Development and Evaluation of Real Time PCR for Detection and Identification of C. jejuni and C. coli from Human Food

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Abstract

The current study aimed to develop, optimize, and evaluate the real-time polymerase chain reaction (qPCR) specificity and sensitivity on pure isolated strains of *Campylobacter* and on enriched broth for direct detection of these cells. To achieve this, *HipO* and *CadF* gene were purchased to target *C. jejuni* and *C. coli*, respectively. Using chessboard titration method, three critical reagents concentration of qPCR were optimized and described. After that, 130 of collected isolates from food samples were confirmed by the developed protocols. Lastly, 27 enriched broths culture-positive for *Campylobacter spp.* were subjected to direct qPCR. The earlier results indicated that the diluted DNA templates improved the amplification plot intensity (ΔRn 225.000) and the amendment of the amount of specific primers, probes, as well as the concentration of $MgCl_2$ on the amplification curves affected positively the fluorescence signal for both *C. jejuni* and *C. coli* established protocols. The confirmation of 130 of *C. jejuni* and *C. coli* isolated from broiler chickens, turkeys, cattle, and handling surface swabs by qPCR optimized protocols demonstrated similar outcomes as phenotypic tests. From 27 broiler chickens' samples, *C. coli* were the most isolated strain from culture method, biochemically confirmed, compared to *C. jejuni*. In contrast, the application of qPCR directly on enriched broth revealed higher percentage (74.07%) of co-existence of *C. coli* and *C. jejuni* from the same analyzed samples. The present study developed and validated reliable, rapid, robust, and specific qPCR method for screening, quantifying, and confirming *Campylobacter* in food safety monitoring.

1. Introduction

Campylobacter is a Gram-negative, microaerobic, non-spore forming, and curved bacilli, that have spiral movement by a polar flagellum present on one or both ends of cells (Gupta, 2006; Facciolà et al., 2017). They normally colonize gastrointestinal tracts of warm-blooded animals such as birds, cattle, sheep, and pigs. This zoonotic genus is transmitted to human through direct contact with animals harboring these germs and also through consumption of contaminated milk, drinking water, and meats especially poultry meats (Bronowski et al., 2014; Chon et al., 2018; El Baaboua et al., 2018). Indeed, *Campylobacter* can grow at temperature of 37°C, but the most dominant and reported species (*C. jejuni* and *C. coli*), in both human stools and food samples, tends to grow at temperature of 42°C (thermophilic organisms), which is usually offered by poultry (Aroori et al., 2013). The ingestion of these micro-organisms lead to an acute enteritis called campylobacteriosis, whose symptoms vary from person to another; like fever, nausea, vomiting, stomach cramps, and bloody diarrhea (Baaboua et al., 2017). The infection evolution is often benign, but post-infectious complications, in case of extra intestinal infections, can occur to provoke Guillain-Barre syndrome, Miller Fisher syndrome in high risk individuals (neonates, elderly, and immune-compromised patients) (Nyati & Nyati, 2013).

Within it taxonomy history, *Campylobacter* was difficult to culture and differentiate from *Vibrio*, *Helicobacter*, and *Arcobacter* genus (Corry et al., 2003). For that purpose, many techniques have been reported to detect, confirm, and characterize *Campylobacter* including phenotypic and genotypic methods (Baaboua et al., 2021; Banowary et al., 2015; Duarte et al., 2016). In fact, the phenotypic methods of these

bacteria have several limitations due to the lack of discriminatory tests between species, disability to detect Viable But Non-Culturable state (VBNC) of *Campylobacter* that may appear under unfavorable conditions, and the fact that these cells are biochemically inert (Duarte et al., 2016; Baaboua et al., 2017). Nowadays, Polymerase Chain Reaction (PCR) is a powerful molecular tool, founded on the use of genetic suitcase to obtain precise information about studied germs throughout the world. Wide variety types of *Campylobacter* PCR were evolved, include conventional, real time or quantitative PCR (qPCR), multiplex, and Reverse Transcription PCR (RT-PCR), regularly used for detection, quantification, identification, and characterization of *Campylobacter* genes (Bui et al., 2012; Banowary et al., 2015; Banting et al., 2016). Interestingly, the international monitoring organizations of pathogens in food, human, and animals such as European Food Safety Authority (EFSA), Food and Drug Administration (FDA), World Health Organization (WHO), and Centers of Diseases Control and Prevention (CDC), and others, have been strongly recommended, their laboratory networks to use efficient approaches like PCR, when reporting prevalence and antibio-resistance profiles of *Campylobacter* (Hurd et al., 2012; CDC, 2019).

