JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

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

Aicha El baaboua[1,2], Mohamed El maadoudi[2], Ayoub Kounnoun[2,3], Hajar Bougtaib[2,3], Abdelhakim Bouyahya[4*], and Jamal Abrini[1]*

Address(es):
[1]Biology and Health Laboratory, Biotechnology and Applied Microbiology Team, Department of Biology, Faculty of Science, Abdelmalek-Essaadi University, Tetouan, Morocco.
[2]Regional Laboratory for Analysis and Research, National Office for Food Safety, Tangier, Morocco.
[3]Laboratory of Applied Biology and Pathology, Department of Biology, Faculty of Science, Abdelmalek-Essaadi University, Tetouan, Morocco.
[4]Laboratory of Human Pathology Biology, Faculty of Sciences, and Genomic Center of Human Pathology, Mohammed V University, Rabat, Morocco.

*Corresponding author: boyahyaa-90@hotmail.fr

https://doi.org/10.55251/jmbfs.4549

ARTICLE INFO

Received 13. 3. 2021
Revised 13. 6. 2022
Accepted 4. 8. 2022
Published 1. 10. 2022

Regular article

OPEN ACCESS

ABSTRACT

Campylobacter remains the leading cause responsible of human gastroenteritis worldwide. The current study aimed to compare the internal DNA extraction protocol with two commercially available kits, using *C. jejuni* (ATCC® 29428™) and *C. coli* (ATCC®*43478*™) and to validate and optimize the internal protocol through artificial contamination and confirmation of *Campylobacter* spp. from broiler chickens, turkeys, and beef meats samples. The extraction processes were carried out following the internal protocols steps and the manufacturer's instructions of PureLink™ Genomic DNA Mini Kit and Wizard® Genomic DNA Purification Kit, respectively. The agarose gel electrophoresis system was used to control the DNA quality. After that, forty *Campylobacter* spp. isolates were confirmed, and finally, an artificial contamination of the aforementioned reference strains at three different concentrations was performed with sterilized minced meats. This is the first work comparing the PureLink™ Genomic DNA Mini Kit and Wizard® Genomic DNA Purification Kit with internal genomic DNA extraction protocol for *Campylobacter spp*. The results indicated that the internal protocol provided similar efficiency to the two kits. All confirmed isolates were successfully amplified, in which28 isolates were *C. coli* and 12 were *C. jejuni*, as revealed by biochemical tests. 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.

Keywords: *Campylobacter spp.*; DNA extraction; DNA quantity; qPCR; PureLink™ Genomic DNA Mini Kit; Wizard® Genomic DNA Purification Kit

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 founded 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® 29428™) and *C. coli* (ATCC®*43478*™) 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% (vol/vol) of defibrinated horse blood and incubated under micro aerophilic conditions (5% O_2, 10% CO_2, 85% N_2) 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 were aliquoted in microcentrifuge tubes and stored at -20 °C until the used.

Internal DNAextraction 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 14000 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	AATGCACAAATTTGCCTTATAAAAGC	123
		Reverse	TNCCATTAAAATTCTGACTTGCTAAATA	
		Probe	**JOE**-ACATACTACTTCTTTATTGCTTG-**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 - **BHQ1**	

DNA amplification

The real-time PCR (qPCR) reaction mixture was evaluated and amended in our laboratory during this study (unpublished data). All qPCR amplification reactions contained, 2 µL of DNA template, 2.5 µL Gold buffer (X10), 0.4 mM of dNTP, 5 mM of $MgCl_2$ 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 completed 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 (C_t). The reaction was considered positive with $C_t \leq 36$ and negative with $C_t > 36$.

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 (Biolife, 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).

