1994; Rodríguez-Lázaro et al. 2007). Real-time PCR (RTi-PCR) is an alternative molecular technique that offers important advantages for the identification and quantification of bacterial agents directly from food and environmental samples and resolves some major disadvantages of the conventional PCR methods (Rodríguez-Lázaro et al. 2007). It allows a specific and rapid quantitative detection with an improved selectivity and reduces the risk of cross-contamination and the analysis time as no post-PCR processing of the samples is required. Previous studies have described RTi-PCR assays for the specific identification of *C. perfringens* in foods and stools in a shorter time than can be achieved by standard culture methods alone (Wise and Siragusa 2005; Dela Cruz et al. 2006; Fu et al. 2006; Skanseng et al. 2006; Albini et al. 2008; Gurjar et al. 2008), although their real implementation in routine food and clinical microbiology laboratories is questionable as most of them use multiplex PCR systems or the sequences selected do not cover completely the *C. perfringens* species and only detect specifically different toxin-producing strains.

The application of PCR for the direct detection of pathogens present in foods and stools has been limited by the complex composition of the biological matrices, which contain inhibitors for PCR amplification (Rodríguez-Lázaro and Hernández 2013; Rodríguez-Lázaro et al. 2005, 2006, 2013; Wise and Siragusa 2005). Milk is an important source of transmission of zoonotic agents such as *C. perfringens*, and its contamination can have several origins: fecal contamination and direct excretion from the udder, from the skin during milking, or from the environment, related with contaminated equipment or with sewage effluents. However, there is no currently available real-time PCR-based method for the detection of this agent in such food matrix. Consequently, the aim of this study was to develop a direct, rapid, and sensitive RTi-PCR method for unambiguously quantitative identification of *C. perfringens* in raw sheep milk without requiring a previous step of isolation on culture media. The real-time PCR method targeted the *plc* gene of *C. perfringens*, a single copy gene that is present in all toxin types of *C. perfringens* and that codifies the alpha toxin (CPA). The CPA is an important virulence factor that plays a key role in the pathogenicity of *C. perfringens* (Rood and Cole 1991; Petit et al. 1999). Consequently, it can detect the different *C. perfringens* irrespective of the toxin type. In addition, it includes an internal amplification control (IAC) for monitoring the PCR performance. *C. perfringens* is a ubiquitous bacterium found in almost all environments examined; consequently, it can be present in a wide range of foods of animal.

Materials and Methods

Bacterial Strains, Culture Media, and Growth Conditions Eighty *Clostridium* strains (including 53 *C. perfringens* isolates and 27 non-*perfringens Clostridium* isolates) and 85 non-*Clostridium* strains were used in this study (Supplementary Material, Tables 1, 2, and 3). All *Clostridium* strains were grown in Reinforced Clostridial Medium (RCM, Oxoid, Hampshire, UK) and incubated at 37 °C in Anaerobic Jars (Oxoid) with a 9–13% CO_2 atmosphere (AnaeroGen™ paper sachets, Oxoid). RCM plates were obtained adding 1.5% (*w*/*v*) of bacteriological agar (Scharlau Microbiology, Barcelona, Spain) to RCM broth. Non-*Clostridium* strains were grown following the recommendations of the Spanish Type Culture Collection (CECT), Valencia, Spain. All the strains were maintained at −80 °C in the suitable broth supplemented with 16% (*v*/*v*) glycerol. *C. perfringens* CECT 563 strain was used as a model *C. perfringens* strain in this study.

Bacterial Genomic DNA Isolation and Quantification Bacterial genomic DNA was extracted with a cetyltrimethylammonium bromide (CTAB)-based standard protocol (Rodríguez-Lázaro et al. 2006). Briefly, bacterial colonies were collected from a half Petri dish with a loop and were suspended in 1 mL of 1× phosphate-buffered saline (PBS) and centrifuged at 4000×*g* for 10 min. The bacterial pellet was resuspended in 567 μL 1× TE buffer and 3 μL 100 mg/mL lysozyme (Sigma-Aldrich Co., Saint Louis, USA) and incubated for 1 h at 37 °C. Then, 30 μL of 10% (*w*/*v*) sodium dodecyl sulfate (SDS) and 3 μL of 20 mg/mL Proteinase K (Sigma) were added, and the mixture was incubated for 1 h at 37 °C. Subsequently, 170 μL of 5 M NaCl, 80 μL of CTAB-NaCl solution (10% (*w*/*v*) in 0.7 M of NaCl), and 5 μL of 100 mg/mL Rnase A (Sigma) were added, and again it was incubated for 30 min at 65 °C. The mixture was cooled to room temperature and subjected to extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/

