COMPARISON, VALIDATION, AND OPTIMIZATION OF INTERNAL GENOMIC DNA EXTRACTION PROTOCOL FOR CAMPYLOBACTER SPECIES

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INTRODUCTION

Campylobacter spp. are Gram-negative, microaerophilic, zoonotic bacteria, and widely known by their pathogenic power that cause human gastroenteritis (campylobacteriosis), with an expensive burden charge on public health (Scharff, 2012). Among Campylobacter species, Campylobacter jejuni (C. jejuni) and Campylobacter coli (C. coli) are the most commonly reported and studied isolates (Del Collo et al., 2017; Gahamanyi et al., 2020). Overall, the disease is self-limiting and can be treated by supportive therapy such as maintenance of hydration and electrolyte balance. Nonetheless, in some cases, the infection may lead to autoimmune syndromes (i.e., Guillain–Barré syndrome and Miller Fisher syndrome), especially in high-risk groups (Skarp et al., 2016; Baaboua et al., 2017). The transmission to human of these thermophilic organisms is made through the consumption of contaminated undercooked meats, drinking water, and raw milk (Budge et al., 2020; Gahamanyi et al., 2020).

For preventing and controlling Campylobacter agents to cause such damages, molecular methods have been widely developed into powerful tools for different applications either in clinical microbiology or in monitoring laboratories for better understanding of their transmission, virulence, surviving factors, and antibiotics resistance mechanisms (Bolton, 2015; Han et al., 2019; Liu et al., 2019). Polymerase Chain Reaction (PCR), Pulse Field Gel Electrophoresis (PFGE), Whole Genome Sequencing (WGS) and others are the typical examples of these techniques (Zou et al., 2010; Chon et al., 2018; Joensen et al., 2020). Indeed, the first common and most important stage of all the aforementioned methods is the genomic DNA extraction. Indeed, numerous DNA extraction protocols and kits commercially available have been described for isolating DNA of Gram-positive and Gram-negative bacteria from biological samples, that were sometimes modified to be more compatible to other microorganisms (Freschi et al., 2005; Dal et al., 2018; Ayana et al., 2019). Basically, the DNA extraction procedure of Gram-negative bacteria is carried out according to four known stages (e.g., Cell lysis, lipids and proteins elimination, DNA wash, and elution) (Bazzicalupo and Fancelli, 1997; Wright et al., 2017).

The efficiency of the DNA extraction protocol was established on the basis of sensitivity of protocols towards the type of bacteria, for example, DNA quality, and purity produced (Leite et al., 2014; Fidler et al., 2020). Moreover, many published PCR-based tests rely on lengthy and expensive methods for isolating the bacterial DNA (Abdelhai et al., 2016). In this context, the present study aimed (1) to evaluate and compare the DNA extraction methods quality yielded from three genomic DNA extraction protocols by using reference strains of Campylobacter, (2) to determine the advantages of the internal DNA extraction protocol in term of rapidness, cost, and efficiency, and eventually (3) to validate and optimize the internal protocol through artificial contamination and confirmation of Campylobacter spp. from broiler chickens, turkeys, and beef meats samples.

MATERIALS & METHODS

Bacterial strains and growth conditions

C. jejuni (ATCC® 29428TM) and C. coli (ATCC®43478TM) were purchased from American Type Culture Collection and cultured as described in type strains section. Briefly, the pellet of each bacterium was inoculated in 7 mL of Bolton broth (Biolife, Italiana, Milano-Italy) supplemented with 5% (v/vol) of defibrinated horse blood and incubated under micro aerobic conditions (5% O2, 10% CO2, 85% N2) at 37 °C for 48 h. A loopful of 10 µL of each broth culture was streaked onto Colombia blood agar plates (Biolife, Italiana, Milano-Italy) and incubated at 37 °C for 48 h. The pure colonies were suspended in Phosphate Buffered Saline (PBS) solution and homogenized by vortex to obtain a turbidity of McFarland tube No. 1.0. In order to prepare a similar range of strains concentration, 1 mL of C. coli and C. jejuni suspension was aliquoted in microcentrifuge tubes and stored at -20 °C until the used.
Internal DNA extraction protocol

Reagents employed

The reagents employed in this study were prepared according to the recommendation provided by cold spring harbor protocols (CSH website, accessed on 6.12.20).