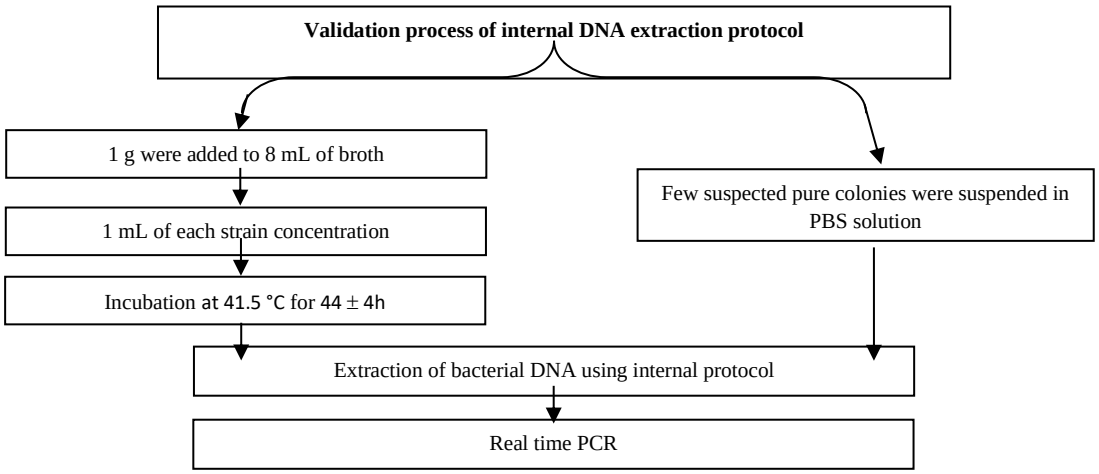

Figure 1 Validation diagram of internal DNA extraction protocol

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, 10^2, and 10 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* inoculums 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 C_t of each artificially contaminated matrices assay for *C. jejuni* and *C. coli*.

RESULTS AND DISCUSSION

This work reported the first comparison of the Pure Link™ 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 Pure Link™ 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.

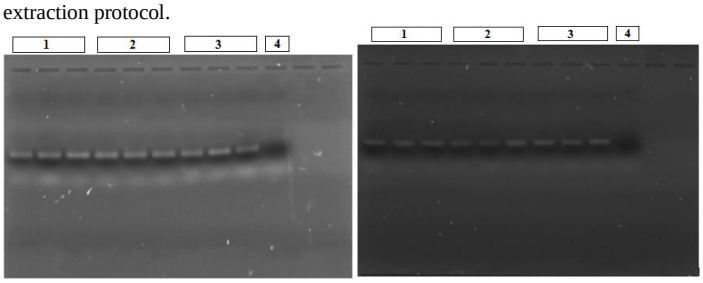

Figure 2 A 1 % agarose gel electrophoresis of *C. coli* (Fig. 2A) and *C. jejuni* (Fig. 2B) DNA extracted in triplicates. 1: Internal protocol, 2: Wizard® Genomic DNA Purification Kit, and3: Pure Link™ Genomic DNA Mini Kit, 4: Negative contro

Table 2 Main indicators comparison of the three DNA extraction protocols

	Internal protocol	Wizard® Genomic DNA Purification Kit	PureLink™ Genomic DNA Mini Kit
Cost	Cheap	Inexpensive	Expensive
Time	1h:30 min	2h	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; $p = .032$) 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)**.

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 phenotypique techniques, in which 70% of isolated strains (28/40) were *C. coli* and 30% (12/40) were *C. jejuni*.

Table 3 Identification of *Campylobacter* spp. isolated from food samples

Strain (N= 40)	Origin	Biochemical confirmation N (%)	qPCR using internal DNA extraction protocol N (%)
C. coli	Broiler chickens, turkeys, and beefs meat.	28 (70)	28 (70)
C. jejuni		12 (30)	12 (30)

The dominant specie reported of *Campylobacter* genus is *C. jejuni* **(Chon *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 attributed 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 suitcase 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 10^2 and 10 CFU/mL. The DNA of *C. coli* was more concentrated compared to *C. jejuni*. Moreover, the negative amplification ($C_t = 0$), in some reactions, was observed for

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_t >36) for the three contaminated matrices at 1 CFU/mL of *C. coli*. The means of threshold C_t observed in beef minced meat were lower than those of poultry samples.

