A Multiplex Quantitative Polymerase Chain Reaction Using Applied Biosystems 7500 Fast System for Simultaneous Identification of Three *Campylobacter* Species with Potential Applications to Food Analysis

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Abstract

Consumption of *Campylobacter*-contaminated food is one of the most common causes of bacterial diarrhea. A previously developed quantitative polymerase chain reaction (qPCR) utilizing the SmartCycler instrument platform for identification of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* had to be modified to address the recent discontinuation of the SmartCycler system. In this study, a multiplex qPCR assay was optimized on the Applied Biosystems 7500 Fast (AB7500F) platform to continue using qPCR for the identification of three target *Campylobacter* spp. AB7500F qPCR efficiencies obtained by testing reference genomic DNA (gDNA) were 90.9%, 86.4%, and 94.6% for *C. jejuni*, *C. coli*, and *C. lari*, respectively, with all correlation coefficient values >0.99. The qPCR results exhibited 100% specificity by testing gDNA samples from 37 non-target reference strains and 86 target strains (50 *C. jejuni*, 27 *C. coli*, and 9 *C. lari* strains) in this study. The lowest detection level using gDNA was 4, 7, and 2 genome copies per reaction for *C. jejuni*, *C. coli*, and *C. lari*, respectively. With a 2-day enrichment procedure, the qPCR method correctly detected target species in a spiked food matrix (frog leg, an aquaculture product). The sensitivity in 25 g food matrix was 4 colony-forming units (CFUs) for *C. jejuni*, 3 CFUs for *C. coli*, and 2 CFUs for *C. lari*. The results suggest that this AB7500F-based qPCR has potential applications for the identification of *C. jejuni*, *C. coli*, and *C. lari* in contaminated food.

Keywords: *Campylobacter*, species identification, multiplex qPCR, food analysis

Introduction

AS A LEADING cause of foodborne bacterial gastroenteritis, *Campylobacter* is responsible for more than 1.3 million infections each year in the United States (CDC, 2020). *Campylobacteriosis*, the infection of *Campylobacter*, is often linked to consumption of contaminated meat, poultry, dairy, aquaculture, and fresh produce (Silva *et al.*, 2011; Gharst *et al.*, 2013; Government of Canada, 2020). Besides animal origins, the environmental reservoirs for *Campylobacter* contamination usually include manure, water, and soil (Whiley *et al.*, 2013). Syndromes of *Campylobacteriosis* range from abdominal cramps, fever, to bloody diarrhea (CDC, 2020). In high-risk groups, such as young children, pregnant women, and elderly, *Campylobacteriosis* may cause more severe or even fatal complications (USDA, 2020).

In the genus *Campylobacter*, *Campylobacter jejuni* is the most common pathogenic species, whereas *Campylobacter coli* and *Campylobacter lari* are recognized with increasing clinical importance (Kaakoush *et al.*, 2015; Saiyudthong *et al.*, 2015). In addition to gastroenteritis, *Campylobacter* infection is sometimes related to other complications. *C. jejuni* is a risk factor of Guillan-Barre syndrome, an autoimmune disease (Neal-McKinney *et al.*, 2018); *C. coli* may cause second trimester intrauterine growth restriction in pregnant women, which leads to abortions (Kuperman-Shani *et al.*, 2015); and *C. lari* infections may result in life-threatening septicemia (Morishita *et al.*, 2013). Therefore, the capability of rapid, specific, and sensitive identification of *C. jejuni*, *C. coli*, and *C. lari* will contribute to the improvement of food safety to protect public health.

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The views presented in this work do not necessarily reflect those of the FDA, nor do we specifically endorse the listed instrumentation or products.

Previously, a multiplex quantitative polymerase chain reaction (qPCR) utilizing the SmartCycler instrument (Cepheid, Inc., Sunnyvale, CA) was developed in this laboratory to simultaneously detect *C. jejuni*, *C. coli*, and *C. lari* by using genomic DNA (gDNA) of pure cultures and artificially spiked raw milk without enrichment (Liu *et al.*, 2017). However, the SmartCycler instrument was recently discontinued by its manufacturer.

