Food Microbiology 77 (2019) 85–92

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Development and application of a real-time polymerase chain reaction method for quantification of *Escherichia coli* in oysters (*Crassostrea gigas*)

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ARTICLE INFO

Keywords:
Escherichia coli
Real-time qPCR
Oyster
Culture-independent method

ABSTRACT

Oysters are important mariculture species worldwide. Because of their filter-feeding behaviors, oysters can accumulate microorganisms, including pathogens, from surrounding water and concentrate bacteria in high numbers. Rapid and suitable methods for quantification of *Escherichia coli* in oysters are necessary considering that oysters are perishable foods often consumed raw and some countries use *E. coli* as the regulatory limit. The objective of this study was to develop a qPCR method for quantification of *E. coli* in oysters. Additionally, different methods were evaluated for DNA extraction from oyster samples and the more reliable method was chosen. Primers and probe were designed targeting *uid*A gene of *E. coli* and shown to specifically amplify DNA from *E. coli*. Standard curves with bacterial DNA extracted from oysters samples artificially inoculated with *E. coli* were conducted. A good correlation was noticed when the qPCR method was compared to a culture method in oyster samples. This is the first report of a method exclusively developed for direct quantification of *E. coli* in oyster, the method showed to be suitable for quantification of *E. coli* in oysters and could be useful in routine analyses, as it requires less time than the culture method.

1. Introduction

Escherichia coli is a facultative anaerobic Gram-negative bacterium, present in the gastrointestinal tract of humans and warm-blooded animals. Although most of these commensal *E. coli* strains are harmless, some are pathogenic and can cause a variety of diseases in humans (Kaper et al., 2004). In fact, they have been widely involved in food-borne outbreaks around the world (Centers for Disease Control and Prevention, 2017). *E. coli* have also been traditionally recognized as an indicator of fecal contamination in water and seafood (Kumaran et al., 2010). Thus, *E. coli* presence in seafood is considered a public health concern, representing a risk to the consumers when pathogenic strains are present (Costa, 2013).

Contamination of shellfish growing water by animal fecal wastes, sewage and rainwater discharges, especially when close to urban and industrial areas, has been considered to be a vehicle for transmission of *E. coli* and other important zoonotic pathogens responsible for cases of food-borne diseases (Abdelzaher et al., 2010). Since bivalve mollusks are filter-feeding organisms, they can accumulate microorganisms, including pathogens, from surrounding water and concentrate bacteria in high numbers (Iwamoto et al., 2010). The problem is increased when considering that oysters are consumed either raw or lightly cooked and are capable of transferring pathogenic microorganisms into the human food chain (Pereira et al., 2017) that can lead to food-borne diseases, which are an important public health problem in both developing and developed countries (Inatsu et al., 2015).

To safeguard against contaminated shellfish products entering the human food chain, many countries have stringent regulation in order to supervise the production of shellfish. Some countries have established regulatory limits and monitoring programs using *E. coli* or fecal coliform counts of bivalve mollusks, as well as their growing areas (Brazil, 2012; FDA, 2015). It has also being emphasized that the detection of the food-borne pathogens plays an important role in order to reduce food-borne disease occurrence (Zhao et al., 2014).

Standard and accepted methods for the detection and quantification of *E. coli* are based on traditional microbiological culture-dependent

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https://doi.org/10.1016/j.fm.2018.08.015
Received 19 March 2018; Received in revised form 30 July 2018; Accepted 31 August 2018
Available online 01 September 2018

methods (ISO, 2015). Those methods still rely on the use of selective media, biochemical reactions and other parameters for bacterial identification which are often limiting, very labor and time consuming and inadequate for food control purposes (Berrada et al., 2006). In order to prevent the spread of infectious diseases, ensure food safety, and thereby to protect public health, there is an increasing demand for more rapid methods of food-borne pathogen detection and quantification (Malcolm et al., 2015; Zhao et al., 2014).

In food matrices, culture-independent methods have been recognized as a valuable alternative to culture-dependent methods. These methods are based on the direct analysis of DNA or RNA extracted from the food matrix with no culturing step. Among culture-independent methods, quantitative real-time polymerase chain reaction (qPCR) represents a powerful tool for the quantification of microbial populations in food (Walker et al., 2018). qPCR allows accurate, automated and quantitative detection of different microorganisms, with the advantage of continuously monitoring the PCR product formation throughout the reaction and offers rapid, simultaneous amplification and sequence-specific-based detection of target genes and is increasingly being applied in food microbiology (Jung et al., 2005). Moreover, the real-time monitoring of the process means no need for post-amplification treatment of the samples, such as gel electrophoresis, reducing the time of analysis and risk of cross-contamination (Zhao et al., 2014).