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

Minced meat	*C. jejuni* (CFU/mL)			*C. coli* (CFU/mL)		
(Mean ± SD)	100	10	1	100	10	1
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 ± 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 sensitivity, culture recovery, and others techniques combined or not with an enrichment step **(Ahmed *et al.*, 2013; El Baaboua *et al.*, 2018)**. In the internal analysis, the quantitative methodology requires enrichment of samples to detect low level of contamination and thus to improve the isolation rates **(Hill *et al.*, 2017)**. That's why; the use of the enrichment stage, in our study, was for purpose to increase cells numbers, particularly at concentration of 1 CFU/mL, in order to check the detection limits of this protocol. **Abdelhai *et al.* (2016)** have studied three rapid methods (physical, boiling, phenol/ethanol and commercial kit) of Gram-positive DNA extraction and concluded that the DNA extraction yields were significantly increased with amount of the initial culture used and thus the quantity of DNA extracted from the same method. This was in accordance with our outcomes of threshold means, from 100 to 1 CFU/mL of each inoculum.

C. jejuni and *C. coli* are the most prevalent species isolated in human food, in particular from broiler chickens and turkeys meat **(Chon *et al.*, 2018; Gahamanyi *et al.*, 2020)**. The low recovery levels of *Campylobacter* in beef were explained, in literature, by the fact that these organisms had less favorable conditions in cattle populations than in poultry **(Zhao *et al.*, 2001)**. Besides to that, the detection limits of decimal dilution series of enrichments of pure cultures were found successively lower in *C. lari* and *C. jejuni* compared to *C. coli*, in the same experimental conditions **(Mayr *et al.*, 2010)**. Base on their results, **Mayr *et al.* (2010)** were demonstrated that the limits of detection for *C. coli* and *C. jejuni* in qPCR assay were from 5 to 10 and 1–5 CFU/250mL, respectively. As well as, the post enrichment bacterial counts (from 5 to 10 CFU/250 mL) of 48 hours was revealed the recovery of 10^7 to 10^8 CFU/mL **(Mayr *et al.*, 2010).** Similar detection limits (at 1 CFU/mL) were noticed in some reactions of *C. coli* and *C. jejuni*. Other than the aforementioned reasons, the low C_t obtained at 1 CFU/mL in turkey minced meat and ground beef from enriched culture can be also attributed to the sampling and/or counting errors before and during serial dilution **(Ben-David and Davidson, 2014).**

Campylobacter genus is the main cause of food borne illnesses that lead to diversity in manifestations and pathogenesis power, depending on species and host susceptibility **(Baaboua *et al.*, 2017)**. In regards to specie ability, the infectious dose plays a critical role. Researchers were confirmed that 15% of human were poisoned after they ingested infectious dose between 8 x 10^2 to 1 x 10^8 of *C. jejuni* **(Black *et al.*, 1988)**.Moreover, others scientists who infected human volunteers by another *C. jejuni* strain, proved that only 80% of the volunteers developed positive stool cultures and half of them fell sick at doses of 10^6 CFU, approximately **(Havelaar *et al.*, 2009)**. As a consequence, the internal DNA extraction method was able to detect low infectious doses (up to 1 CFU/mL after enrichment) and can be used in human stool and food specimens for all the Gram-negative bacteria.

Optimization of the internal DNA extraction protocol

The internal DNA extraction protocol was amended when extracting DNA from enriched matrice safter the used of Bolton enrichment broth supplemented with defibrinated horse blood. Indeed, the heme compounds, hemoglobin derivative, inactivate reversibly polymerase in qPCR, which must be removed before starting the DNA extraction procedure. This is the reason why, the cell pellet obtained after centrifugation was washed several times with the PBS solution. **Akane *et al.* (1994)** have presented an efficient alternative molecule named Bovine Serum Album (BSA) that can be added to the PCR mixture to enhance the amplification reaction in blood specimens. Likewise, to ensure that DNA extracted from *Campylobacter* strains were cleaned from salts (mainly Na^+), additional wash step was carried out to the DNA pellet, twice and more, with ethanol70% at room temperature.