In this study, a multiplex assay was optimized on the Applied Biosystems 7500 Fast (AB7500F) platform (Thermo Fisher Scientific, Inc., Waltham, MA) to continue using qPCR for simultaneous identification of the target species. Performance of the method on AB7500F was evaluated, including the efficiency, specificity, and sensitivity. In addition to gDNA of pure bacterial cultures, the AB7500F qPCR in combination with an enrichment procedure was also tested with frog leg, a widely consumed aquaculture product regulated by FDA, to assess the applications to the analysis of *Campylobacter*-contaminated food.

Materials and Methods

Bacterial strains, culture conditions, and gDNA extraction

All glycerol stocks were kept frozen at -70°C until use. Thirty-seven non-target reference strains in 33 species were obtained from the American Type Culture Collection (ATCC) and cultured under appropriate conditions following the FDA Bacteriological Analytical Manual (BAM) (FDA, 2020a) for DNA extraction. Fourteen target reference strains (four *C. jejuni*, five *C. coli*, and five *C. lari* strains) were obtained from ATCC. Seventy-two *Campylobacter* field strains (46 *C. jejuni*, 22 *C. coli*, and 4 *C. lari* strains) were from the laboratory collection. They had been previously characterized following BAM (FDA, 2020a) and by VITEK® MS, an automated mass spectrometry system for microbial identification (bioMérieux, Inc., Marcy-l'Étoile, France).

Campylobacter strains were cultured on BBL™ *Campylobacter* Agar (Cat. No. B21727X; Thermo Fisher Scientific, Inc.) at $37-42^{\circ}\text{C}$ for 2 days under microaerophilic condition before gDNA extraction. gDNA was extracted by using the QIAamp® DNA Mini Protocol on a QIAcube® automated instrument (QIAGEN Company, Hilden, Germany) and subsequently quantified with the double-stranded DNA (dsDNA) BR Assay Kit on a Qubit® 3.0 fluorimeter (Thermo Fisher Scientific, Inc.).

Oligo sequences and qPCR conditions

For the detection of *C. jejuni*, *C. lari*, and the internal amplification control (IAC), sequences were adopted from the SmartCycler qPCR method previously published (Liu *et al.*, 2017). For *C. coli* detection, new oligos were designed by using the PrimerQuest tool (IDT, 2020) to improve qPCR efficiency. Sequences of all primers and probes are listed in Table 1. The IAC template is a 140 bp fragment, including a 114 bp green fluorescence protein (GFP) amplicon and protection bases on both ends: 5'-GTGCTGCTGCCCCGAC AACCCTACCTGAGCACCCAGTCCGCCCTGAGCAA AGACCCCAACGAGAAGCGCGATCACATGGTCTCTGC TGGAGTTTCGTGACCGCCGCGGGATCACTCTCGGC ATGGACGAGCTGTACAAGTA-3'. The oligos were

TABLE 1. PRIMERS AND PROBES USED IN THE MULTIPLEX QUANTITATIVE POLYMERASE CHAIN REACTION ON APPLIED BIOSYSTEMS 7500 FAST

Oligo ID	5' to 3' Sequences	Working concentration (nM)	Target and amplicon size	Design or references
Cj_qF	TGCACGAGTACTATGAATAACGA	200	<i>Campylobacter jejuni</i>	Published sequences (Liu <i>et al.</i> , 2017) with fluorophore modification.
Cj_qR	TCCAAAATCCTCAGCTTGCCATT	200	<i>hipO</i> and 124 bp	Designed in the present study.
Cj_probe2	/TAMRA/TTCGAACCTCAGTCAAAAATCCACAGCT/BHQ2/	200	<i>Campylobacter coli</i>	
Cc_qF2	ACTTCCATGCCCTAAGAC	200	and 102 bp	
Cc_qR2	TCCACCTATACTAGGCTTGTC	200	<i>Campylobacter lari</i>	
Cc_probe2	/FAM/AGTTGCAGG/ZEN/AGTTCCAGCTAAAACCTTG/IBFQ/	150	<i>pep</i>	
Cl_qF	TTAGATTGTTGTGAAATAGCGAGTT	200	and 86 bp	Published sequences (Liu <i>et al.</i> , 2017) with fluorophore modification.
Cl_qR	TGAGCTGATTGCTCTATAAATTCG	200	GFP and 114 bp	Published sequences (Liu <i>et al.</i> , 2017) with fluorophore modification.
Cl_probe2	/VIC/TGAAAATTGGAACGCGAGGTG/MGB-NFQ/	200		
IAC_qF	GCACAACCACTACCTGAGC	160		
IAC_qR	TCCATGCCGAGAGTGATC	160		
IAC_probe	/Cy5/TGAGCAAG/TAO/ACCCCAACGAGAAAGC/IBRQ/	160		

IAC targeting the GFP.