The qPCR method has shown excellent performance being rapid and sensitive for quantifying microorganisms, including those in the viable but non-culturable (VBNC) state. It has been established as a valuable alternative to traditional culture methods and may be expected to replace the culture methods in the food industry (Truchado et al., 2016). Taminiau et al. (2014) have reported a PCR method for detection of six major pathogens, including *E. coli* O157:H7, in different seafood matrices. Other studies were conducted with PCR for quantification of *E. coli* in vegetable, salad, meat and water samples (Elizaquível et al., 2011, 2012) as well in seafood, including raw oysters (Takahashi et al., 2009). However, rapid and reliable method is not available about the quantification of *E. coli* in oyster. Therefore, standardized rapid methods for *E. coli* in oyster should be established by an appropriate validation method (Kagkli et al., 2011). Since oysters are perishable foods, rapid and suitable methods are needed to assure the quantification of *E. coli*, particularly because some countries use *E. coli* as the regulatory limit. In order to replace the culture methods in the routine analyses, some parameters of the development methods should be determined, such as, the limit of detection, the dynamic range, the qPCR efficiency, the inclusivity and exclusivity tests, as well as a matrix of study (AOAC, 2012; ISO, 2011). In this study, we addressed these data gaps and a qPCR method for quantification of *E coli* in oysters was developed.

2. Material and methods

2.1. Preparation of *E. coli* suspensions

Escherichia coli (ATCC 25922) Lab-Elite Certified Reference Material (Microbiologics Inc. Saint Cloud, Minnesota, USA) was grown to the stationary phase, overnight at 35 °C in brain heart infusion broth (BHI, Himedia, Mumbai, India). Ten-fold serial dilutions of the culture were prepared in 0.1% (w/v) peptone water to obtain suspensions of *E. coli* at numbers between 10^1 and 10^8 colony forming units (CFU) per mL. In duplicate, plate count agar (PCA; Oxoid, Mississauga, Ontario, Canada) and tryptone bile x-glucuronide selective medium (TBX; Oxoid, Mississauga, Ontario, Canada) were spread with 0.1 mL of the *E. coli* dilutions. The plates were incubated at 44 ± 1 °C for 24 h, and the results were expressed as CFU/mL. This step was determined in duplicate.

2.2. Oyster samples

All the oysters (*Crassostrea gigas*) used for DNA extraction and artificial inoculation experiments in this study were purchased from a local producer and harvested no more than 3 h before starting the experiments. In order to assure the absence of *E. coli*, firstly the oysters were analyzed by culture method (ISO/TS 16649-3:2015) (ISO, 2015) and qPCR. Oyster samples with no growth using ISO/TS 16649-3:2015 method and no amplification on PCR were used in the study.

2.3. DNA extraction

For bacterial genomic DNA extraction, 1 mL aliquots of medium containing 10^7 CFU/mL of *E. coli* were centrifuged (4000 × g for 5 min). The resulted pellets were used for DNA extraction using DNeasy blood & tissue kit (Qiagen, Missinauga, Ontario, Canada) automated on the Qiacube System (Qiagen, Hilden, Germany), according to the manufacturers protocol for bacterial pellet. Genomic DNA of oyster (*C. gigas*) was extracted from 1 mL of oyster homogenate using the same kit but with the protocol for tissue sample. DNA concentrations were estimated from measurements at 260 and 280 nm on Thermo Scientific NanoDrop 1000 spectrophotometer (Wilmington, DE, USA).

2.4. Primers and probe design

Forward and reverse primers and the hydrolysis probe targeting the *uidA* gene of *E. coli*, that codes for β-glucuronidase (Blanco et al., 1985) were designed using the program Primer3Plus (Untergasser et al., 2007) and sequence information found in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov; accession number S69414). The primers and probe were analyzed using the OligoCalc Program (Kibbe, 2007) for verification of secondary structures. In silico analytical specificity of the primers was tested with the Primer Blast (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers and probe sequences with respective melting temperatures (T_m) are presented in Table 1. The amplicon size was 84 bp and the primers and probe were synthesized by Sigma Aldrich (Saint Louis, MO, USA).