Internal DNA extraction protocol

Because of the cost and the limited number of reactions provided by commercially available DNA extraction kits, an internal protocol for Gram-negative bacteria within the Regional Laboratory for Analysis and Research (RLAR) in Tangier, Morocco was investigated. The internal protocol was proposed according to several research papers (Chen and Kuo, 1993; Freschi et al., 2005; Green and Sambrook, 2017) with slight modifications. In brief, 1 mL of each pure culture was centrifuged for 3 min at 12 000 rpm. The cell pellet was resuspended and lysed in 200 µL of lysis buffer (40 mM of tris acetate (pH: 7.8); 20 mM of sodium acetate; 1 mM of EDTA, and 1% of SDS) by vigorous pipetting. Briefly, 66 µL was added of 5M sodium chloride (NaCl) and mixed well. The mixture was centrifuged for 5 min at 14 000 rpm. The floating solution was transferred into a new vial, and 266 µL of chloroform was added. The mixture was vortexed until a milking solution was formed and centrifuged for 2 min at 14 000 rpm. The aqueous phase containing DNA was transferred in new vial, in which 40 µL of 3M sodium acetate (pH: 5.2) and 600 µL of isopropanol were respectively added and mixed gently by inversion. After that, the solution was centrifuged for 2 min at 14 000 rpm. The pellet was washed by adding 600 µL of 70% of ethanol, and centrifuged at 14000 rpm for 7 min. The pellet obtained was dried at room temperature and finally, the bacterial DNA was eluted in 100 µL of Tris-EDTA (TE) buffer (10 mM of tris-HCl (pH: 8) and 1 mM of EDTA (pH: 8)) and stored at -20 °C.

Comparison of the internal protocol with commercial kits

From the same batch of strains, C. jejuni and C. coli were also extracted, in triplicate, using Pure Link™ Genomic DNA Mini Kit (Invitrogen corporation, Carlsbad, California, USA) and Wizard® Genomic DNA Purification Kit (Promega, cat no. A1125, Madison, USA), following the manufacturer's instructions.

For each DNA extraction protocol, the DNA quality control was checked by using standard agarose gel electrophoresis.20 µL of extracted DNA were loaded in 1% agarose gel containing ethidium bromide (so to have0.5 µg/mL), and running in Tris-Acetate-EDTA (TAE) running buffer for 45 min at 85V. The gel was visualized immediately, after electrophoresis, by Vilber Lourmat TM ultra-violet trans illuminator (Labindia Instruments, Mumbai, India) at 254 nm wavelength.

Real time PCR

qPCR probes and primers

The extracted DNA was amplified using CadF gene encodes for outer membrane fibronectin-binding protein of C. coli and HipO gene encodes for C. jejuni hippurate hydrolase. The oligonucleotides primers and probes used in this study, listed in Table 1, have been provided by Oligonucleotide Information, Bio Basic Canada Inc.

Table 1 Oligonucleotides probes and primers used for C. jejuni and C. coli (Oligonucleotide Information, Bio Basic Canada Inc.)

| Strain | Gene | Oligonucleotide | Sequence (5’→ 3’) | Product Size (bp) |
|--------|------|-----------------|-------------------|------------------|
| C. jejuni | HipO | Forward | AATGCAAAAATTTGCCTATAAAAAGC | 123 |
|         |      | Reverse | TNCCATTTAAATCTGACTGTCGAAATA |   |
|         |      | Probe   | JOE-ACATACTACTTCTTTATCGTT-BHQ1 |   |
| C. coli  | CadF | Forward | GAG AAA TTT TAT TTT TAT GGT TTA GCT GGT | 103 |
|         |      | Reverse | ACC TGC TCC ATA ATG GCC AA |   |
|         |      | Probe   | CY3- CCT CCA CTT TTA TTA TCA AAA GCG CCT TTA GAA A - BBQ1 |   |

DNA amplification

The real-time PCR (qPCR) reaction mixture was evaluated and amended in our laboratory during the study (unpublished data). All qPCR amplification reactions contained, 2 µL of DNA template, 2.5 µL of Gold buffer (X10), 0.4 mM of dNTP, 5 mM of MgCl₂, and 1.25U/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, respectively, for C. jejuni, while 0.8 µM and 0.12 µM of C. coli-specific primers and probe for C. coli. The reaction mixture was prepared by Clinical Laboratory Reagent Water, for a total volume of 25 µL.