GFP, green fluorescence protein; IAC, internal amplification control; ID, identification.

synthesized by Applied Biosystems of Thermo Fisher Scientific, Inc., (Waltham, MA) and Integrated DNA Technologies (Coralville, IA).

AB7500F qPCR reactions were run under fast mode. Each reaction contained 12.5 μ L TaqMan™ Universal PCR Master Mix (Cat. No. 4304437; Thermo Fisher Scientific, Inc.), 2.875 μ L primers and probes targeting the three *Campylobacter* spp., 1.8 μ L IAC oligos (0.4 μ L primers, 0.4 μ L probe, and 1.0 μ L IAC template), 0.2 μ L Platinum™ Taq Hot Start DNA Polymerase (Cat. No. 10966018; Thermo Fisher Scientific, Inc.), and molecular biology grade nuclease-free water to reach a final volume of 25 μ L. Working concentrations of primers and probes are listed in Table 1. The final concentration of IAC template was 0.6 pM, containing $\sim 3.6 \times 10^5$ copies of the GFP fragment.

The thermocycling conditions included an initial heat activation step at 95°C for 20 s, followed by 45 cycles of denaturation at 95°C for 3 s, and annealing and extension at 62°C for 30 s with optics on. The threshold and baseline were automatically calculated by the AB7500F software version 2.3.

Determination of qPCR efficiency and correlation coefficient

To determine qPCR efficiency and correlation coefficient, 10-fold serial dilutions of gDNA of ATCC strains were prepared across five orders of magnitude. The amplification efficiency of each target was calculated with an online calculator (Agilent, 2020). The correlation coefficient, R^2 , was determined with Microsoft Excel 2010 by using triplicated qPCR data at each template concentration from a single qPCR run. Copy numbers were calculated with an online tool (URI, 2020). The genome size of *Campylobacter* was ~ 1.7 Mb according to the National Center for Biotechnology Information (NCBI, 2020) and published results (Miller *et al.*, 2014; Zhao *et al.*, 2016).

Exclusivity and inclusivity studies

The exclusivity study was performed by using gDNA of 30 non-*Campylobacter* reference strains from 29 species in 23 genera, as well as 7 ATCC strains from 4 non-target species in the genus *Campylobacter* (*Campylobacter fetus*, *Campylobacter iguaniorum*, *Campylobacter hyointestinalis*, and *Campylobacter upsaliensis*). The inclusivity study was performed by using gDNA of 50 *C. jejuni*, 27 *C. coli*, and 9 *C. lari* strains, including ATCC references, characterized field isolates from food and environment, and archived clinical isolates transferred from hospitals. Triplicate qPCR experiments were carried out with ~ 2 pg gDNA per reaction, which contained ~ 1000 *Campylobacter* genome copies following a standard protocol (FDA, 2020b). The IAC cycle thresholds were usually no more than 30 by using 2 pg gDNA templates. If both targets and IAC were negative, the results were not valid and experiments were repeated. Mean and standard deviation were calculated from qPCR triplicates.

Determination of fractional detection levels

Tenfold serial dilutions of gDNA were prepared for high concentrations of template (~ 300 genome copies per reaction and above), and twofold serial dilutions were prepared

for template concentrations lower than 300 genome copies per reaction. Five qPCR replicates were carried out at each concentration. If the qPCR results of five replicates were partially positive (i.e., 1–4 positives), the concentration of the template was determined to be the fractional detection level (FDA, 2020c).

The spike-and-recovery study

Bone-in frog legs were purchased at a local grocery store. Aliquots of 25 g of frog legs (bone-in) were sterilely placed in Combibag™ (No. 30551; BioMerieux SKU) and kept frozen until use. The spiking, aging, and recovery experiments were designed following FDA guidelines (FDA, 2020c) and BAM (FDA, 2020a) with minor modifications.