2.5. Selectivity test

Firstly, the selectivity of the primers and probe were tested against target and non-target DNA extracted from bacterial species, including those commonly found in oyster and marine environments and/or phylogenetically related to the target specie. For exclusivity test, 14 bacterial strains were used, as follows: *Enterococcus faecalis* (ATCC 29212), *Salmonella* Typhimurium (ATCC 14028), *Proteus vulgaris* (ATCC 8427), *Clostridium perfringens* (ATCC 12924), *Bacillus subtilis* (ATCC 6533), *Bacillus cereus* (ATCC 11778), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC, 19115), *Klebsiella pneumoniae* (ATCC 13882), *Citrobacter freundii* (ATCC 43864), *Vibrio parahaemolyticus* (ATCC 17802), *Yersinia enterocolitica* (ATCC 9610), *Aeromonas hydrophila* (ATCC 7966), *Staphylococcus aureus* (ATCC 25923). For inclusivity test, *E. coli* (ATCC 25922), *E. coli* (ATCC 35218), *E. coli* (ATCC 43894), and *E. coli* isolated from mussel (n = 20), oyster (n = 40) and fish (n = 10) were also used. The strains were grown in nutrient broth (Himedia, Mumbai, India) at 35 ± 1 °C for 24 h, except *V. parahaemolyticus* which was grown in nutrient broth enriched with

Table 1
Primers and probe sequences designed for *uid*A gene of *E. coli*.

Oligonucleotide	Sequence 5′- 3′	T_m (°C)
Forward primer	CGGAAGCAACGCGTAAACTC	66.7
Reverse primer	TGATGGTATCGGTGTGAGCG	67.1
Probe	HEX-ACCCGACGCGTC CGATCACCT-BHQ1	76.0

3% NaCl.

2.6. Quantitative PCR conditions

The performance of qPCR was realized in Real Time Rotor-Gene Q® (Qiagen, Hombrechtikon, Switzerland) and the reaction was performed using the Rotor-Gene Probe PCR kit (Qiagen, Hilden, Germany). The amplification reactions were carried out in a final volume of 25 µL containing: 5.5 µL of RNase free-water; 12.5 µL of 2 × Rotor-Gene Probe master mix; 0.4 µM of each primer; 0.2 µM of probe and 2 µL of DNA. All reactions were carried out in duplicate under the following cycling conditions: 3 min at 95 °C; 45 cycles of 3 s at 95 °C and 10 s at 60 °C (ON-HEX). All samples were tested by qPCR with a concentration of 50 ng of DNA per reaction. In the present study, each reaction included a negative qPCR control (DNA-free water with no template control (NTC) and a positive control with 50 ng of *E. coli* (ATCC 25922) DNA, as described by ISO 22174 (ISO, 2005).

2.7. Standard curves with E. coli DNA

Standard curves were prepared with serial dilutions of genomic DNA extracted from *E. coli.* The number of genome copies was calculated on the basis of the size of the *E. coli* (GenBank accession number CP_009072.1) genome (5.13 Mbp) using the Avogadro's constant (6.023×10^{23}) and the molecular weight of DNA (660 Da/bp). Genomic DNA was ten-fold serially diluted in ultrapure water to final concentrations ranging from 10^7 to 10^0 genome copies per 2 µL, equivalent to concentrations of 50.62 ng to 5.62 fg. The reliability criteria were the correlation coefficient (R^2) and the amplification efficiency. The amplification efficiencies were obtained according to Bustin et al. (2009), using the following equation: $E = (10^{-1/s} - 1) \times 100$, where E is the efficiency (%) and *s* is the slope obtained from the standard curve. Each standard curve run was performed in triplicate. The repeatability assay was performed with three different DNA extracts from *E. coli*, while qPCR runs were performed in 3 days by the use of the three DNA extracts. The limit of detection (LOD) and limit of quantification (LOQ) were determined through the repeatability test. It is noteworthy that samples with quantification cycle (Cq) values higher than 40 were excluded from further analyses.

2.8. Standard curves with oyster DNA background

Bacterial DNA standard curves were also prepared in the presence of background DNA of oyster (50.62 ng per reaction) in order to evaluate the effect of background oyster DNA in qPCR LOD and efficiency and to determine the sensitivity of the test. For the construction of standard curve, each dilution was tested in triplicate.