Thermal cycling conditions

The amplification was performed with the following thermal cycling conditions: initial denaturation at 95°C for 10 min and 45 cycles; in which each cycle consisting of denaturation at 95°C for 15s, annealing at60°C for 1 min, and followed by elongation at 72°C for 30s. The reaction was conducted in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster city, USA) and quantitative results of real-time PCR were assessed on threshold cycle values (Ct). The reaction was considered positive with C<sub>t</sub>≤36 and negative with C<sub>t</sub> > 36.

Validation of internal protocol

Confirmation of Campylobacter species

From February to June 2018, in Northern of Morocco, forty suspicious Campylobacter spp. isolates were collected according to the Moroccan standard NM ISO: 10272-1 (2008) from Campylobacter blood base agar Plates (Biofile, Italiana, Milano-Italy) containing 5% defibrinated horse blood and antimicrobial supplement (Polymyxin B, cycloheximide, rifampicin and trimethoprim). The suspected colonies recovered from broiler chikens, turkeys, and beef meats samples, were biochemically confirmed as C. jejuni and C. coli, respectively following the recommended tests in the NM ISO: 10272-1 (2008). The pure isolated strains underwent molecular confirmation using the internal DNA extraction process followed by the qPCR procedure as reported previously (Figure 1).

Validation process of internal DNA extraction protocol

Validation diagram of internal DNA extraction protocol

Figure 1 Validation diagram of internal DNA extraction protocol

1 g were added to 8 mL of broth

1 mL of each strain concentration

Incubation at 41.5 °C for 44 ± 4h

Extraction of bacterial DNA using internal protocol

Few suspected pure colonies were suspended in PBS solution

Real time PCR
Artificial contamination of food matrices

Further validation was also examined using enriched matrices artificially contaminated. Beef, turkey, and broiler chicken minced meat samples were purchased from market, randomly, and were sterilized by freezing and ultraviolet exposition methods. Using a PBS solution, 10-fold serial dilution of C. jejuni and C. coli was performed to obtain the concentrations of 10^3, 10^4, and 10^5 CFU/mL. Then, in triplicate, 8 mL of Bolton enrichment broth containing 5% defibrinated horse blood and selective supplement (Cefoperazone, Vancomycin, Trimethoprim lactate, and amphotericin B) was mixed with 1 g of sterilized minced meat, and 1 mL of each reference strains concentrations (1:10), so as to obtain 100, 10 and 1 CFU/mL as final Campylobacter incoomuls concentration in each tube before enrichment. The incubation was achieved under microaerophilic conditions for 44h ± 4h at 41.5 °C. Finally, 1 mL of each enriched broths was subjected to extraction and qPCR as mentioned above (Figure 1).

Optimization of internal protocol

For confirmation of the pure isolates, after culture method, no necessary optimization was needed. Nevertheless, in the artificial contamination of food matrices, three stages in the aforementioned internal DNA extraction protocol were amended and optimized. Firstly, the bacterial pellet was washed three times with PBS solution. Secondly, the lysis step was incubated for additional time at room temperature and lastly, the washing stage was repeated up to three times with ethanol 70%.

Data analysis

Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA, 2007) was used to calculate rate of strains identified by qPCR, the means, and also standard deviation of amplification threshold Ct of each artificially contaminated matrices assay for C. jejuni and C. coli.

![Image](image_url)

Table 2 Main indicators comparison of the three DNA extraction protocols

| Internal protocol | Wizard® Genomic DNA Purification Kit | PureLink™ Genomic DNA Mini Kit |
|-------------------|-------------------------------------|--------------------------------|
| Cost              | Inexpensive                         | Expensive                      |
| Time              | 1h:30 min                            | 1h:35min                       |
| Efficiency        | Similar                              |                                |

The comparison of handling steps of the three DNA extraction methods was closely similar between the three protocols. However, the internal protocol was clearly yielded more advantages than the kits examined in this work. Ruiz-Fuentes et al. (2015) were compared four DNA extraction methods for the detection of Mycobacterium leprae from Ziehl–Neelsen-stained microscopic slides and concluded that the DNA concentration from the Wizard® Genomic DNA Purification Kit was the lowest (1.00±0.18 ng/μL, p ≤ 0.052) among Chelex 100 resin procedure, phenol–chloroform–isoamyl alcohol method, and QIAamp DNA Mini Kit tested. Similar results were observed by Assenmacher et al. (2020). At the best of our knowledge, no published research data, other than the information offered by user guide of this kit, was found describing and/or comparing the advantages or gaps of PureLink™ Genomic DNA Mini Kit for Gram negative or positive bacteria. Despite the appropriate information offered by DNA quality control, this tool consumes a significant amount of DNA (<1–4 ng) (Nikolaev et al., 2018).