Briefly, fresh *Campylobacter* cells grown on BBL *Campylobacter* agar plates were suspended in sterile 1 $\times$ phosphate-buffered saline. Quantification of cell suspensions was carried out by optical density measurement on a NanoDrop™ 2000 spectrophotometer. Suspensions were diluted between 10^{-1} and 10^{-8} times in triplicates. Ten-time *E. coli* ATCC 35150 were added to one aliquot of *Campylobacter* suspension at each concentration as the background flora. Frog legs spiked with suspensions containing *Campylobacter* and background flora were subsequently aged at 4°C for 2 days following a standard protocol (FDA, 2020c). Meanwhile, the remaining two aliquots of *Campylobacter* suspension at each concentration (without background flora) were independently spread on two BBL *Campylobacter* agar plates for colony counting.

After aging, 225 mL blood-free Bolton Broth was added to each Combibag containing spiked frog legs. The samples were incubated under microaerophilic condition at 37°C without shaking for 2 h, and they were then transferred to 42°C for 2 days with a shaking speed of 80 revolutions per minute. After enrichment, 1.0 μ L filtered culture was directly tested by the AB7500F qPCR method. Another 1.0 μ L filtered enrichment culture was streaked on selective Bolton Broth with 5% sheep blood agar and grown under microaerophilic condition as mentioned earlier. Two isolated colonies from each enrichment, which were morphologically like *Campylobacter*, were examined by using the VITEK MS microbial identification (ID) system with IVD database v3.2.0 (bioMérieux, Inc.) following the manufacturer's instructions to verify the species of recovered bacteria. For each target species, the spike-and-recovery experiment was independently carried out by using two ATCC strains.

Results

In this study, a multiplex qPCR method was optimized on the AB7500F platform to continue the simultaneous identification for *C. jejuni*, *C. coli*, and *C. lari* after the discontinuation of SmartCycler. The amplification efficiencies of qPCR were 90.9%, 86.4%, and 94.6% for *C. jejuni*, *C. coli*, and *C. lari*, respectively. The correlation coefficients of linearity were all >0.99 with template concentrations across five orders of magnitude (Fig. 1).

Detection limit of the qPCR method was first evaluated by using gDNA of two ATCC strains from each target species, and similar results were obtained within the same species (Table 2). The lowest fractional level of detection was 4 genome copies for *C. jejuni*, 7 genome copies for *C. coli*, and 2 genome copies for *C. lari* gDNA per reaction.

FIG. 1. The qPCR efficiency and correlation coefficient of standard curves for (A) *Campylobacter jejuni*, (B) *Campylobacter coli*, and (C) *Campylobacter lari* determined by using gDNA of ATCC strains. X axis, $\log_{10}$ of genome copies per qPCR reaction. Y axis, qPCR Ct. Each error bar represents the standard deviation of five experimental replicates. Inset, linear regression, correlation coefficient (R^2), and efficiency (E). ATCC, American Type Culture Collection; Ct, cycle threshold; gDNA, genomic DNA; qPCR, quantitative polymerase chain reaction.

Using gDNA of 37 non-target ATCC strains (Table 3) as templates, this qPCR method showed 100% exclusive specificity. There was no false positive detected, clearly showing that the method did not cross-react with the non-target species tested in the study. As all IAC results were acceptable, false negatives caused by PCR inhibition could be excluded. Notably, qPCR results were all negative when testing gDNA of seven ATCC strains from non-target *Campylobacter* spp., *C. fetus*, *C. hyointestinalis*, *C. iguaniorum*, and

TABLE 2. DETECTION LIMIT DETERMINED BY USING GENOMIC DNA OF AMERICAN TYPE CULTURE COLLECTION STRAINS

Target species	ATCC No. of strains	Lowest detection level (genome copies per reaction)
<i>Campylobacter jejuni</i>	33291	4
	33560	6
<i>Campylobacter coli</i>	33559	7
	43133	10
<i>Campylobacter lari</i>	35222	2
	43675	3

Genome copies were rounded up to the next nearest integers. ATCC, American Type Culture Collection.

C. upsaliensis. The results demonstrate that this method is species specific within the genus *Campylobacter*.

Both ATCC strains and previously characterized field strains were used in the inclusivity study. Results showed that this qPCR method achieved 0 false negative by testing gDNA of 50 *C. jejuni*, 27 *C. coli*, and 9 *C. lari* strains (Table 4).