2.9. Evaluation of different DNA extraction kits

Three different commercial DNA extraction kits were tested for the direct quantification of *E. coli* in oyster using qPCR. For that purpose, seven oyster samples were inoculated with *E. coli* (ATCC 25922) in concentrations ranging from 10^1 to 10^8 CFU/g. Thus, genomic DNA was extracted from each sample by the following methods: DNeasy Blood & Tissue Kit (TK) (Qiagen, Missinauga, Ontario, Canada); DNeasy Mericon Food Kit (FK) (Qiagen, Missinauga, Ontario, Canada) and Axygen® AxyPrep™ Bacterial Genomic Miniprep Kit (MK) (Axygen Biosciences, Union City, California, USA), according to manufacturer's protocol. One milliliter of each sample (inoculated samples and negative controls) were used for DNA extraction. Before the extraction with DNeasy blood & tissue kit and AxyPrep™ Bacterial Genomic Miniprep Kit, the samples were centrifuged (5000 × g for 5 min). After DNA extraction, measurements in triplicate of total DNA yield and quality were obtained using a Thermo Scientific NanoDrop 1000 spectrophotometer. Extracted DNA was stored at - 20 ± 1 °C until qPCR assays.

The corresponding *E. coli* CFU/g was calculated using plate agar counts following ISO 16649-2:2001 method (ISO, 2001). TBX agar plates were inoculated, in duplicate, with decimal dilutions of each sample and incubated at 44 ± 1 °C for 24 h. The Cq values (y-axis) for each set of reactions were plotted against log CFU/g (x-axis) to obtain a standard curve. The standard curves were accessed to determine the most suitable extraction method and to define the sensitivity, linearity, range of use, LOD and LOQ of the qPCR method. These results allow the choice of the best extraction method for qPCR and to construct a standard curve considering the LOQ and the linearity.

2.10. Comparison of qPCR with the culture method in enumerating E. coli in oyster samples

For evaluation of the qPCR method developed, bacterial counts obtained by the qPCR method were compared with those obtained by the culture method in artificially and naturally contaminated oysters. A total of 30 oyster samples were artificially inoculated with *E. coli* in concentrations ranging from 2 log to 6 log CFU/g, with six replicates in each concentration. Each oyster sample was used for the enumeration of *E. coli* following the ISO standards (ISO, 2001). Concurrently, DNA was extracted from aliquots of 1 mL from each oyster sample and then subjected to qPCR. DNA was subjected to qPCR in triplicate, and the Cq average values were used for *E. coli* enumeration. These results were also used for repeatability testing.

For additional evaluation of the qPCR method, a total of 60 oyster samples were harvested and analyzed in different days. The amount of *E. coli* was estimated using the qPCR method through the standard curve and the enumeration was concurrently conducted using ISO method, as described previously.

2.11. Data analysis

The mean, standard deviation (SD), coefficient of variation (CV), repeatability standard deviation (RSDr) and linear regression were calculated using Statistica 12.0® software (Stat-Soft, Inc., USA). The CV and RSDr was evaluated for comparison of the variability between qPCR and culture method in natural and artificially contaminated oyster samples and were also used for the repeatability test.

3. Results and discussion

3.1. Selectivity test

Selectivity is defined as the measure of the inclusivity (detection of the target microorganism) and exclusivity (non-detection of non-target microorganisms) (AOAC, 2012; ISO, 2011). The selectivity of the primer pair and probe was assessed by qPCR amplification of DNA from a total of 87 strains, covering bacteria that are phylogenetically related with *E. coli* or possibly found in shellfish, as well as, some *E. coli* strains. The primers and probe specifically amplified genomic DNA from *E. coli* ATCC 25922, ATCC 35218, ATCC 43894 and the *E. coli* isolates from oyster, mussel and fish samples. Non-target DNA samples showed no Cq because the amplification plot did not cross the threshold fluorescence level (Table 2).

The qPCR assay was shown to be specific for *E. coli* and similar Cq values, with a mean of 16.22 (SD = 0.977) were obtained (Table 2). The *uidA* gene, that encodes β-glucuronidase, has a highly conserved nature and is found in the majority of *E. coli* strains (Kaspar et al., 1987). The *uidA* gene is also found in *E. coli* 0157:H7 that do not exhibit β-glucuronidase enzymatic activity (Monday et al., 2001) and, according to Maheux et al. (2008) can lead to false-negative results. Therefore, in our study, the primers targeting gene *uidA* discriminated DNA extracted from different species and showed to be suitable for specific amplification of *E. coli* DNA, including O157:H7 serotype

Table 2
Bacterial strains used in this study for selectivity assessment and results of PCR assay.