Table 3 Identification of Campylobacter spp. isolated from food samples

| Strain (N= 40) | Biochemical confirmation N (%) | qPCR using internal DNA extraction protocol N (%) |
|----------------|-------------------------------|-----------------------------------------------|
| C. coli        | 28 (70)                       | 28 (70)                                       |
| C. jejuni      | 12 (30)                       | 12 (30)                                       |

The dominant specie reported of Campylobacter genus is C. jejuni (Chou et al., 2018). However, additional investigations found that C. coli was more frequent compared to C. jejuni in meat samples (Guirin et al., 2019; Liu et al., 2019). These findings were similar to those indicated in the present study. Indeed, researchers suggested that the difference in the recovery of Campylobacter spp. can be atributed to culture conditions and method used, phenotypic techniques, seasons, or also types and stage of samples treatment (Butzler, 2004; Iannetti et al., 2020). Owing to the inert biochemical profile of this genus, few phenotypic tests were described to differentiate, mainly, between C. jejuni and C. coli such as hippurate hydrolysis. Despite the false negative and/or positive results that occasionally come across, this test remain useful for some research laboratories (Nakari et al., 2008; Adzitey and Corry, 2011).

Today, molecular methods are the leaders of the most relevant information provided in epidemiological and clinical studies, in which come to overcome the inconveniences of culture and phenotypic techniques, and thus speed up the outcomes (Acke et al., 2009). Real time PCR is one of these methods that use the genetic suitecase to identify and distinguish Campylobacter species worldwide. In their comparative study between hippurate hydrolysis and multiplex PCR for differentiating C. coli and C. jejuni, Adzitey and Corry, (2011) have demonstrated that 17 of the 18 strains were in agreement with both methods used. It was also noticed in the present work, that biochemical tests used to identify Campylobacter species and qPCR using HipO and CadF genes for C. jejuni and C. coli respectively, showed similar results and therefore both tests had proportional sensitivity and efficiency.

Artificial contamination of food matrices

In order to validate internal DNA extraction protocol, an artificial contamination of broiler chicken, beef, and turkey minced meats at different concentrations of Campylobacter reference strains, after enrichment, was carried out. Table 4 demonstrated that DNA templates of C. jejuni and C. coli extracted by the internal protocol were successfully amplified in the three contaminated matrices at 10^3 and 10^5 CFU/mL. The DNA of C. coli was more concentrated compared to C. jejuni. Moreover, the negative amplification (C= 0), in some reactions, was observed for

RESULTS AND DISCUSSION

This work reported the first comparison of the PureLink™ Genomic DNA Mini Kit and Wizard Genomic DNA Purification Kit with internal genomic DNA extraction protocol for obtaining the DNA from Campylobacter species.

Comparison of the internal protocol with commercial kits

The current study compared the DNA quality control after extraction process in 1% agarose gel electrophoresis, using the internal DNA extraction protocol, Wizard® Genomic DNA Purification Kit, and PureLink™ Genomic DNA Mini Kit for C. coli and C. jejuni in triplicate. The results showed in Figure 2 indicated that the DNA integrity was similar in the three compared protocols for both tested strains, since the intensity of the bands was clearly the same. Indeed, the internal DNA genomic extraction protocol was simplest, much cheaper, and faster (1h: 30 min) among the Wizard® Promega kit, which took 2hours, and the Pure Link® Invitrogen kit that was the most expensive (Table 2). This latter had similar processing time (1h: 35 min) as internal protocol, since the purification step based on the use of centrifuge columns (Pure Link® Spin Column) to speed up the extraction protocol.