TABLE 3. REFERENCE STRAINS USED IN THE EXCLUSIVITY STUDY

Genus	Species	ATCC No.
<i>Acinetobacter</i>	<i>baumanni</i>	19606
<i>Aeromonas</i>	<i>hydrophila</i>	7965
<i>Alcaligenes</i>	<i>faecalis</i>	8750
<i>Bacillus</i>	<i>cereus</i>	14579
<i>Bacillus</i>	<i>subtilis</i>	19659
<i>Bordetella</i>	<i>bronchiseptica</i>	10580
<i>Burkholderia</i>	<i>cepacia</i>	25608
<i>Enterobacter</i>	<i>aerogenes</i>	13048
<i>Enterococcus</i>	<i>faecalis</i>	29212
<i>Enterococcus</i>	<i>durans</i>	6056
<i>Escherichia</i>	<i>coli</i>	25922
<i>Escherichia</i>	<i>coli</i>	35150
<i>Hafnia</i>	<i>alvei</i>	13337
<i>Klebsiella</i>	<i>pneumoniae</i>	13883
<i>Leclercia</i>	<i>adecarboxylata</i>	23216
<i>Listeria</i>	<i>monocytogenes</i>	19111
<i>Listeria</i>	<i>seeligeri</i>	35967
<i>Pasteurella</i>	<i>aerogenes</i>	27883
<i>Plesiomonas</i>	<i>shigelloides</i>	51903
<i>Proteus</i>	<i>mirabilis</i>	7002
<i>Providencia</i>	<i>rettgeri</i>	35565
<i>Providencia</i>	<i>alcalifaciens</i>	51902
<i>Salmonella</i>	<i>choleraesuis</i>	6539
<i>Salmonella</i>	<i>bongori</i>	43975
<i>Serratia</i>	<i>liquifaciens</i>	27592
<i>Staphylococcus</i>	<i>xylosus</i>	29971
<i>Streptococcus</i>	<i>bovis</i>	9809
<i>Streptococcus</i>	<i>pyogenes</i>	19615
<i>Vibrio</i>	<i>cholerae</i>	15748
<i>Yarrowia</i>	<i>lipolytica</i>	9773
<i>Campylobacter</i>	<i>fetus</i>	25936
<i>Campylobacter</i>	<i>fetus</i>	19438
<i>Campylobacter</i>	<i>fetus</i>	33561
<i>Campylobacter</i>	<i>fetus</i>	27374
<i>Campylobacter</i>	<i>iguaniorum</i>	BAA2680
<i>Campylobacter</i>	<i>hyointestinalis</i>	35217
<i>Campylobacter</i>	<i>upsaliensis</i>	49816

ATCC, American Type Culture Collection.

TABLE 4. AMERICAN TYPE CULTURE COLLECTION REFERENCE STRAINS AND FIELD ISOLATES USED IN THE INCLUSIVITY STUDY FOR *CAMPYLOBACTER JEJUNI*, *CAMPYLOBACTER COLI*, AND *CAMPYLOBACTER LARI* IDENTIFICATION