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isoamyl alcohol (24:1), followed by DNA precipitation with isopropanol and washing with 70% (*v*/*v*) ethanol (Sambroock and Russell 2001). DNA was resuspended in 100 μL of 10 mM Tris-HCl, pH 8.0. The quantity of the DNA obtained was determined spectrophotometrically using the ND-100 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The ratios of optical density at 260 nm to that at 280 nm and at 260 nm to that at 230 nm were calculated in order to determine the quality of the DNA samples.

Oligonucleotides The primers and fluorogenic probes that comprise the *CPplc-IAC* RTi-PCR system (*CPplcF*, *CPplcR*, *CPplcP*, and *CPIACP*) and the primers needed for the internal amplification control (IAC) construction (*CPIACF* and *CPIACR*) are listed in Table 1. They were designed using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA). All the primers were purchased from MWG-Biotech AG (Ebensburg, Germany) whereas *CPplcP* and *CPIACP* TaqMan Minor Groove Binding (MGB) probes were acquired from Applied Biosystems (Warrington, UK). The *CPplcP* and the *CPIACP* TaqMan MGB probes were labeled on the 5′ ends with the fluorescent 6-carboxyfluorescein (FAM) and the fluorescent VIC reporter dyes, respectively, and with non-fluorescent quencher dyes at the 3′ ends.

IAC Construction The internal amplification control is a chimeric DNA fragment of 118 bp that included a portion (70 bp) of the listeriolysin-positive regulatory (*prfA*) gene from *Listeria monocytogenes* CECT 911 strain which is flanked by the specific *CPplc* forward and reverse primers. The IAC did not display significant similarity to any DNA sequence deposited in public databases, with the exception of its target sequence in the *L. monocytogenes prfA* gene, as was determined by BLAST-N searches. The IAC amplicon, 118 bp in size, was longer than the 71-bp *plc*-specific amplicon, facilitating the differentiation of the two PCR products by gel electrophoresis. The IAC was generated by two rounds of PCR as previously described by Rodríguez-Lázaro et al. (2005) using the *CPIAC* and the *CPplc* forward and reverse primers, respectively. The IAC PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into the pCR® 2.1 TOPO® vector (Invitrogen, Carlsbad, CA) and transformed in One Shot® MAX Efficiency® DH5α™-T1® competent *E. coli* cells (Invitrogen) following the manufacturers' recommendations in all the protocols. Finally, the plasmidic DNA was purified with the Qiagen Plasmid purification Kit (Qiagen) and was quantified and diluted to the appropriate concentration in double-distilled water containing 0.1 mg/mL of bovine serum albumin (BSA) as blocking agent.

RTi-PCR PCRs were performed in a 20-μL final volume containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 100 nM of *CPplcF* primer, 300 nM of *CPplcR* primer, 200 nM of *CPplcP* TaqMan MGB probe, and 2 μL of the target DNA solution. The RTi-PCRs including the IAC also contained 50 nM of *CPIACP* TaqMan MGB probe and 100 genome equivalents of IAC. Real-time PCR amplifications were run in a 7500 Real-Time PCR System Platform (Applied Biosystems). The cycling parameters consisted of a 95 °C incubation temperature for 10 min for enzyme activation and DNA denaturation, followed by 50 PCR amplification cycles consisting of 95 °C for 15 s and 60 °C for 1 min. Fluorescence acquisition was at the end of the annealing stage of each cycle. The PCR products were analyzed by using the Sequence Detection System software version 1.2.3 (Applied Biosystems). Unless otherwise stated, all reactions were carried out in triplicate and all the assays included a positive control (DNA from *C. perfringens* CECT 563) and a no template control (NTC) where the DNA was replaced by sterile water.