Figure 2 A 1% agarose gel electrophoresis of C. coli (Fig. 2A) and C. jejuni (Fig. 2B) DNA extracted in triplicates: 1, Internal protocol, 2, Wizard® Genomic DNA Purification Kit, and3: PureLink™ Genomic DNA Mini Kit, 4: Negative control

Validation of internal protocol

Confirmation of Campylobacter species

Between February and June 2018, the forty Campylobacter spp. pure colonies were recovered from food samples (Broiler chicken, turkey, and beef meats.), biochemically confirmed as C. coli and C. jejuni, were further identified by qPCR using the internal DNA extraction protocol. The findings summarized in Table 3 showed that qPCR identified similar Campylobacter species as confirmed by phenotypyque techniques, in which 70% of isolated strains (28/40) were C. coli and 30% (12/40) were C. jejuni.

Table 4 Strains identification of Campylobacter jejuni

| Strain | Origin | N (%) |
|--------|--------|-------|
| C. coli | Broiler chickens, turkeys, and beefs meat. | 28 (70) |
| C. jejuni | | 12 (30) |

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C. jejuni, particularly, in turkey (11.67 ± 20.21) and beef (11.33 ± 19.63) minced meat at concentration of 1 CFU/mL. Likewise, it was noticed some other negative amplifications (C >36) for the three contaminated matrices at 1 CFU/mL of C. coli. The means of threshold C observed in beef minced meat were lower than those of poultry samples.

Table 4 DNA means observed according to C, value of C. jejuni and C. coli

| Minced meat    | C. jejuni (CFU/mL) | C. coli (CFU/mL) |
|----------------|-------------------|-----------------|
| (Mean ± SD)    | 100               | 10              | 1              | 100           | 10               |
| Broiler chicken| 28.47 ± 7.05      | 26.83 ± 7.82    | 16.33 ± 15.57  | 16.83 ± 1.76  | 29.5 ± 1.5      | 34.67 ± 2.08    |
| Turkey         | 23.67 ± 3.21      | 32 ± 18.50      | 11.67 ± 20.21  | 18.33 ± 2.08  | 29.5 ± 0.87     | 37.67 ± 5.86    |
| Beef           | 16 ± 2.4          | 29.5 ± 0.86     | 11.33 ± 19.63  | 23.67 ± 3.21  | 31.17 ± 1.26    | 36.33 ± 1.53    |

SD: Standard deviation.

The artificial contamination with serial dilution of reference strains is usually used in validation procedures of PCR. It is not a standard method. The reactivity of the DNApolymerase in the presence of 7.0 to 8.0% (v/v) propaan-1-ol.They results are in accordance with those attended by Bass et al. (2008) that show a high degree of non-specific amplification when DNA was overstocked in ethanol or isopropanol. Nevertheless, and despite the use of ethanol up to three times before the last step in the internal DNA extraction protocol, all concentrated DNA templates of C. coli and C. jejuni were amplified correctly in our study.

Besides all these strengths findings, there are some limitations that should be noted. Due to the lack of equipments, the DNA purity (A260/A280) and quantity yielded from the present internal genomic DNA extraction protocol were not evaluated and compared with the studied kits. Also, further assays should be performed to determine concentration on food and food samples at different strains concentrations (i.e. 10^2, 5, and 1 CFU/mL) as well as on other Gram-negative bacteria to verify our suggestions.

CONCLUSION

Campylobacter remains the leading cause responsible of human gastroenteritis worldwide. The infections are constantly increasing from consumption of contaminated poultry meats and therefore representing a heavy public health burden. The current work clearly established that the internal genomic DNA extraction protocol provided similar efficiency compared to the two commercially available kits. The confirmation of Campylobacter pure colonies shown that 28 isolates were C. coli and 12 were C. jejuni, similar to phenotypic methods. Furthermore, a positive amplification was also observed in the three contaminated food matrices, after enrichment, at all examined doses. Except some reactions that were negative at 1 CFU/mL of C. jejuni and C. coli. This was explained by the detection limits of both internal protocol and qPCR. Based on our findings, three crucial steps in determining the extraction of DNA quality of this protocol were amended. Hence, the study highlighted the importance of validating simpler, cheaper, and faster DNA extraction protocol, for each laboratory, as part of future risk assessment, control and monitoring programs of Campylobacter frequency required in molecular studies.

Conflicts of interest: The authors declare that they have no competing interests.

Availability of data and materials: The data used in this study are included within the article.

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