In Morocco, the interest in *Campylobacter* species started very recently, whereof it seems to be necessary, in both clinical and microbiological laboratories, to develop reliable, sensible, and fast methods for their own purposes. Therefore, the aim of the present study was (**i**) to develop and optimize real-time PCR technique in the Regional Laboratory for Analysis and Research (RLAR) in Tangier, Morocco by using reference strains of *C. coli* and *C. jejuni*, (**ii**) to confirm *Campylobacter spp.* isolates during this project, and eventually (**iii**) to apply directly the developed protocols on enriched human food samples.

2. Materials And Methods

2. 1. Bacterial Strains And Growth Conditions

So as to test the specificity and selectivity of the primers, *C. jejuni* (ATCC® 29428™) and *C. coli* (ATCC®43478™) were initially purchased from the American Type Culture Collection and revitalized following the description in type strains technical sheet. The pure colonies were stored at -70°C in brain heart infusion (BHI) broth (Biolife, Italiana, Milano-Italy) containing 25% (v/v) of glycerol (BDH, Poole, United Kingdom). The cells were recovered from − 70°C and grown on Columbia blood agar plates (Biolife, Italiana, Milano-Italy) supplemented with 5% (v/v) defibrinated horse blood and incubated microaerobically (5% O_2, 10% CO_2, 85% N_2) at 42°C for 48 h.

2.2. Genomic DNA extraction

Few fresh colonies of *C. jejuni* and *C. coli* were removed from Columbia blood agar plates and placed into in microcentrifuge tubes, separately, containing 1 mL of Phosphate Buffered Saline (PBS) solution in order to obtain a turbidity of McFarland tube No. 1.0. The mixture was homogenized by vortexing for minute. The aliquots of 1 mL of cultures were concentrated at 14 000 rpm for 3 min. After that, the total genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, cat no. A1125, Madison, USA) following the manufacturer's instructions.

2.3. Real-time PCR Assays

2.3.1. Probes and primers

Two primers sets were evaluated in this study, which were previously designed and provided by *Oligonucleotide Information, Bio Basic Canada Inc*, for detection and confirmation of two *Campylobacter* species. The *HipO* primers (123 pb) were customed to target the *HipO* gene encodes for hippurate hydrolase enzyme of *C. jejuni* and *CadF* primers (103 pb) were chosen to hunt the *CadF* gene, outer membrane fibronecting binding protein of *C. coli* (Table 1). The lyophilized primers and probes were rehydrated by adding deionized water diethyl pyrocarbonate (DEPC), following the manufacturer's recommendations to obtain 100 µM primary concentration of primers and probes. Each oligonucleotide was aliquoted and stored at -20°C for future use.

Table 1
Characteristics of primers and probes used for detection of *C. jejuni* and *C. coli*

Oligonucleotide	**Sequence (5'◊3')**	**T_m (°C)**	**GC (%)**
CJ_HipO_Forward	AAT GCA CAA ATT TGC CTT ATA AAA GC	52.31	30.77
CJ_HipO_Reverse	TNC CAT TAA AAT TCT GAC TTG CTA AAT A	50.73	26.79
CJ_HipO_Probe	**JOE**-ACA TAC TTC TTT TTT ATT GCT TG-**BHQ1**	47.11	30.43
CC_ CadF_Forward	GAG AAA TTT TAT TTT TAT GGT TTA GCT GGT	51.45	26.67
CC_ CadF_Reverse	ACC TGC TCC ATA ATG GCC AA	56.73	50
CC_ CadF_Probe	**CY3**-CCT CCA CTT TTA TTA TCA AAA GCG CCT TTA GAA A-**BHQ2**	58.23	35.29

CJ: *Campylobacter jejuni*; **CC**: *Campylobacter coli*.