Campylobacter spp.	ATCC or PNL No.
<i>C. jejuni</i>	
<i>C. jejuni</i>	ATCC 29428
<i>C. jejuni</i>	ATCC 33291
<i>C. jejuni</i>	ATCC 33560
<i>C. jejuni</i>	ATCC 700819
<i>C. jejuni</i> ^a	GH82419
<i>C. jejuni</i> ^a	GH67493
<i>C. jejuni</i> ^a	GH18401
<i>C. jejuni</i> ^a	NW1230:420/2
<i>C. jejuni</i> ^a	GH96803
<i>C. jejuni</i> ^a	GH04009
<i>C. jejuni</i> ^a	GH84500
<i>C. jejuni</i> ^a	GH36987
<i>C. jejuni</i> ^a	GH05184
<i>C. jejuni</i> ^a	GH84244
<i>C. jejuni</i> ^a	GH72740
<i>C. jejuni</i> ^a	GH66566
<i>C. jejuni</i> ^a	GH78808
<i>C. jejuni</i> ^a	GH49803
<i>C. jejuni</i> ^a	GH10197
<i>C. jejuni</i> ^a	GH49484
<i>C. jejuni</i> ^a	GH13350R
<i>C. jejuni</i> ^a	GH53960
<i>C. jejuni</i> ^a	GH67493
<i>C. jejuni</i> ^a	GH05391
<i>C. jejuni</i> ^a	GH36669
<i>C. jejuni</i> ^a	GH31300
<i>C. jejuni</i> ^a	GH20281
<i>C. jejuni</i>	R4B2-02
<i>C. jejuni</i>	R4B2-05
<i>C. jejuni</i>	R4B2-07
<i>C. jejuni</i>	R4B2-08
<i>C. jejuni</i>	R4B2-09
<i>C. jejuni</i>	R4B2-27
<i>C. jejuni</i>	R4B2-28
<i>C. jejuni</i>	R4B2-34
<i>C. jejuni</i>	R4B2-38
<i>C. jejuni</i>	R4B2-42
<i>C. jejuni</i>	R4B4-09
<i>C. jejuni</i>	R4B4-10
<i>C. jejuni</i>	R4B4-11
<i>C. jejuni</i>	R4B4-13
<i>C. jejuni</i>	R4B4-15
<i>C. jejuni</i>	R4B4-19
<i>C. jejuni</i>	R4B4-20
<i>C. jejuni</i>	R4B4-25
<i>C. jejuni</i>	R4B4-26
<i>C. jejuni</i>	R4B4-31
<i>C. jejuni</i>	R4B4-32
<i>C. jejuni</i>	R4B4-37
<i>C. jejuni</i>	R7B1-01
<i>C. coli</i>	
<i>C. coli</i>	ATCC 33559
<i>C. coli</i>	ATCC 43133
<i>C. coli</i>	ATCC 43473
<i>C. coli</i>	ATCC 43485
<i>C. coli</i>	ATCC BAA1061

(continued)

TABLE 4. (CONTINUED)

Campylobacter spp.	ATCC or PNL No.
<i>C. coli</i> ^b	20G12
<i>C. coli</i>	21A-34
<i>C. coli</i>	21F-42
<i>C. coli</i>	20A64
<i>C. coli</i>	20A55
<i>C. coli</i>	21B14
<i>C. coli</i>	21B52
<i>C. coli</i>	21B53
<i>C. coli</i>	21B62
<i>C. coli</i>	21F34
<i>C. coli</i>	R11B1-06
<i>C. coli</i>	R11B1-13
<i>C. coli</i>	R11B1-19
<i>C. coli</i>	R11B1-21
<i>C. coli</i>	R11B5-33
<i>C. coli</i>	R4B2-10
<i>C. coli</i>	R4B2-15
<i>C. coli</i>	R4B2-19
<i>C. coli</i>	R4B2-31
<i>C. coli</i>	R7B1-44
<i>C. coli</i>	R7B3-75
<i>C. coli</i> ^c	N41652
<i>C. lari</i>	
<i>C. lari</i>	ATCC 35221
<i>C. lari</i>	ATCC 35222
<i>C. lari</i>	ATCC 43675
<i>C. lari</i>	ATCC BAA1060
<i>C. lari</i>	ATCC 35223
<i>C. lari</i>	R7B2-71
<i>C. lari</i>	R7B5-45
<i>C. lari</i>	20A31
<i>C. lari</i>	R11B5-11

^aSpeciation of the field strains was previously published (Gharst *et al.*, 2013).

^bSpeciation of the field strain was previously published (Liu *et al.*, 2016).

^cSpeciation of the field strain was previously published (Zhao *et al.*, 2016).

ATCC, American Type Culture Collection; PNL, Pacific Northwest Laboratory.

The AB7500F qPCR method was also evaluated by using artificially mixed gDNA of the target species (*C. jejuni* ATCC 33291, *C. coli* ATCC 43479, and *C. lari* ATCC 35222 mixed at 1 pg/species/reaction). Positive qPCR results with acceptable IAC demonstrated that this method simultaneously identified gDNA of all three target species in a single reaction (Table 5). A set of representative multiplex amplification plots is shown in Figure 2.

After assessing the multiplex qPCR with gDNA of pure cultures, artificially contaminated frog legs were used to mimic real food contaminations. Results of the spike-and-recovery study showed that this qPCR method was able to correctly identify spiked *C. jejuni*, *C. coli*, and *C. lari* in frog legs. The lowest detection level was 4, 3, and 2 spiked colony-forming units (CFUs) per 25 g food for *C. jejuni*, *C. coli*, and *C. lari*, respectively. Culture of frog legs spiked with background flora but without *Campylobacter* spiking (blank control) yielded negative qPCR results with acceptable IAC, clearly showing that the positive qPCR signals detected in spiked food matrix came from target *Campylobacter* spp.