The purification was carried out, herein, to eliminate the contaminants that could disrupt the DNA amplification. For example, a higher concentration of EDTA (> 0.5mM) can reduce the fluorescence to 46% **(Al-Soud and Rådström, 2001)** and can be an inhibitory through depleting the divalent cations such as magnesium (Mg_2^+), necessary for Taq polymerase functioning **(Datta and LiCata, 2003)**. More than 5mM and 25mM of sodium acetate and NaCl respectively in DNA extracts, affect negatively the qPCR reaction **(Watson and Blackwell, 2000)**. Concerning alcohols (e.g. isopropanol and ethanol), a concentration lower than 1% does not affect the qPCR cycles, and a higher concentrations become progressively inhibitor leading to several polymerase errors. **Claveau *et al.* (2004)** have worked on the relationship between PCR errors and alcohols, which observed that alcohols involved conformational loosening due to a decrease in the hydrophobic effect and also mutation occurrence (up to 9.8 x $10^{(-3)}$ mutation/bp/PCR) with Vent(r) (exo-) DNA polymerase in the presence of 7.0 to 8.0% (v/v) propan-1-ol.Their results are in accordance with those attended by **Bass *et al.* (2008)** that shown 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 from direct extraction on stool and food samples at determined strains concentrations (i.e. 10^2, 10, 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.

Fundingstatement: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements: We thank the Ministry of Higher Education, Scientific Research and Management Training, Rabat, Morocco, for the financial support of the doctoral studies of one of the authors.

REFERENCE

Abdelhai, M. H., Hassanin, H. A. M., & Sun, X. (2016). Comparative Study of Rapid DNA Extraction Methods of Pathogenic Bacteria. *Am. J. Biosci. Bioeng.*, 4, 1. https://doi.org/10.11648/j.bio.20160401.11.

Acke, E., McGill, K., Golden, O., Jones, B. R., Fanning, S., & Whyte, P. (2009). A comparison of different culture methods for the recovery of Campylobacter species from pets. *Zoonoses Public Health.*, 56, 490–495. https://doi.org/10.1111/j.1863-2378.2008.01205.x.

Adzitey, F., and Corry, J. (2011). A Comparison between Hippurate Hydrolysis and Multiplex PCR for Differentiating *Campylobacter coli* and *Campylobacter jejuni*. *Trop. Life Sci. Res.*, 22, 91–98. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3819090/.

Ahmed, M. F. M., Schulz, J., & Hartung, J. (2013). Survival of *Campylobacter jejuni* in naturally and artificially contaminated laying hen feces. *Poult. Sci.* 92., 364–369. https://doi.org/10.3382/ps.2012-02496.

Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., & Kimura, K. (1994). Identification of the Heme Compound Copurified with Deoxyribonucleic Acid (DNA) from Bloodstains, a Major Inhibitor of Polymerase Chain Reaction (PCR) Amplification. *J. Forensic Sci.*, 39, 362–372.https://doi.org/10.1520/jfs13607j.

Al-Soud, W. A., & Rådström, P. (2001). Purification and Characterization of PCR-Inhibitory Components in Blood Cells. *J. Clin. Microbiol.*, 39, 485–493. https://doi.org/10.1128/jcm.39.2.485-493.2001.

Assenmacher, D. M., Fields, S. D., & Crupper, S. S. (2020). Comparison of Commercial Kits for Recovery and Analysis of Bacterial DNA From Fingerprints. *J. Forensic Sci.*, 2020. https://doi.org/10.1111/1556-4029.14280.

Ayana, M., Cools, P., Mekonnen, Z., Biruksew, A., Dana, D., Rashwan, N., et al. (2019). Comparison of four DNA extraction and three preservation protocols for the molecular detection and quantification of soil-transmitted helminths in stool. *PLoS Negl. Trop. Dis.*, 13, e0007778. https://doi.org/10.1371/journal.pntd.0007778.