Spore Induction and Preparation

Spore Induction The medium described by Duncan and Strong (1968) and modified by Jong et al. (2002) was used as sporulation medium (m-DS). A 5% (*v*/*v*) inoculum was routinely used to obtain spores. Inocula for the spore induction

Table 1 Oligonucleotides used in this study

Target	Name	Type	Sequence
Clostridium perfringens plc gene	CPplcF	Forward primer	5′-GCTAATCTTACTGCCGTTGATAG(CT)-3′
	CPplcR	Reverse primer	5′-TGTTCTTTCCTTTC(CT)TCTGCAAAA-3′
	CPplcP	TaqMan MGB probe	5′-FAM-CAGGACATGTTAAGTTTG-MGB-NFQ-3′
IAC chimeric DNA	CPIACP	TaqMan MGB probe	5′-VIC-CCATACACATAGGTCAGG-MGB-NFQ-3′
Listeria monocytogenes prfA gene	CPIACF	Forward primer	5′-GCTAATGTTACTGCCGTTGATAG(CT)GGCTCTATTTGCGGTC-3′
	CPIACR	Reverse primer	5′-TGTTCTTTCCTTTC(CT)TCTGCAAAATCTTGATGCCATCAGGA-3′

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were prepared from stock cultures of *C. perfringens* CECT 563 strain (stored at −80 °C in 16% (*v*/*v*) glycerol) by inoculating 5 mL of RCM broth and incubated at 37 °C for 24 h in anaerobic conditions. Subsequently, the cultures were plated on Perfringens Agar Base (Oxoid) without egg yolk emulsion and selective agents and plates were incubated in the same conditions. One separate colony was used to inoculate an overnight preculture in 5 mL of RCM broth which was used to inoculate 10 mL of m-DS sporulation media that was incubated overnight at 37 °C in anaerobic conditions. The m-DS preculture was used to inoculate 200 mL of fresh m-DS media in order to obtain enough quantity of spores for further preparation and was incubated at 37 °C for 24 h in anaerobic conditions.

Spore Preparation Spores were prepared as indicated in Herman et al. (1995). Vegetative cells were killed by heating for 30 min at 63 °C and digested by incubation with 30 mg of lysozyme (Sigma) and 2 mg of DNase I (Sigma) in 0.067 M phosphate buffer (pH 7) and 15 mM $MgCl_2$ for 1 h at 37 °C, to ensure the degradation of vegetative cell DNA. Then, 10 mg of trypsin (Sigma) was added and the mixture was incubated for a further 90 min. The suspension obtained was heated for 10 min at 90 °C to inactivate the DNase. The spores were concentrated by centrifugation at 12,000×*g* and 4 °C for 30 min, washed three times with sterile water, and stored at 4 °C in sterile water. To quantify the number of spores obtained, serial dilutions from the spore solution were made in sterile water and 1 mL of each dilution was heat treated at 70 °C for 20 min and cooled in ice water. One hundred microliters was plated by triplicate in Perfringens Agar Base (Oxoid) without egg yolk emulsion and selective agents and with an overlayer of the same medium, and plates were incubated overnight at 37 °C in anaerobic conditions.

Quantitative Detection of *C. perfringens* in Artificially Contaminated Raw Sheep Milk The raw sheep milk was collected from a local sheep farm in Castilla y León (Spain) and microbiologically analyzed following the guidelines of the US Food and Drug Administration (FDA 2001) in order to ensure that the milk sample did not contain *C. perfringens* and then was stored at −20 °C until use. In three independent experiments, 1 mL of serial tenfold dilutions, which included approximately 3×10^7, 3×10^6, 3×10^5, 3×10^4, 3000, 300, 30, and 3 *C. perfringens* spores, was added into 50-mL centrifuge tubes containing 25 mL of raw sheep milk. The mixture was homogenized and pre-treated with 1% (*w*/*v*) trypsin (Sigma) and 12% (*v*/*v*) Triton X-100 (Sigma) at 37 °C for 1 h. Then, it was centrifuged at 4600×*g* for 30 min and the bacterial pellet obtained was subjected to DNA extraction as described by López-Enríquez et al. (2007). The resulting bacterial DNA was eluted in 50 μL of 10 mM Tris-HCl, pH 8.0. A non-contaminated sample (blank) in which 1 mL of sterile water was added was also performed.