TABLE 5. QUANTITATIVE POLYMERASE CHAIN REACTION RESULTS BY USING ARTIFICIALLY MIXED GENOMIC DNA OF THE THREE TARGET SPECIES

Sample	Mean C. j Ct	SD C. j Ct	Mean C. c Ct	SD C. c Ct	Mean C. l Ct	SD C. l Ct	Mean IAC Ct	SD IAC Ct
Mixed gDNA	28.80	0.12	25.81	0.06	28.05	0.18	27.36	0.37
qPCR negative control (ddH ₂ O)	NA	NA	NA	NA	NA	NA	27.42	0.15

Mean and SD were calculated from triplicates.

C. c., *Campylobacter coli*; *C. j.*, *Campylobacter jejuni*; *C. l.*, *Campylobacter lari*; Ct, cycle threshold; gDNA, genomic DNA; IAC, internal amplification control; NA, not applicable; qPCR, quantitative polymerase chain reaction; SD, standard deviation.

instead of the matrix itself. In addition, the results by using the VITEK MS microbial ID system confirmed that the *Campylobacter*-like isolates post-enrichment came from the spiked species whereas there was no colony isolated from the blank control (Table 6).

Discussion

qPCR has great advantages over traditional PCR (Valasek and Repa, 2005). We have previously developed a SmartCycler-based qPCR method, which allows simultaneous identification for *C. jejuni*, *C. coli*, and *C. lari* in a single reaction (Liu *et al.*, 2017). To address the recent discontinuation of the SmartCycler by its manufacturer, a follow-up study is reported here with an optimized multiplex qPCR on AB7500F as the replacement. Widely used in laboratories, the AB7500F system has greater capacity than the SmartCycler (96 wells vs. 16 wells per instrument). Here, a unique com-

bination of target genes was employed for *Campylobacter* spp. identification (*hipO* for *C. jejuni*, *ceu* for *C. coli*, and *pep* for *C. lari*). Performance of the new method was similar to the SmartCycler method regarding qPCR specificity (100% exclusivity and inclusivity on both platforms with the stains tested) and sensitivity (4, 7, and 2 genome copies for *C. jejuni*, *C. coli*, and *C. lari*, respectively, on the AB7500F platform vs. 2, 3, and 2 genome copies on the SmartCycler platform).

In this study, the strains used for exclusivity test were the same as those used for the SmartCycler method (30 non-*Campylobacter* ATCC strains from 29 species in 23 genera, as well as 7 ATCC strains from 4 non-target *Campylobacter* spp. *C. fetus*, *C. iguaniorum*, *C. hyointestinalis*, and *C. upsaliensis*). Notably, compared with the 32 *Campylobacter* strains used for evaluation of the SmartCycler qPCR, more field strains were used for the inclusivity test in this study, resulting in a total of 86 target strains (50 from *C. jejuni*, 27 from *C. coli*, and 9 from *C. lari*). No cross-reactivity was

FIG. 2. Representative multiplex qPCR amplification plots using 1:1:1 artificially mixed gDNA of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* strains. X axis, qPCR cycle number. Y axis, delta Rn, normalized fluorescence value subtracted by baseline. Arrows indicate the Ct values of *C. jejuni*, *C. coli*, *C. lari*, and IAC in this experiment. Ct, cycle threshold; gDNA, genomic DNA; IAC, internal amplification control; qPCR, quantitative polymerase chain reaction. Color images are available online.

TABLE 6. RESULTS OF A REPRESENTATIVE SPIKE-AND-RECOVERY EXPERIMENT

Sample description	Spiked strains	Lowest detection level (CFUs/25 g)	Target Ct	IAC Ct	Correct ID by qPCR?	Confirmation by VITEK ® MS
25 g Frog leg spike	<i>Campylobacter jejuni</i> 33291	4	<i>C. j</i> =28.24	30.05	Yes	<i>C. jejuni</i>
25 g Frog leg spike	<i>Campylobacter coli</i> 43485	3	<i>C. c</i> =21.45	30.09	Yes	<i>C. coli</i>
25 g Frog leg spike	<i>Campylobacter lari</i> 35223	2	<i>C. l</i> =22.93	32.21	Yes	<i>C. lari</i>
25 g Frog leg (spike blank control)	NA	NA	Undetermined	31.17	NA	NA
qPCR positive control (ATCC gDNA)	NA	NA	<i>C. j</i> =24.02; <i>C. c</i> =23.01; <i>C. l</i> =24.18	34.99	Yes	NA
qPCR negative control (ddH ₂ O)	NA	NA	Undetermined	29.12	NA	NA

Lowest detection level was rounded to the next integer.