2.3.2. qPCR assays for *C. coli* and *C. jejuni*

To set up *C. jejuni* and *C. coli* amplification mixture, we followed some descriptions established by Toplak et al. (2012). Briefly, for total amplification volume of 25 µL, two reactions were performed; each of them contained 1 X of PCR gold buffer (10 X), 0.4 mM of deoxyribonucleoside triphosphates dNTPs (10 mM), 4 mM of magnesium chloride $MgCl_2$ (25 mM), 1 µM of *C. jejuni*-specific primers (10 µM), 0.2 µM of probe, 1.25 U/µL *Taq* polymerase. Only the DNA concentration was different in this first assay (2 µL of pure DNA extracted and 2 µL of 1/200 diluted DNA). The final volume was completed by Clinical Laboratory Reagent Water. Regarding the protocol of *C. coli*, the optimized concentrations of *C. jejuni* protocol were used for the first assay of *C. coli* and modified thereafter.

2.3.3. Thermal cycling conditions

The choice of thermal cycling conditions was carried out taking into account the notes in the paper named “the basic protocol plus troubleshooting and optimization strategies” introduced by Lorenz, (2012). Thus, the initial denaturation was performed at 95°C for 10 min and 45 cycles; in which each cycle consisting of denaturation at 95°C for 15s, annealing at 60°C for 1 min, and followed by elongation at 72°C for 30s. Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster city, USA) was used to collect the residual fluorescence emitted from hydrolysis of *Joe* and *Cy3* dyes and the quantitative results of real-time PCR were assessed on threshold cycle values (C_t). The reaction was considered positive with $C_t \leq 36$ and negative with $C_t > 36$.

2.4. Optimization of qPCR protocol

2.4.1. *HipO* gene for *C. Jejuni* detection

After the success of the first amplification assay of *C. jejuni*, and in order to enhance the amplification plots and also to economize the qPCR reagents, the chessboard titration method was performed to establish the correct working concentrations of each reagent (Gunson et al., 2003). Three critical points were optimized, in which buffer, 1/200 diluted *C. jejuni* DNA template, and dNTPs concentrations were the same. However, in the second assay, we checked out the concentrations of *C. jejuni*-specific forward and reverse (1.2; 0.8; and 0.4 µM) depending on *C. jejuni*-specific probes (0.08; 0.06; 0.04 µM), the amount of $MgCl_2$ (4 and 5 mM), as well as the probe concentrations of *C. jejuni*-probe (0.06; 0.08; and 0.12 µM).

2.4.2. *CadF* gene for *C. Coli* detection

The amendment of *C. coli* qPCR protocol was involved in two critical steps, applying chessboard titration to assess the suitable concentrations of *C. coli*-specific primers and probes, as mentioned in Table 2.

Table 2
Chessboard titration for amendment of *C. coli* qPCR protocol

Reagents	1st assay	2nd assay	3rd assay	4th assay
PCR gold buffer (10 X)	1 X			
dNTPs (10 mM)	0.4 mM			
$MgCl_2$ (25 mM)	5 mM			
Taq polymerase (5U/µL)	1.25 U/µL			
C. coli-specific forward (10 µM)	0.4 µM	0.8 µM		
C. coli-specific reverse (10 µM)	0.4 µM	0.8 µM		
C. coli-specific probe (1 µM)	0.04 µM	0.04 µM	0.08 µM	0.12 µM

2.5. Validation of *C. jejuni* and *C. coli* qPCR developed protocols

2.5.1. Confirmation of *Campylobacter* pure isolates

From July 2015 to June 2018, 464 of meat samples, in Northern of Morocco, were analyzed according to the horizontal method for detection and enumeration of *Campylobacter* in foods, following Moroccan standard NM ISO: 10272-1 (2008) with slight modifications (El Baaboua et al., 2021). 130 suspicious *Campylobacter spp.* isolates were collected, mainly, from *Campylobacter* blood base agar plates (Biolife, Italiana, Milano-Italy) containing 5% defibrinated horse blood and antimicrobial supplement (polymyxin B, cycloheximide, rifampicin and trimethoprim). Using the phenotypic tests recommended by NM ISO: 10272-1 (2008), the suspected colonies were biochemically confirmed as *C. jejuni* and *C. coli*, and subjected to molecular confirmation using the selected profile of qPCR protocols for each strain.