Results

Design and Optimization of the *C. perfringens*-Specific RTi-PCR Assay Suitable regions for the design of *C. perfringens*-specific PCR primers were identified by aligning all phospholipase C (*plc*) gene sequences deposited in public databases, using the CLUSTALW multiple-alignment tool (European Bioinformatics Institute, EMBL; www.ebi.ac.uk). PCR primers, *CPplcF* and *CPplcR*, were designed to amplify a 71-bp fragment corresponding to positions 1822 to 1892 from the *C. perfringens plc* gene (GenBank accession no. X17300; Saint-Joanis et al. 1989). The specificity of the primers and probe designed and the PCR amplicon generated were analyzed in silico using the BLAST-N program (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) by demonstrating that any other microbial DNA sequence deposited in GenBank was not recognized by them. The concentration of the reagents and the PCR conditions described above (RTi-PCR; "Materials and Methods" section) were optimized following the general guidelines (Anonymous 1999).

Optimization of Duplex *CPplc*-IAC RTi-PCR Assay The optimal concentration of the IAC probe (*CPIACP*) and the optimal number of genome equivalents of IAC were determined experimentally. Different quantities of *CPIACP* (50, 100, 150, and 200 nM) were tested in a RTi-PCR assay including 200 nM of *CPplcP*, 10,000 genome equivalents of IAC, and no bacterial genomic DNA of *C. perfringens*. The optimal concentration of the *CPIACP* TaqMan MGB probe was 50 nM. Subsequently, different amounts of IAC (1000, 100, 10, and 1 genome equivalents) were tested in the presence of decreasing amounts of genomic DNA of *C. perfringens* (100, 10, and 1 genome equivalents). The concentration of IAC without affecting the detection limit of the *C. perfringens plc* system was 100 genome equivalents per reaction.

Selectivity of the *CPplc*-IAC Assay Evaluation of the specificity of the *CPplc*-IAC RTi-PCR system was performed using 1 ng of DNA from an ample range of bacterial species: 53 *C. perfringens* strains, 27 *Clostridium* spp. strains that contained 20 different species of this genus, and 85 non-*Clostridium* strains (Supplementary Material Tables 1, 2, and 3). Among the selected strains, we included members of the five toxin types of *C. perfringens* (A, B, C, D, and E) which can be subtyped in this species, other pathogenic *Clostridium* species, and other foodborne and environmentally related species. All *C. perfringens* strains analyzed showed a positive

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FAM fluorescence signal, demonstrating the amplification of the *plc* gene target. The non-*perfringens* Clostridia and non-*Clostridium* strains showed a negative FAM signal, but a positive VIC fluorescence signal, indicating the amplification of the IAC. The amplification of IAC (VIC signal) excluded the possibility of the absence of signal due to failure in the PCR. Consequently, the *CPplc*-IAC RTi-PCR system was 100% inclusive for *C. perfringens* and 100% exclusive for non-target bacteria.