ATCC, American Type Culture Collection; *C. c*, *C. coli*; *C. j*, *C. jejuni*; *C. l*, *C. lari*; CFU, colony-forming unit; Ct, cycle threshold; gDNA, genomic DNA; IAC, internal amplification control; ID, identification; NA, not applicable; qPCR, quantitative polymerase chain reaction.

detected at the species level by using gDNA of a wide range of other bacterial genera or non-target species in the same genus *Campylobacter*. All the 86 target strains, including 14 ATCC references and 72 field strains, were all correctly identified by the AB7500F-based multiplex qPCR method, indicating potential applications of this method to surveillance studies and outbreak investigations associated with *Campylobacter* food poisoning.

Typically, qPCR efficiency lies between 90% and 110%. In this study, after optimization on target gene, primer concentration, annealing temperature, and concentration of Taq polymerase, the efficiency of *C. coli* amplification was 86.4% (Fig. 1B, inset). However, satisfactory results were obtained for specific and sensitive identification of the target species by using gDNA from pure cultures or spiked food. Better qPCR performance might be achieved in future studies with higher efficiency after more systematic optimizations while keeping detection of the other two species uncompromised.

In addition to the assessment with gDNA of pure cultures, artificially contaminated frog legs were used for method evaluation to mimic real conditions. Edible frog is an aquatic animal product regulated by FDA (2017) and is widely consumed by people in Europe, Asia, Africa, and the United States (Onadeko *et al.*, 2011; Akinyemi and Ogaga, 2015; NOAA, 2017). In 2017, 3080 metric tons of edible frogs were imported to the United States for food consumption with a value of \$12.5 million (NOAA, 2017).

Campylobacter-like bacteria have been isolated from northern leopard frogs (Gossling *et al.*, 1982). A *C. fetus* infection is associated with consumption of frog meat (Patrick *et al.*, 2013). *Campylobacter* spp. are also found in surface water (Nilsson *et al.*, 2017, 2018; WHO, 2019) and other aquacultures (Soonthornchaikul and Garelick, 2009). Therefore, there is possibility that edible frogs are contaminated by *C. jejuni*, *C. coli*, and *C. lari*, which may result in sporadic infections and outbreaks threatening public health. The AB7500F-based qPCR assay and the enrichment procedure developed here have laid the foundation to identify *C.*

jejuni, *C. coli*, and *C. lari* from contaminated foods and ensure the United States' food safety.

As background flora in food matrices have been shown to inhibit growth of target bacteria (Vold *et al.*, 2000; Lardeux *et al.*, 2015; Biesta-Peters *et al.*, 2019), enrichment procedures specific for *Campylobacter* recovery are important for food examinations. In this study, an effective procedure was developed to recover *C. jejuni*, *C. coli*, and *C. lari* at low abundance from refrigerated bone-in frog legs. Even after aging at 4°C for 2 days, the AB7500F qPCR method was able to correctly detect the target species from 10-time background flora. The lowest detection levels were 2–4 CFUs per 25 g food matrix, which were similar to those obtained by using gDNA of pure cultures (Table 6 vs. Table 2). In addition, the VITEK MS results confirmed that the *Campylobacter*-like colonies isolated from enrichment cultures were the spiked species (Table 6), showing that this enrichment procedure facilitates the recovery of target *Campylobacter* spp. more than bacterial flora in spiked frog legs. The unique combination of GasPak™ sachet for microaerophilic growth, Combibag for food handling, and BBL *Campylobacter* Agar for colony isolation ensured the traceability and improved the ease of use in food safety laboratories.

In summary, this qPCR method in combination with the enrichment procedure shows great potential as a rapid, specific, and sensitive diagnostic tool for both pure cultures and foods contaminated with *C. jejuni*, *C. coli*, and *C. lari*.

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