Baaboua, A. E., Maadoudi, M. E., Bouyahya, A., & Abrini, J. (2017). Intestinal Infections of Campylobacter: A Review. *Microbiol. Res. J. Int.*, 18, 1–8. https://journalmrji.com/index.php/MRJI/article/view/5145.

Bass, C., Nikou, D., Blagborough, A. M., Vontas, J., Sinden, R. E., Williamson, M. S., et al. (2008). PCR-based detection of Plasmodium in *Anopheles mosquitoes*: a comparison of a new high-throughput assay with existing methods. *Malar. J.*, 7, 177. https://doi.org/10.1186/1475-2875-7-177.

Bazzicalupo, M., & Fancelli, S. (1997). DNA Extraction from Bacterial Cultures," in *Fingerprinting Methods Based on Arbitrarily Primed PCR* Springer Lab Manuals., eds. M. R. Micheli and R. Bova (Berlin, Heidelberg: Springer), 41–45. https://doi.org/10.1007/978-3-642-60441-6_7.

Ben-David, A., & Davidson, C. E. (2014). Estimation method for serial dilution experiments. *J. Microbiol. Methods.*, 107, 214–221. https://doi.org/10.1016/j.mimet.2014.08.023.

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P., & Blaser, M. J. (1988). Experimental *Campylobacter jejuni* Infection in Humans. *J. Infect. Dis.*, 157, 472–479. https://doi.org/10.21236/ada265410.

Bolton, D. J. (2015). Campylobacter virulence and survival factors. *Food Microbiol.*, 48, 99–108. https://doi.org/10.1016/j.fm.2014.11.017.

Budge, S., Barnett, M., Hutchings, P., Parker, A., Tyrrel, S., Hassard, F., et al. (2020). Risk factors and transmission pathways associated with infant *Campylobacter* spp. prevalence and malnutrition: A formative study in rural Ethiopia. *PloS One.*, 15, e0232541. https://doi.org/10.1371/journal.pone.0232541.

Butzler, J.-P. (2004). *Campylobacter*, from obscurity to celebrity. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.*, 10, 868–876. https://doi.org/10.1111/j.1469-0691.2004.00983.x.

Chen, W., & Kuo, T. (1993). A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res.*, 21, 2260–2260. https://doi.org/10.1093/nar/21.9.2260.

Chon, J.-W., Lee, S.-K., Yoon, Y., Yoon, K.-S., Kwak, H.-S., Joo, I.-S., et al. (2018). Quantitative prevalence and characterization of *Campylobacter* from chicken and duck carcasses from poultry slaughterhouses in South Korea. *Poult. Sci.*, 97, 2909–2916. https://doi.org/10.3382/ps/pey120.

Claveau, S., Sasseville, M., & Beauregard, M. (2004). Alcohol-Mediated Error-Prone PCR. *DNA Cell Biol.* 23, 789–795. https://doi.org/10.1089/dna.2004.23.789.

CSH Cold Spring Harbor Protocols. Available at: http://cshprotocols.cshlp.org/ [Accessed June 12, 2020].

Dal, T., Açıkgöz, Z. C., Başyiğit, T., Zeybek, H., & Durmaz, R. (2018). [Comparison of two commercial DNA extraction kits and PCR master mixes for the detection of Brucella from blood samples and blood culture bottles]. *Mikrobiyol. Bul.*, 52, 135–146. https://doi.org/10.5578/mb.66742.

Datta, K., & LiCata, V. J. (2003). Salt dependence of DNA binding by Thermus aquaticus and *Escherichia coli* DNA polymerases. *J. Biol. Chem.*, 278, 5694–5701. https://doi.org/10.31390/gradschool_dissertations.2142.