2.5.2. Direct application of qPCR on positive enrichment broths for *Campylobacter spp.*

Parallel to the isolation step, the enriched broiler chickens' broths samples were stored at -20°C. After biochemical and molecular confirmation of *Campylobacter* species, 1 mL of 27 stored broths positive samples for *Campylobacter* underwent DNA extraction as previously described. The DNA templates were tested, for the presence of *C. jejuni* and *C. coli*, at the same time, according to the aforementioned amplification procedures.

2.6. Data analysis

Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA, 2007) was used to calculate percentages of strains identified and recovered from culture and qPCR methods.

3. Results And Discussion

The present study aimed to develop and optimize real-time PCR technique in the regional laboratory for analysis and research in Morocco, useful for laboratory practice, to confirm *Campylobacter spp.* isolates, as well as direct application of these developed protocols on enriched food samples broths.

3.1. Real-time PCR Assays

Campylobacter species are slow-growing, in which their recovery procedure can take place up to one week, hence the need for evaluation of time-saving, specific, and sensitive technique such as qPCR. The first assay of *C. jejuni* qPCR intended to establish the initial amplification mixture that included two reactions (Fig. 1). The results showed the pure extracted DNA of *C. jejuni* started to be amplified from the cycle of 11 (Ct_1), while the 1/200 diluted DNA (Ct_2) begun the amplification from the cycle of 19. The diluted DNA template improved more the amplification intensity (ΔRn 225.000) than that noticed in the initial DNA concentration (ΔRn = 180.000). Experimentally, the dilution treatment of extracted DNA was carried to remove PCR chemical inhibitors (e.g. ethanol, isopropanol, chloroform, phenol, etc.) left in after DNA extraction operation and therefore enhance the efficacy of PCR amplification plot. McCord et al.

(2014) were analyzed a variety of PCR parameters like dilution, increasing polymerase, Bovine Serum Albumin (BSA), and magnesium concentration on the amplification curves inhibitory effects and found that dilution decreased the concentration of the DNA sampled at the same time as the inhibitors. Other than the effect on C_t value, the DNA serial dilutions demonstrated an improvement in DNA detection sensitivity, which is approximately 1 ng/µL of concentration (Fontanot et al., 2014).

3.2. Optimization of qPCR

3.2.1. *HipO* gene for *C. jejuni* detection

A PCR effectiveness value is reflected by the relative increase in fluorescence in the exponential phase of the amplification curve that can be varied through the concentration of one or more of PCR components. During the optimization tests, three parameters were evaluated; the impact of the amount of *C. jejuni* specific primers, probe, as well as the concentration of $MgCl_2$ on the amplification curves. The Fig. 2 indicates that the increase in concentrations of *C. jejuni*-specific forward, reverse, and probe (1.2; 1.2; 0.08 µM) affected positively the fluorescence signal, with the same threshold cycle (C_t = 19) of the 1/200 diluted DNA template of *C. jejuni*. Moreover, the optimization of the amount of $MgCl_2$ (5 mM), primers (0.4 µM), and the probe (0.12 µM) showed a great amplification profile compared to amplification curve illustrated in Fig. 2A (ΔRn = 325.000 versus ΔRn = 120.000, respectively). In their comparative investigation about fluorescent-increase kinetics of different fluorescent reporters used for qPCR depend on monitoring chemistry, targeted sequence, type of DNA input, and PCR efficiency, Ruijter et al. (2014) found that the PCR efficiency of hydrolysis probes and LUX primers, compared to DNA-binding dyes do not require a correction of the observed quantification cycle, in which the results were directly visualized without need to an over estimation. In this study, the technology of hydrolysis probes was used and proved similar efficiency.

Obviously, magnesium ion (Mg^{2+}) is required co-factors for DNA polymerase and concentration threshold of this ion has an inhibitory impact on DNA polymerase enzyme. An excessive amount of $MgCl_2$ may prevent a complete denaturation of DNA template through stabilizing the duplex strand DNA and subsequently reduce the amplicons produced (Lorenz, 2012). Hence, an adequate concentration of this divalent cation (between 0.5 to 5.0 mM) is crucial for successful DNA detection (Datta & LiCata, 2003; Cai et al., 2018). Indeed, the appropriate amount of Mg^{2+} in each amplification protocol depends, particular, upon master mix reagents (i.e. type of polymerase, dNTPs concentration, primers, and impurities in DNA used) (Gouvea et al., 1990; Roux, 2009). In regards of our situation, for both *HipO* and *CadF* gene, 5 mM of magnesium chloride ion was the optimal concentration related to our reagents used in this study to obtain a sigmoid curve of amplification.