Analytical Sensitivity and Quantification Range of the *CPplc*-IAC RTi-PCR Assay *C. perfringens* CECT 563 colonies were collected from a half Petri dish, and the bacterial pellet obtained was subjected to DNA extraction as described above (bacterial genomic DNA isolation and quantification; "Materials and Methods" section). Tenfold serial dilutions from *C. perfringens* genomic DNA obtained were made in sterile water with 0.1 mg/mL of BSA covering a linearity range of eight orders of magnitude from approximately 1×10^7 to 1 genome equivalents per reaction. They were analyzed in an RTi-PCR assay where all the DNA concentrations were tested by triplicate except the DNA concentrations that contained 10 and 1 genome equivalents per reaction which were tested ten times. This experiment was carried out by triplicate and in the presence or absence of IAC in order to determine the effect of the internal control about the detection limit of the RTi-PCR assay. The mean C_T values ± standard error of these values in the three assays are shown in Table 2. The limit of detection (LOD), which is defined as the lowest copy number that exhibits positive results in 95% of the replicates (Hernández et al. 2004), represents the analytical sensitivity of the system (Bustin et al. 2009). A positive fluorescence FAM signal was detected in all the replicates down to 10 genome equivalents and 1 genome equivalent which was detected in 23 and 10% of the replicates in the presence or absence of IAC in the PCR reaction, respectively (Table 2). To determine the capacity of quantification of the *CPplc* and *CPplc*-IAC RTi-PCR systems, the mean C_T values produced by the analysis of the serial dilutions of the *C. perfringens* DNA were plotted against the $\log_{10}$ genome equivalents per reaction, and the standard curves were constructed in order to calculate the slopes(s) and the correlation coefficients (R^2), which define the efficiency (E) and the linearity of the system (Rodríguez-Lázaro et al. 2005; Rodríguez-Lázaro et al. 2006). The efficiency was calculated from the slopes obtained according with the equation $E = 10^{(-1/\text{slope})} - 1$. The overall slopes of the regression curves were −3.7300 and −3.7739, indicating a PCR efficiency of 0.854 and 0.841 for the *CPplc* and *CPplc*-IAC RTi-PCR systems, respectively, and the R^2 values obtained were 0.9995 and 0.9990, respectively. Both the efficiency and R^2 values were close to the theoretical values ($E = 1$ and $R^2 = 1$) demonstrating that the capacity of the PCR to duplicate target molecules (E) and the linearity (R^2) between the generated results (C_T values) and the amount of analyte present in the sample were very high. The limit of quantification (LOQ) was determined as the lowest copy number that exhibits linear correlation with C_T, with R^2 values above 0.99 and non-overlapping standard deviation values among adjoining DNA dilutions (Hernández et al. 2004). Statistical analysis (ANOVA and Tukey or Games-Howell post hoc methods, $\alpha = 0.01$) showed no overlapping C_T values from the concentrations 1×10^7 to 10 genome equivalents per reaction. Taking into account the statistical and experimental results obtained, the *CPplc* and *CPplc*-IAC RTi-PCR systems demonstrated an accurate capacity of quantification over a linear dynamic range of 7 logs, being 10 genome equivalents of the LOQ.

Quantitative Detection of *C. perfringens* in Raw Sheep Milk Twenty-five milliliters of raw sheep milk was artificially contaminated with different concentrations of *C. perfringens* CECT 563 spores, which were calculated by a standard plating count method (FDA 2001). Each 25 mL of contaminated milk was subjected to DNA isolation procedure, and 50 μL of bacterial DNA was obtained. The DNA sample was tenfold diluted, and 5 μL of each dilution was analyzed. It was possible to detect 300 spores of *C. perfringens* in 25 mL of milk with 78% probability and 30 spores with 50% probability (Table 3). The mean C_T values obtained for each milk sample contaminated with a different amount of spores were plotted against the $\log_{10}$ concentration of genome equivalents per reaction, and the standard curve was constructed. The overall efficiency (E) was 0.858, and the linearity (R^2) was 0.991. The *C. perfringens* spore values were calculated extrapolating the C_T values obtained in the corresponding standard regression curve, and those values obtained with standard plating techniques were compared in order to estimate the degree of correspondence, i.e., the relative accuracy. Relative accuracy values ranged between 83.03 and 151.18% demonstrating a good degree of correspondence between the results obtained by both methods, i.e., the standard plating method and the *CPplc*-IAC RTi-PCR method (Table 3).

Discussion

C. perfringens is an important pathogenic bacterium that is the causal agent to an ample variety of diseases, including food poisoning, which affects human and domestic animals. The ubiquitous nature of this bacterium and its spores makes possible its presence in a wide range of foods and makes it a frequent problem for the food industry (Andersson et al. 1995). Industries related with the production of foods from animal origin require the development of simple and rapid methods, for the control of transmission of foodborne pathogens for ensuring food safety and preventing food poisoning

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Table 2 Determination of the detection and quantification limits of the RTi-PCR assay with genomic DNA from *C. perfringens* strain CECT 563[a]