Bacterial strain	Source	Cq value
Inclusivity test		
E. coli	ATCC 25922	15.8
E. coli	ATCC 35218	16.2
E. coli	ATCC 43894	16.9
E. coli	Oyster	18.3
E. coli	Oyster	16.3
E. coli	Oyster	21.3
E. coli	Oyster	17.7
E. coli	Oyster	15.6
E. coli	Oyster	16.4
E. coli	Oyster	16.7
E. coli	Oyster	18.3
E. coli	Oyster	19.0
E. coli	Oyster	20.7
E. coli	Oyster	16.8
E. coli	Oyster	16.5
E. coli	Oyster	15.4
E. coli	Oyster	19.7
E. coli	Oyster	16.0
E. coli	Oyster	17.8
E. coli	Oyster	17.5
E. coli	Oyster	17.7
E. coli	Oyster	15.3
E. coli	Oyster	20.2
E. coli	Oyster	19.3
E. coli	Oyster	16.9
E. coli	Oyster	17.7
E. coli	Oyster	16.2
E. coli	Oyster	16.6
E. coli	Oyster	16.8
E. coli	Oyster	18.7
E. coli	Oyster	15.3
E. coli	Oyster	14.3
E. coli	Oyster	21.2
E. coli	Oyster	20.8
E. coli	Oyster	19.3
E. coli	Oyster	16.4
E. coli	Oyster	16.0
E. coli	Oyster	16.3
E. coli	Oyster	16.7
E. coli	Oyster	16.4
E. coli	Oyster	17.2
E. coli	Oyster	15.7
E. coli	Oyster	16.1
E. coli	Mussel	16.6
E. coli	Mussel	18.5
E. coli	Mussel	15.1
E. coli	Mussel	14.2
E. coli	Mussel	21.1
E. coli	Mussel	20.7
E. coli	Mussel	19.0
E. coli	Mussel	16.1
E. coli	Mussel	15.7
E. coli	Mussel	16.0
E. coli	Mussel	16.4
E. coli	Mussel	16.4
E. coli	Mussel	17.2
E. coli	Mussel	15.7
E. coli	Mussel	16.1
E. coli	Mussel	16.6
E. coli	Mussel	18.5
E. coli	Mussel	15.1
E. coli	Mussel	14.2
E. coli	Mussel	21.1
E. coli	Fish	16.3
E. coli	Fish	16.4
E. coli	Fish	16.0
E. coli	Fish	17.3
E. coli	Fish	16.9
E. coli	Fish	15.3
E. coli	Fish	18.3
E. coli	Fish	19.7

Table 2 (*continued*)

Bacterial strain	Source	Cq value
Inclusivity test		
E. coli	Fish	19.2
E. coli	Fish	16.2
Exclusivity Test		
Enterococcus faecalis	ATCC 29212	–
Salmonella Typhimurium	ATCC 14028	–
Proteus vulgaris	ATCC 8427	–
Clostridium perfringens	ATCC 12924	–
Bacillus subtilis	ATCC 6533	–
Bacillus cereus	ATCC 11778	–
Salmonella enteritidis	ATCC 13076	–
Listeria monocytogenes	ATCC 19115	–
Klebsiella pneumoniae	ATCC 13882	–
Citrobacter freundii	ATCC 43864	–
Vibrio parahaemolyticus	ATCC 17802	–
Yersinia enterocolitica	ATCC 9610	–
Aeromonas hydrophila	ATCC 7966	–
Staphylococcus aureus	ATCC 25923	–

(ATCC 43894). Blast analyses showed suitable alignment with *E. coli* strains with 100% identity and no secondary structures were revealed by OligoCalc Program. The qPCR method proved to be advantageous over the methods based on β-glucuronidase enzyme activity, especially for detection of some pathogenic *E. coli*.

3.2. Standard curves with E. coli DNA

Bustin et al. (2009) related that PCR amplification efficiency must be established by means of calibration curves, because such calibration provides a simple, rapid, and reproducible indication of its efficiency, analytical sensitivity, and robustness of the assay. Therefore, in the present work the reaction parameters (efficiency, slope, and correlation coefficient) of the qPCR assay were determined based on standard curves obtained from tenfold serial dilutions of three different *E. coli* DNA extractions. The reaction parameters were calculated by plotting the Cq values against the log of the genome copy number. Four independent standard curves were determined for the primers and probe (Table 3).