3.2.2. *CadF* gene for *C. coli* detection

Based on *C. jejuni* founded protocol, the chessboard titration was carried out in amendment of *C. coli* qPCR reaction mix. The findings in Table 3 indicated negative amplification, when using small

concentrations of *C. coli*-specific primers and the probe (0.4 µM and 0.04 µM respectively). An increased in *C. coli*-specific forward and primers (0.8 µM of each sequence and 0.04 µM of probe) presented a positive qPCR reaction, reflected by the fluorescence signal reached at ΔRn equal to 3500. In addition, more that the amount of probe introduced was higher (up to 0.12 µM), more that the sigmoid curve of aplification was observed (ΔRn 7000).The comparison of *C. jejuni* and *C. coli* amplification curves found that the specific primers of *C. jejuni* offered a higher level of fluorescence compared with *CadF* primers of *C. coli* (ΔRn = 325.000 against ΔRn 7000, respectively).

Table 3
Amendment results of *C. coli* specific primers and probes

	1st assay	2nd assay	3rd assay	4th assay
Ct	28			
Fluorescence level (ΔRn)	N/A	3500	5000	7000

Basically, *HipO* and *CadF* primers sets were designed to hunt and identify exclusively *C. jejuni* and *C. coli* respectively among other bacterial species, in which the real time PCR data will serve for further analysis. Primers characteristics (length, CG contents, coverage, 3' end contain strong bases (C or G), and melting temperature (T_m)) are additional criteria that should be taken into consideration when studying the qPCR efficiency (Ricke et al., 2019). Forward and reverse primers must present a greater content of CG (40–60%) to form strong hydrogen bonds between the primer and the DNA template (Iwai et al., 2011; Jaric et al., 2013). Based on the comparison of the primer's quality of *C. jejuni* and *C. coli* target genes in this investigation, the findings revealed incompatible content of CG between reverse and forward of *CadF* gene (Table 1). The quality of *CadF* primers may be one of the reasons affected the fluorescence level of *C. coli* obtained despite the doubled amount of primers introduced in the amplification mixture. Gardès et al. (2012) strongly recommended the bioinformatic analyses of primers, before their use, to validate the choice and thus avoid or improve their specify use in molecular detection tests.

Moreover, PCR works efficiently only within the overlapping of temperature range defined by the primers and thermal cycling conditions used. If these temperatures were not respected, secondary structures may occur at primer-template binding at higher temperature and also mismatch interactions that could be favored when primers with high T_m values in PCR reactions run at low annealing temperatures (Naqib et al., 2019). In our case, the use of 1% agarose gel electrophoresis for the qPCR products (data not shown) indicated the presence of specific and intense bands of each qPCR products of *CadF* and *HipO* gene, which means that the primers were bound perfectly with the DNA of each target.

3.3. Validation of *C. jejuni* and *C. coli* qPCR developed protocols

3.3.1. Confirmation of *Campylobacter spp.* isolates

After optimization, the qPCR retained protocols of *C. jejuni* and *C. coli* were as follow; 2 µL of diluted DNA solution, 1X PCR gold buffer (10X), 0.4 mM of dNTPs, 5 mM of $MgCl_2$ and 1.25 U/µL of *Taq* polymerase. The concentrations of primers were different for each strain, so that 0.4 µM and 0.12 µM of each primer and *C. jejuni*-specific probe in *C. jejuni* reaction mixture, while, 0.8 µM and 0.12 µM of *C. coli*-specific primers were the most favorable amount in the amplification mixture of *C. coli*. The application of qPCR optimized protocols on 130 of *C. jejuni* and *C. coli* isolated from variety type of food (broiler chickens, turkeys, cattle, and handling surface swabs) were 100% positive for *C. coli* (108/108) and for *C. jejuni* (22/22), similar outcomes as found by phenotypic tests (Table 4). The results proved also the dominance frequency of *C. coli* than *C. jejuni* in Moroccan food samples.