CPplc				
Approx. genome equivalents/reaction	Confidence interval limit[b] Lower	Upper	Signal ratio[c]	C_T[d]
1×10^7	9,986,951	10,065,795	9/9	14.66 ± 0.05
1×10^6	997,693	1,004,372	9/9	18.79 ± 0.10
1×10^5	99,643	100,358	9/9	22.52 ± 0.04
1×10^4	9887	10,113	9/9	25.89 ± 0.06
1×10^3	964	1036	9/9	29.58 ± 0.07
100	89	111	9/9	33.63 ± 0.13
10	7	14	28/30	37.23 ± 0.18
1	0	2	7/30	39.54 ± 0.13
CPplc-IAC				
Approx. genome equivalents/reaction	Confidence interval limit[b] Lower	Upper	Signal ratio[c]	C_T[d]
1×10^7	9,986,951	10,065,795	9/9	14.69 ± 0.04
1×10^6	997,693	1,004,372	9/9	19.02 ± 0.03
1×10^5	99,643	100,358	9/9	22.70 ± 0.06
1×10^4	9887	10,113	9/9	26.13 ± 0.03
1×10^3	964	1036	9/9	29.73 ± 0.09
100	89	111	9/9	33.67 ± 0.13
10	7	14	30/30	37.81 ± 0.26
1	0	2	3/30	39.94 ± 0.10

[a] No template controls (NTCs) for both RTi-PCR systems were negative (C_T values of 50 in all the replicates). The overall slope (s) of the regression curve was −3.7300 and −3.7739 for the *CPplc* and *CPplc*-IAC RTi-PCR systems, respectively, indicating a PCR efficiency (E) of 0.854 and 0.841, respectively, and the regression coefficients (R^2) were 0.9995 and 0.9990, respectively

[b] Calculated for the expected number of template molecules at each dilution, at the 95% confidence level

[c] Positive results out of 9 reactions, 30 reactions for those containing 10 and 1 genome equivalents per reaction

[d] Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.01$), taking into account the unavoidable error associated with serial dilutions

(Kanakarag et al. 1998). Moreover, the clinical diagnoses of human and animal diseases need rapid and specific methods for detection and identification of pathogen microorganisms (Wang et al. 1994). The standard methods applied for identification and quantification of *C. perfringens* are tedious and time-consuming and require the confirmation with biochemical tests of a number of representative colonies (FDA 2001). However, one of the more important advantages of PCR in microbiology diagnostics is the rapidity that it offers for pathogen diagnosis (Belanger et al. 2003; Rodríguez-Lázaro et al. 2007).

The *plc* is a single copy gene that is located in a conservated region on the bacterial chromosome in all the toxin types of *C. perfringens*. This gene codifies the alpha-toxin (CPA), which is a phospholipase C sphingomyelinase, which hydrolyzes phospholipids and promotes membrane disorganization playing an important role in the gas gangrene disease (Petit et al. 1999). The CPA toxin has been described as a cytolytic, hemolytic, dermonecrotic, and lethal toxin that is specific of this clostridial species (Tsutsui et al. 1995). Therefore, the *plc* gene is a good candidate target for the specific identification of this pathogen by PCR-based detection methods. The selectivity of the oligonucleotides was tested in silico using the BLAST-N bioinformatic tool and was confirmed to be experimentally 100% selective by the screening of 53 *C. perfringens* strains, 20 non-*perfringens* *Clostridium* spp. Strains, and 85 non-*Clostridium* strains. Consequently, the described method could be a rapid alternative to the confirmatory test subsequent to isolation of suspicious or presumptive colonies on selective media from food, stools, or environmental samples.

A major limitation to the application of RTi-PCR-based tests in diagnostic laboratories, for the detection of pathogens from biological matrices, is the underestimation due to false-negative results relating with the presence of PCR inhibitors in the sample (Rodríguez-Lázaro et al. 2006; Rodríguez-Lazaro et al. 2007), which is common in milk samples (Romero et al. 1995) and in fecal samples (Wise and Siragusa 2005) particularly at low pathogen concentrations. For this purpose, the *CPplc* RTi-PCR system included an internal amplification

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Table 3 Quantitative detection of *C. perfringens* CECT 563 in artificially contaminated raw sheep milk[a]