The standard curves presented a suitable linear correlation (mean $R^2 = 0.99$) with a mean slope value of −3.3576, corresponding to a qPCR efficiency of 99%. These correlation coefficients demonstrated that qPCR assays were linear over a range of 10^1 genome copies. A reliable standard curve should present a R^2 value higher than 0.95 and a slope between −3.9 and −3.0, corresponding to qPCR efficiencies between 80 and 115%, respectively, as reported by Zhang and Fang (2006). It should be noted that European Network of Genetically Modified Organism Laboratory (ENGL) (ENGL, 2015) defined for genetically modified organisms (GMO) analysis that the parameters of the standard curve should be more restrictive, presenting a R^2 higher than

Table 3
Parameters of qPCR standard curves for *E. coli* detection using bacterial DNA serial dilutions.

qPCR run	DNA sample	Efficiency (%)	Slope	R^2	Range (genome copies of *E. coli*)
1	A	0.999	−3.3363	99%	10^1–10^7
2	B	0.998	−3.4128	96%	10^1–10^7
3	C	0.996	−3.3546	99%	10^1–10^7
4	B	0.999	−3.3265	100%	10^1–10^7
Mean		0.998	−3.3576	99%	10^1–10^7
SD		0.002	0.039	1.550	
CV (%)		0.17	−1.15	1.57	

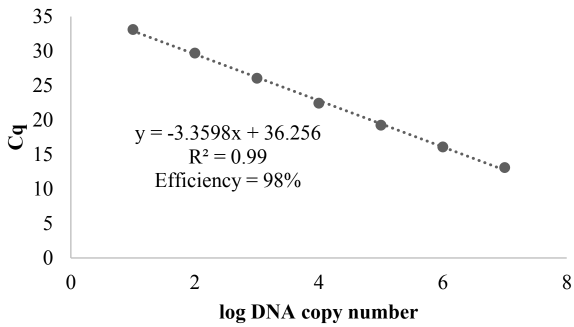

Fig. 1. qPCR standard curve for *E. coli* quantification generated using 4 DNA extractions from *E. coli* (ATCC 25922) as template DNA.

0.98 and a slope between −3.6 and −3.1, corresponding to PCR efficiency between 90 and 110%.

The y-intercept is an indication of the sensitivity of the assay and how accurately the template has been quantified, the y-intercept value of a sensitive method should be 33 to 37 cycles (Adams, 2006). In our case, the mean y-intercept found was equal to 36.256 (Fig. 1).

The LOD for *E. coli* was 10^1 genome copies (corresponding to 56.2 fg) in all of the reactions, although 1 genome copy (corresponding to 5.62 fg) was detected in 55.6% of the reactions (mean Cq = 38.02). Since 1 genome copy was not detected in all reactions, it was not used for the construction of the standard curve. ENGL (ENGL, 2015) indicated that the sensitivity of a qPCR method can be determined through the LOD, i.e., the lowest amount of sample that can be reliably detected. The qPCR assay developed in the present study ensured the reliably detection of amounts of *E. coli* DNA ranging from 10^1 genome copies (56.2 fg) to 10^7 genome copies (50.62 pg), showing the high sensitivity of this method. The efficiency of the reaction found in the present study are more suitable than the efficiency of 112% reported on a study conducted with *E. coli* O157:H7 by Elizaquível et al. (2011), using primers targeting with the same *uidA* gene. It is noteworthy that a LOD of 1 genome copy was found by those authors, while the LOD in the present study was 10 genome copies.

3.3. Standard curves with oyster DNA background

In order to evaluate the effect of background oyster DNA in qPCR quantification of *E. coli*, amplification efficiencies were determined by the construction of a standard curve with serial dilution of bacterial DNA in the presence of a constant amount of DNA extracted from oyster sample (50.62 ng per well). The results obtained from the standard curve constructed with mixed DNA showed a reduction of the reaction efficiency. In the presence of oyster DNA background, the amplification efficiency was 80% (slope = 3.9257), the correlation coefficient was 0.99 and the LOD increased to 10^2 copies (mean Cq = 34.81). Andersen et al. (2006) affirmed that the presence of large amounts of background DNA could also have some effect on the target DNA amplification. It is generally assumed that changes in PCR efficiency may occur due to substances in the isolated DNA, such as enhancers and inhibitors of the PCR reaction, originating either from the sample matrix or from the DNA extraction solutions (Cankar et al., 2006).

Since a slightly reduction on the efficiency was observed with oyster background DNA, the construction of standard curves with bacterial DNA extracted from oysters samples inoculated with *E. coli* were necessary to evaluate if the matrix could have some influence on the qPCR efficiency.