Table 4
qPCR confirmation of *Campylobacter spp.* isolated from food samples

Samples	Biochemical identification N (%)		qPCR confirmation N (%)	
	C. coli (N = 108)	C. jejuni (N = 22)	C. coli (N = 108)	C. jejuni (N = 22)
Broiler chickens	71 (65.74)	13 (59.09)	71 (65.74)	13 (59.09)
Turkeys	25 (23.15)	4 (18.18)	25 (23.15)	4 (18.18)
Cattle	7 (6.48)	3 (13.64)	7 (6.48)	3 (13.64)
Swabs	5 (4.63)	2 (9.09)	5 (4.63)	2 (9.09)

According to the interpretive summary of food samples analyzed, indicated in Moroccan standard (NM 10272-1: 2008), the presence of *Campylobacter* colonies make specimens unsuitable for human consumption. *C. jejuni* is the dominant reported specie of *Campylobacter* genus, mainly in broiler chickens and turkeys meat samples (Chon et al., 2018). However, further investigations confirmed that *C. coli* was more frequent compared to *C. jejuni* in meat samples (Guirin et al., 2019; D. Liu et al., 2019). These findings were similar to those indicated in the present project. Indeed, scientists suggested that the difference in the recovery of *Campylobacter spp.* can be awarded to culture conditions, seasons, types and stage of samples treatment, or also phenotypic techniques used (Butzler, 2004; Iannetti et al., 2020). Owing to the *Campylobacter* inert biochemical profile, few phenotypic tests were described to differentiate those species (Baaboua et al., 2017). From the recommended biochemical tests in NM ISO 10272-1 (2008), hippurate hydrolysis was used and showed similar sensitivity with our molecular results (the presence of *hipO* gene in suspected *C. jejuni* isolated strains), which makes this test remains useful phenotypic criterion to differentiate *C. jejuni* from other species (Liu et al., 2020). Similar findings were also noticed for the confirmation of *C. coli*. Consequently, both methods had proportional sensitivity and efficiency in this study.

3.3.2. Direct application of qPCR on positive enrichment broths for *Campylobacter*

The developed protocols of qPCR were directly applied on 27 enriched broths samples positive for *Campylobacter*, the findings were summarized in Table 5. From 27 broiler chickens' samples, *C. coli* (77.78%) were the most isolated strain from culture method, biochemically confirmed, compared to *C. jejuni* (22.22%). Nonetheless, the application of qPCR directly on enriched broth revealed a higher percentage (74.07%) of co-existence of *C. coli* and *C. jejuni* in the same analyzed samples, which means that the positive samples, for only *C. coli* by culture method, could be also positive for *C. jejuni*, whose was not selected and/or isolated. It is undeniable that culture technique has a less sensitivity than real time PCR method (Singh et al., 2011; Bessède et al., 2018). This was also noticed in the present work, whereby the qPCR detected higher percentage of both researched genes comparable to culture technique that recovered importantly *C. coli*. Thus far, real-time PCR is the most sensitive and reliable tool for the detection of *Campylobacter* genus. In the light of these findings, we suggested the evaluation of multiplex qPCR to include *HipO*, *CadF*, and other genes for an easy and appropriate routine use (El Baaboua et al., 2021). Unlike, the replacement of the culture approach seems to be difficult (Liang et al., 2018; Baaboua et al., 2021).

Table 5
Percentage of *C. jejuni* and *C. coli* recovery from culture and real time PCR

Strain detected	**NM ISO: 10272-1 (2008) N (%)**	**qPCR N (%)**
Only C. jejuni	6 (22.22)	4 (14.81)
Only C. coli	21(77.78)	8 (29.63)
C. jejuni **&** C. coli	0 (0)	20 (74.07)

In order to control and prevent *Campylobacter* infections, real time PCR was used as culture-independent diagnostic test and have proven the ability to detect the VBNC state of *C. jejuni*, commonly recognized as hidden risk factor for campylobacteriosis (Liu et al., 2018; Lv et al., 2020). Likewise, Fontanot et al. (2014) were studied the sensitivity of qPCR method, through artificial contamination of broth inoculated with the same amount of *C. coli* and *C. jejuni* (alone or in a mixture) and concluded that the qPCR detected 10 CFU/mL for both species. Also, the use of this technique in combination with standard method allowed researchers to detect *Campylobacter spp.* in broths at 24h, matter that reduced the time needed using the official ISO :10272-1B (2008) technique (Fontanot et al., 2014). Beyond all of this, recently the technological innovations have surpassed the physicochemical barriers in order to access to the microbial genome easily and faster. Soejima et al., (2016) suggested a novel master mix for direct qPCR to detect genes in a single bacterium with solid cell walls, applied in research and diagnostics without need to DNA extraction.