Approx. *C. perfringens* CFUs/25 mL	Approx. *C. perfringens* genome equivalents/reaction[b]	Signal ratio[c]	C_T value[d]	Relative accuracy[e]
3×10^7	3×10^5	9/9	23.27 ± 0.05	100.19
3×10^6	3×10^4	9/9	26.82 ± 0.14	101.23
3×10^5	3×10^3	9/9	30.39 ± 0.10	102.74
3×10^4	300	9/9	35.04 ± 0.17	93.74
3×10^3	30	9/9	39.11 ± 0.31	83.03
300	3	7/9	40.99 ± 0.36	151.18
30	<1	5/9	41.35 ± 0.13	NA
3	0	0/9	>50	NA
0[f]	0	0	>50	NA

NA not applicable

[a] Raw milk directly collected from a sheep farm. Results of three independent experiments with three PCR replicates used in each. The overall efficiency was 0.858, and the linearity (R^2) was 0.991

[b] Estimated number of *C. perfringens* genome equivalents in each PCR run, assuming 100% DNA extraction efficiency (each reaction contained 5 μL of a tenfold diluted DNA preparation of 50 μL extracted from 25 ml milk)

[c] Positive results out of nine reactions

[d] Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.01$), taking into account unavoidable error associated with serial dilutions

[e] Degree of correspondence between the results obtained with the standard plating technique (log *C. perfringens* CFUs/mL) and those obtained with the *CPplc*-IAC RTi-PCR method (log *C. perfringens* genome equivalents/reaction)

[f] Non-contaminated sample

control (IAC). The *CPplc* IAC is a non-target DNA that is coamplified with the same primers (*CPplcF* and *CPplcR*). It is constructed by introducing *C. perfringens plc*-specific primer sites as 5′ overhanging ends of another set of primers which are specific to a portion of the *L. monocytogenes prfA* gene previously validated as an RTi-PCR probe target (unpublished data). Therefore, the chimeric DNA is hybridized by a second fluorescent probe (*CPIACP*) permitting the simultaneous detection of the target DNA and the IAC in the same reaction. No significant differences were found between the results obtained with the *CPplc*- and the *CPplc*-IAC RTi-PCR assays, demonstrating that the IAC inclusion does not affect at the performance of the assays. To our knowledge, the CP*plc*-IAC RTi-PCR system is the first that included a competitive IAC to enhance the reliability of the PCR assay.

The capacity of the *CPplc*-IAC RTi-PCR method for detection and quantification of *C. perfringens* was evaluated with a DNA model using the *C. perfringens* CECT 563 strain. It was possible to detect 1 target genome equivalent in at least 23% of the replicates, and 10 genome equivalents were detected in all the cases (Table 2), with 10 genome equivalents being the limit of detection of the system. The efficiency and the R^2 values of an RTi-PCR assay define its ability to determine accurately the number of targets present in the sample. The calculated R^2 (*CPplc*, 0.995; *CPplcIAC*, 0.9990) and E (*CPplc*, 0.854; *CPplcIAC*, 0.841) values highlighted the quantification linearity over a range of 7 logs, down to approximately 10 *C. perfringens* genome equivalents, and a very good RTi-PCR performance. *C. perfringens* is an important intestinal pathogen. Changes in the composition and function of the intestinal microflora play an important role on gut morphology, nutrition, immune responses, and protection against colonization of pathogens. Therefore, the development of techniques as *CPplc*-IAC RTi-PCR for accurate enumeration of intestinal bacterial population is critical in order to understand nutritional effects and intestinal malfunctions related with the bacterial gut load (Fu et al. 2006; Skanseng et al. 2006). On the other hand, the inevitable presence of *C. perfringens* in foods or water could be monitored in order to prevent the increased contamination of this pathogen in the food industry and in the water supply (Novak and Juneja 2002; Shannon et al. 2007).