3.4. Evaluation of different DNA extraction kits

When PCR is applied to food samples the amplification reaction can be inhibited or its sensitivity reduced severely (Dickinson et al., 1995) by the presence of compounds from the food matrix. An important requirement of DNA-based method for bacterial quantification in food is an efficient DNA extraction method (Garcia et al., 2013). The choice of the DNA extraction method should consider costs, optimal yield of DNA and removal of substances that could influence the PCR reaction (Cankar et al., 2006; Oliveira et al., 2013). Results related to DNA yield obtained in our study for each of the three DNA extraction kits are summarized in Table 4.

Table 4
Comparison of the extraction purity and yield of the different kits tested for the extraction of bacterial DNA from oyster samples.

Log CFU/g	Purity (ratio A260/280)			Yield (ng/μl)		
	MK	FK	TK	MK	FK	TK
2	1.9	1.7	2.0	14.0	29.6	105.3
3	1.9	1.6	2.1	13.2	30.0	120.4
4	1.9	1.7	2.1	14.6	23.4	118.5
5	1.9	1.7	2.1	17.1	21.8	92.6
6	1.9	1.6	2.1	15.4	23.1	73.5
7	2.1	1.5	2.1	19.2	16.6	53.1
8	2.1	1.4	2.1	13.4	21.8	59.8
Mean	2.9	1.6	2.1	15.3	23.8	89.0

MK: axyprep™ bacterial genomic miniprep kit; KF: dneasy mericon food kit; TK: dneasy blood and tissue kit.

The MK and FK protocols provided a low DNA yield especially when compared with the TK protocol that provides DNA concentrations ranging for 53.1–120.45 ng/μL. Using MK and FK, DNA concentrations varied from 13.2 to 19.2 ng/μL and 16.6–29.6 ng/μL, respectively (Table 4). The DNA yields presented in our results are lower than observed by Garcia et al. (2013) where the method with the highest yield achieved 188.0–317.0 ng/uL of DNA; however, it is possible that some DNA from the sample may have contaminated the bacterial DNA. The UV absorbance (A260/A280 ratio) was used to assess DNA quality. Oliveira et al. (2013) highlighted that the deviation from the theoretical value of approximately 1.8 for DNA extracts indicates the presence of proteins, phenols, or contaminants absorbing strongly in the 280 nm region. The extracts obtained by the tested protocols presented a range of 260/280 ratios of 1.4–2.1. The 260/280 absorbance measurements were as follows: MK, 1.9 to 2.1; FK, 1.4 to 1.7; and TK. 2.0 to 2.1. These results indicate contamination of DNA with protein in FK protocol and with RNA in MK and TK protocols (Table 4).

A range of 260/280 larger than the present study was observed on a previous study conducted by Oliveira et al. (2013) with bacterial DNA extracted from mussel samples using six different extraction protocols, the purity values found were 0.9–2.1. Considering the purity results (2.0–2.1), the high DNA yield observed with TK method could be due a presence of DNA from oyster tissue, which was also observed by Oliveira et al. (2013).

Regarding qPCR (Fig. 2) with the DNA obtained from the three different kits, the quantification was specific with a LOQ value of 4 log of *E. coli* by FK and MK and 2 log of *E. coli* by TK. The differences between the LOQ values, which is 2 log lower for TK method than others, could be due to differences observed in the DNA yield.

The assays with DNA extracted with FK and MK protocols performed in a linear manner between 4 log and 8 log CFU/g of *E. coli* and the R^2 value were 0.99 for both FK and MK assays. The efficiency were 120% (slope = −2.9146) for MK method, which is out of the acceptable range (ENGL, 2015; Zhang and Fang, 2006) and 100% (slope = −3.3117) for FK method. With respect to the standard curve constructed with DNA from TK, the assay performed in a linear manner between 2 log and 8 log CFU/g of *E. coli*, the R^2 value was 0.99 corresponding to a PCR efficiency of 100% (slope = −3.3102) (Fig. 2). The LOQ of 2 log was the lowest LOQ between the three methods.