Besides all these strengths, there are some limitations to note. The detection limits of this method through artificial contamination, DNA quantification when diluted templates, and specificity of *CadF* and *HipO* genes against others microorganisms should be examined in future research.

4. Conclusion

C. jejuni is the main foodborne pathogen implicated in campylobacteriosis that threatens the public health. Hence, the implicated species should be determined as soon as possible, in particular *C. jejuni* and *C. coli*. The earlier results in the current study showed that the diluted DNA templates improved the amplification plot intensity and the amendment of the amount of specific primers, probes of *HipO*, and *CadF* gene, as well as the concentration of $MgCl_2$ upon the amplification curves affected positively the fluorescence signal for both *C. jejuni* and *C. coli* established protocols. Also, it was observed that the amplifications curves of *C. jejuni* primers offered a higher level of fluorescence compared with *CadF* primers of *C. coli* (ΔRn = 325.000 against ΔRn 7000, respectively). This was explained by the high quality of *HipO* primers compared to *CadF* primers. The qPCR confirmation of 130 *C. jejuni* and *C. coli* strains isolated from variety type of food, indicated similar outcomes as found by phenotypic tests. Nevertheless, the application of qPCR directly on enriched broth revealed a higher percentage of co-existence of *C. coli* and *C. jejuni* in the same analyzed samples, which means that the positive samples, for only *C. coli* by culture method, were also positive for *C. jejuni*, whose was not recovered. The present study developed and validated reliable, rapid, robust, and specific qPCR method for screening, quantifying, and confirming *Campylobacter* in food safety monitoring.

Declarations

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This study has not received funding.

Data availability

All used data in this study were cited in the manuscript.