The routine method used to detect and approximately enumerate spore-forming anaerobe microorganisms as *Clostridium tyrobutyricum* in dairy products is the most probable number (MPN) that is based on the lactate fermentation capacity of cells from gas positive tubes and additional confirmatory tests. Recently, molecular studies in our laboratory (unpublished data) based on real-time PCR and denaturing gradient gel electrophoresis (DGGE) demonstrated the presence of *C. perfringens* in positive MNP tubes from the analysis of different raw milk samples. Microbiological testing

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shows *C. perfringens* capable of fermenting lactose, and thus, it the growth of this microorganism is possible in the selective media and under the conditions used to develop the MPN assays from raw milk samples analyzed. In 1981, in England and Wales, one incident with 77 cases associated with milk and dairy products was reported (Public Health Laboratory Service Communicable Disease Surveillance Centre, 1984), and cheese and cheese sauces have been reported as susceptible foods to contamination by *C. perfringens* (Nakamura and Kelly 1968). Accordingly, *C. perfringens* spores present in raw milk could be the origin of contamination of several dairy products and the origin of food poisoning. To determine the performance of the *CPplc*-IAC RTi-PCR assay from milk samples, artificial contaminations of raw sheep milk, with spores of *C. perfringens* CECT 563, were carried out in the laboratory. Taking into account that 25 mL of contaminated milk was subjected to DNA isolation obtaining 50 μL of DNA sample and that 5 μL of a tenfold dilution from this DNA sample was used as template in the PCR, the practical detection limit for *C. perfringens* was around 300 spores per 25 mL of contaminated milk (or around three *C. perfringens* genome equivalents per reaction) with 78% probability and the practical quantification limit was 3000 spores per 25 mL of milk (or approximately 30 genome equivalents per reaction).

The relative accuracy is defined as the closeness of agreement between the result obtained by an accepted method and that obtained by an alternative method (Anonymous 2003). To validate the *CPplc*-IAC RTi-PCR system, the results obtained with our RTi-PCR method were compared with the standard plating method to enumerate *C. perfringens* in food (FDA 2001). The values of relative accuracy obtained ranged between 83.03 and 151.18% indicating the reliability of the designed RTi-PCR method to count *C. perfringens* in real samples. Therefore, the assay described here provides an alternative for the quantitative detection of *C. perfringens* in dairy products.

Currently, several laboratory criteria are accepted by the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) for confirming the occurrence of a *C. perfringens* type A food poisoning outbreak: (a) demonstrating the presence of 10^5 *C. perfringens* organisms per gram of stool from two or more persons, (b) demonstrating the presence of 10^5 *C. perfringens* organisms per gram of epidemiologically implicated food, or (c) demonstrating the presence of *cpe* gene in the feces of several people involved in a food poisoning outbreak (Olsen et al. 2000). The presence of *C. perfringens* in normal foods as well as in the intestinal tract as normal flora results in that non-implicated foods and asymptomatic individuals show substantial amounts of *C. perfringens* spores or cells. Thus, in a food poisoning outbreak, the confirmation of enterotoxigenicity by in vitro CPE detection is recommended, in addition to pathogen determination by microbiological or molecular methods. The *CPplc*-IAC RTi-PCR system could be used as a rapid screening tool in order to allow establishing the presence of *C. perfringens* in the implicated food and stools as quickly as possible, while the presence of the enterotoxin is confirmed. Loh et al. (2008) reported the ability of real-time PCR methods to reduce the diagnosis time and to allow rapid adoption of preventive and control measures in *C. perfringens* food-borne outbreaks.

In conclusion, our results demonstrate that *CPplc*-IAC RTi-PCR assay targeting the *C. perfringens plc* gene is a promising tool for direct detection of this pathogen from raw milk samples. To our knowledge, this is the first real-time PCR assay for the detection of *C. perfringens* directly from milk and this is the first assay that includes a competitive IAC which could be extrapolated to other real-time PCR detection methods in order to improve the effectiveness of the systems. This assay has potential for application in food safety and in clinical diagnosis of human and animal diseases related with the CPA toxin.

Compliance with Ethical Standards

Funding This work was supported by the European Union's Marie-Curie mobility program (FP7-PEOPLE-2007-2-2-ERG ref. 209050; FoodPath). L.L.-E. received a Ph.D. studentship from the INIA.

Conflict of Interest Marta Hernández declares that she has no conflict of interest. Lorena López-Enríquez declares that she has no conflict of interest. David Rodríguez-Lázaro declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants.

Informed Consent Not applicable.

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