Del Collo, L. P., Karns, J. S., Biswas, D., Lombard, J. E., Haley, B. J., Kristensen, R. C., et al. (2017). Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter* spp. in bulk tank milk and milk filters from US dairies. *J. Dairy Sci.*, 100, 3470–3479. https://doi.org/10.3168/jds.2016-12084.

El Baaboua, A., El Maadoudi, M., Bouyahya, A., Belmehdi, O., Kounnoun, A., Zahli, R., et al. (2018). Evaluation of Antimicrobial Activity of Four Organic Acids Used in Chicks Feed to Control *Salmonella typhimurium:* Suggestion of Amendment in the Search Standard. *Int. J. Microbiol.*, https://doi.org/10.1155/2018/7352593.

Fidler, G., Tolnai, E., Stagel, A., Remenyik, J., Stundl, L., Gal, F., et al. (2020). Tendentious effects of automated and manual metagenomic DNA purification protocols on broiler gut microbiome taxonomic profiling. *Sci. Rep.*, 10, 3419. https://doi.org/10.1038/s41598-020-60304-y.

Freschi, C. R., Carvalho, L. F. de O. e S., & Oliveira, C. J. B. de (2005). Comparison of DNA-extraction methods and Selective Enrichment broths on the detection of *Salmonella Typhimurium* in swine feces by polymerase chain reaction (PCR). *Braz. J. Microbiol.*, 36, 363–367. https://doi.org/10.1590/s1517-83822005000400011.

Gahamanyi, N., Mboera, L. E. G., Matee, M. I., Mutangana, D., & Komba, E. V. G. (2020). Prevalence, Risk Factors, and Antimicrobial Resistance Profiles of Thermophilic *Campylobacter* Species in Humans and Animals in Sub-Saharan Africa: A Systematic Review. *Int. J. Microbiol.*, 2020. https://doi.org/10.1155/2020/2092478.

Green, M. R., & Sambrook, J. (2017). Isolating DNA from Gram-Negative Bacteria. *Cold Spring Harb. Protoc.*, 2017. https://doi.org/10.1101/pdb.prot093369.

Guirin, G. F., Brusa, V., Adriani, C. D., and Leotta, G. A. (2019). Prevalence of *Campylobacter jejuni* and *Campylobacter coli* from broilers at conventional and kosher abattoirs and retail stores. *Rev. Argent. Microbiol.*, https://doi.org/10.1016/j.ram.2019.07.002.

Han, X., Guan, X., Zeng, H., Li, J., Huang, X., Wen, Y., et al. (2019). Prevalence, antimicrobial resistance profiles and virulence-associated genes of thermophilic *Campylobacter* spp. isolated from ducks in a Chinese slaughterhouse. *Food Control.*, 104, 157–166. https://doi.org/10.1016/j.foodcont.2019.04.038.

Havelaar, A. H., Pelt, W. van, Ang, C. W., Wagenaar, J. A., Putten, J. P. M. van, Gross, U., et al. (2009). Immunity to *Campylobacter:* its role in risk assessment and epidemiology. *Crit. Rev. Microbiol.*, 35, 1–22. https://doi.org/10.1080/10408410802636017.

Hill, G. N., Bratcher, C. L., Wang, L., Singh, M., Tapp, W. N., & McKee, S. R. (2017). Optimizing Enrichment of *Campylobacter* on poultry. *J. Appl. Poult. Res.*, 26, 307–315. https://doi.org/10.3382/japr/pfw075.

Iannetti, S., Calistri, P., Di Serafino, G., Marotta, F., Alessiani, A., Antoci, S., et al. (2020). *Campylobacter jejuni* and *Campylobacter coli*: prevalence, contamination levels, genetic diversity and antibiotic resistance in Italy. *Vet. Ital.*, 56. https://pubmed.ncbi.nlm.nih.gov/32343091/.

Joensen, K. G., Kiil, K., Gantzhorn, M. R., Nauerby, B., Engberg, J., Holt, H. M., et al. (2020). Whole-Genome Sequencing to Detect Numerous *Campylobacter jejuni* Outbreaks and Match Patient Isolates to Sources, Denmark, 2015-2017. *Emerg. Infect. Dis.*, 26, 523–532. https://doi.org/10.3201/eid2603.190947.