Competing interests

The authors declare that they have no competing interests

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Figures

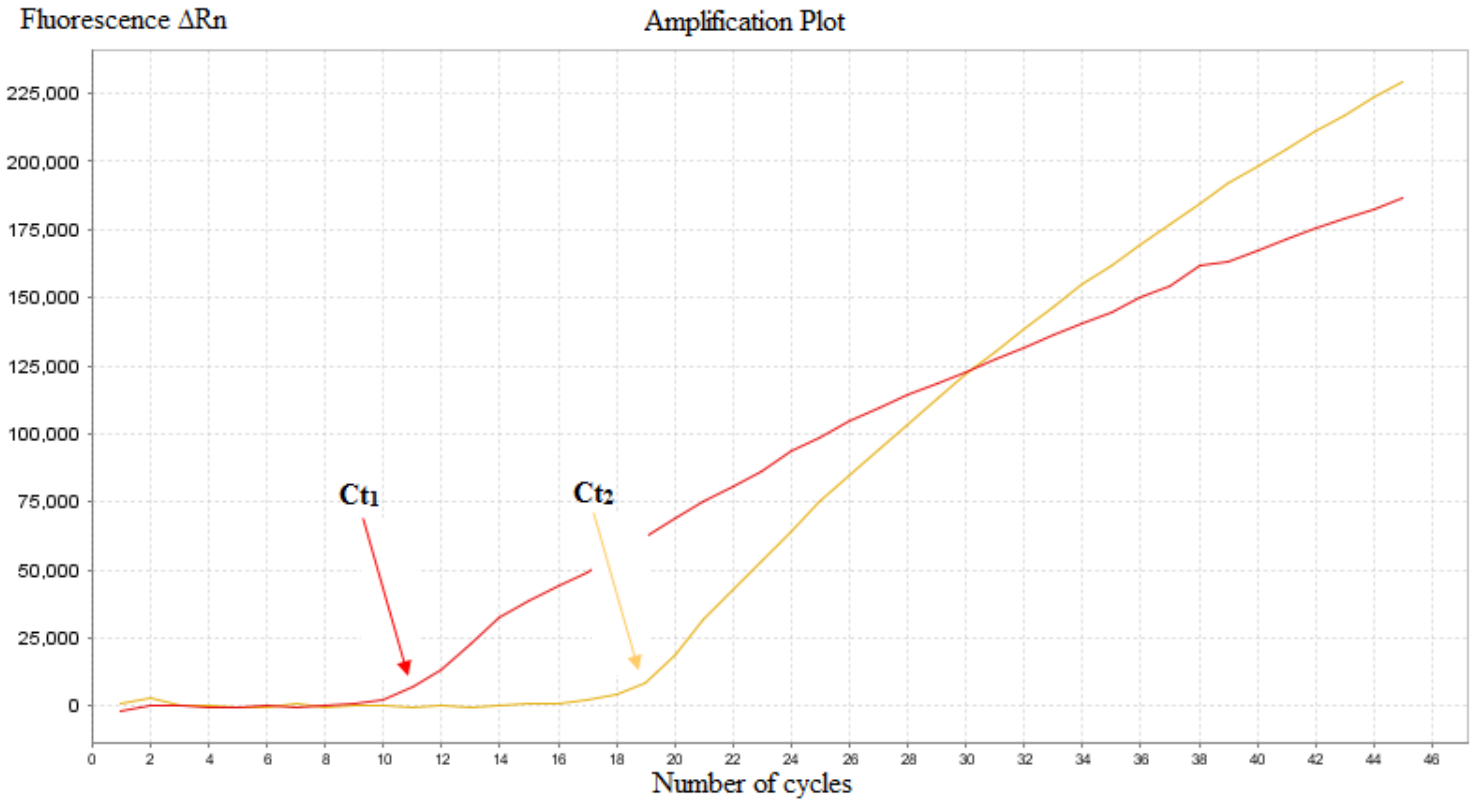

Figure 1

C. jejuni first assay results. **Ct_1**: Concentrated DNA template**, Ct_2:** 1/200 diluted DNA of *C. jejuni.*

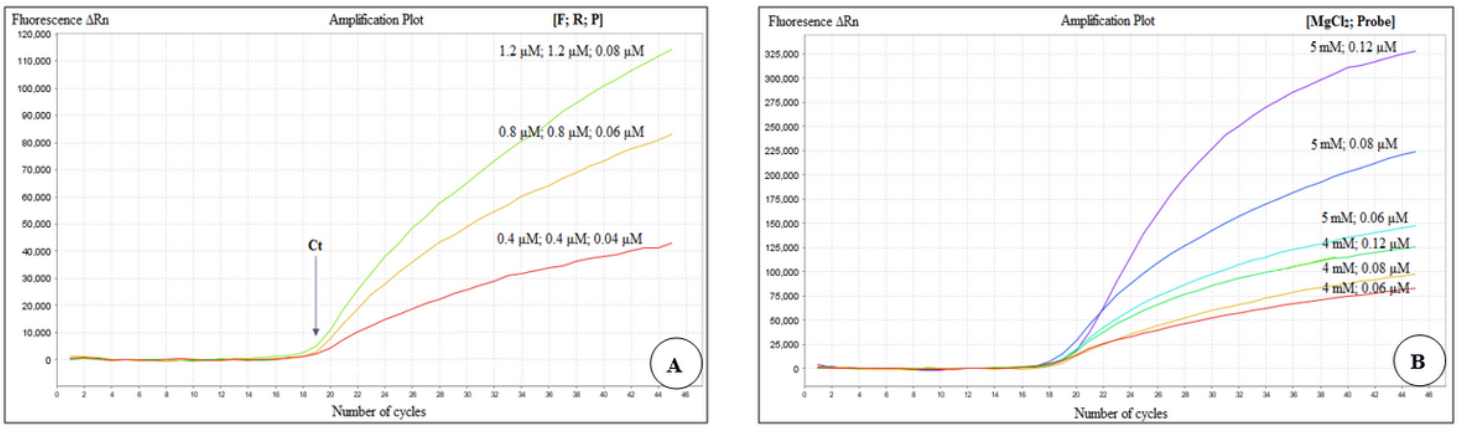

Figure 2

C. jejuni optimization qPCR protocol, according to the impact of primers and probes concentrations (Fig. 2A), and also according to $MgCl_2$ concentrations (Fig. 2B). **F:** forward**; R:** reverse**; P:** probe**.**