Leite, D. C. A., Balieiro, F. C., Pires, C. A., Madari, B. E., Rosado, A. S., Coutinho, H. L. C., et al. (2014). Comparison of DNA extraction protocols for microbial communities from soil treated with biochar. *Braz. J. Microbiol.*, 45, 175–183. https://doi.org/10.1590/s1517-83822014000100023.

Liu, D., Liu, W., Li, X., Yao, H., Shen, Z., Wang, Y., et al. (2019). Presence and Antimicrobial Susceptibility of RE-cmeABC-Positive Campylobacter Isolated from Food-Producing Animals, 2014–2016. *Engineering*., https://doi.org/10.1016/j.eng.2019.10.013.

Mayr, A. M., Lick, S., Bauer, J., Thärigen, D., Busch, U., & Huber, I. (2010). Rapid Detection and Differentiation of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* in Food, Using Multiplex Real-Time PCR. *J. Food Prot.*, 73, 241–250.https://doi.org/10.4315/0362-028x-73.2.241.

Nakari, U.-M., Puhakka, A., & Siitonen, A. (2008). Correct identification and discrimination between *Campylobacter jejuni* and C. coli by a standardized hippurate test and species-specific polymerase chain reaction. *Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol.*, 27, 513–518. https://doi.org/10.1007/s10096-008-0467-9.

Nikolaev, S., Lemmens, L., Koessler, T., Blouin, J.-L., & Nouspikel, T. (2018). Circulating tumoral DNA: Preanalytical validation and quality control in a diagnostic laboratory. *Anal. Biochem.*, 542, 34–39. https://doi.org/10.1016/j.ab.2017.11.004.

Ruiz-Fuentes, J. L., Díaz, A., Entenza, A. E., Frión, Y., Suárez, O., Torres, P., et al. (2015). Comparison of four DNA extraction methods for the detection of *Mycobacterium leprae* from Ziehl–Neelsen-stained microscopic slides. *Int. J. Mycobacteriology*., 4, 284–289. https://doi.org/10.1016/j.ijmyco.2015.06.005.

Scharff, R. L. (2012). Economic Burden from Health Losses Due to Foodborne Illness in the United States. *J. Food Prot.*, 75, 123–131. https://doi.org/10.4315/0362-028x.jfp-11-058.

Skarp, C. P. A., Hänninen, M.-L., & Rautelin, H. I. K. (2016). Campylobacteriosis: the role of poultry meat. *Clin. Microbiol. Infect.*, 22, 103–109. https://doi.org/10.1016/j.cmi.2015.11.019.

Watson, R. J., & Blackwell, B. (2000). Purification and characterization of a common soil component which inhibits the polymerase chain reaction. *Can. J. Microbiol.*, 46, 633–642. https://doi.org/10.1139/w00-043.

Wright, M. H., Adelskov, J., & Greene, A. C. (2017). Bacterial DNA Extraction Using Individual Enzymes and Phenol/Chloroform Separation. *J. Microbiol. Biol. Educ.*, 18. https://doi.org/10.1128/jmbe.v18i2.1348.

Zhao, C., Ge, B., De Villena, J., Sudler, R., Yeh, E., Zhao, S., et al. (2001). Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella Serovar*s in Retail Chicken, Turkey, Pork, and Beef from the Greater Washington, D.C., Area. *Appl. Environ. Microbiol.*, 67, 5431–5436. https://doi.org/10.1128/aem.67.12.5431-5436.2001.

Zou, W., Lin, W.-J., Foley, S. L., Chen, C.-H., Nayak, R., & Chen, J. J. (2010). Evaluation of Pulsed-Field Gel Electrophoresis Profiles for Identification of *Salmonella Serotypes*. *J. Clin. Microbiol.*, 48, 3122–3126. https://doi.org/10.1128/jcm.00645-10.