In fact, the results of our study are in agreement with Cankar et al. (2006) who reported that the DNA extraction methods should ensure

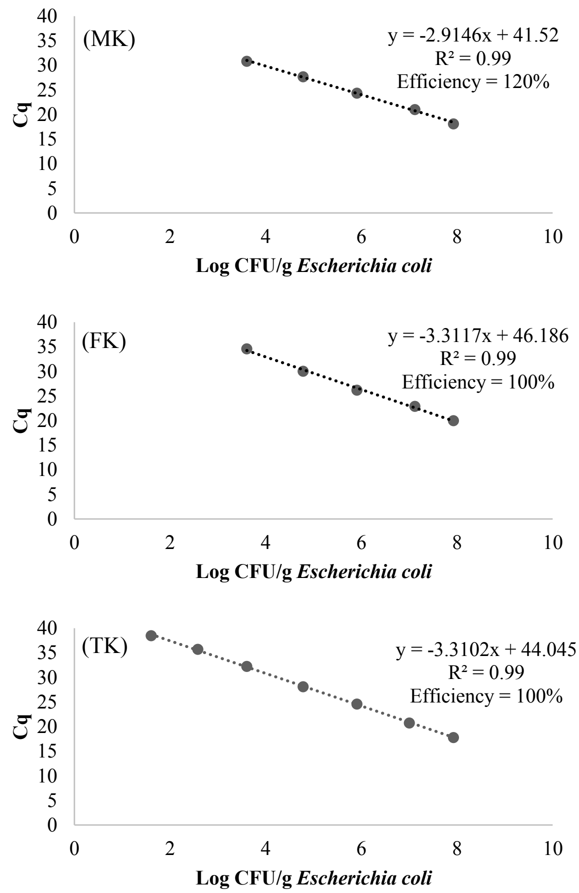

Fig. 2. Standard curves generated from the quantification cycle (Cq) values plotted against the enumeration of *E. coli* in oyster samples using Axygen® AxyPrep™ Bacterial Genomic Miniprep Kit (MK); DNeasy Mericon Food Kit (FK) and DNeasy Blood & Tissue Kit (TK).

high DNA yield and quality and minimize interference with PCR reactions and also, that the best indicator of DNA quality is functionality in the application of interest (ThermoScientific, 2011).

Considering the results of efficiency, range of use and LOQ, ease of use, chemical toxicity and storage advantages, the TK protocol was selected for all subsequent experiments trials with oyster samples. The possibility of total automation which makes the DNA extraction standardized and fast avoiding cross-contamination was also considered.

3.5. Standard curve with inoculated oyster samples

A new standard curve (Cq values *versus* log CFU/g of *E. coli*) was constructed using the TK method for DNA extraction. The standard curve was linear from 10^2 to 10^7 *E. coli* CFU/g. The R^2 was 0.99 and the slope of the curve was −3.3354 corresponding to an efficiency of 99%, demonstrating the strong correlation between qPCR method and CFU/g counts.

The LOQ of 2 log is lower than LOQ reported in previous studies but still could be enhanced by a pre-enrichment treatment. The parameters found here were different from a previous study conducted by Takahashi et al. (2009) where a LOD of 3 log CFU/g of *E. coli* and efficiency of 111% were reported, emphasizing the suitability of the method of the present study for *E. coli* quantification in oyster samples. The linearity of the standard curve and the fact that the qPCR operates with constant efficiency confirmed that the assay was well suited to quantitative measurements of *E. coli* in oyster samples. The concentration of *E. coli* CFU/g obtained by the proposed qPCR can be described by the following equation: Cq = - 3.3696 log CFU/g + 44.63, where CFU/g $= 10^{(Cq-44.421/-3.3354)}$.

Table 5
Repeatability of qPCR assay in different days.

	Mean Cq of each run				
E. coli Log CFU/g	10^2	10^3	10^4	10^5	10^6
	36.63	33.33	29.35	27.37	24.86
	37.38	32.99	29.69	26.37	23.83
	37.16	33.52	31.12	27.14	23.40
	37.28	33.21	29.99	26.38	23.63
	37.62	32.76	28.06	26.70	22.85
	37.18	31.94	29.14	26.23	22.43
Mean	37.21	32.96	29.56	26.70	23.50
SD	0.33	0.56	1.01	0.46	0.84
% RSDr	0.89	1.71	3.42	1.73	3.59

3.6. Comparison of qPCR with the culture method in enumerating E. coli in oyster samples

The 30 oyster samples artificially inoculated with *E. coli* in different concentrations were used for repeatability testing and to compare the qPCR method with the culture method using the equation CFU/g $= 10^{(Cq-44.421/-3.3354)}$, determined previously.

The RSDr values were determined for the Cq values from each of the six repetitions with the five different concentrations (10^2–10^6 CFU/g) of *E. coli* in oyster samples (Table 5). The RSDr values were below 4%, ranging between 0.89% and 3.59%. According to ENGL (ENGL, 2015) guidelines, the repeatability of qPCR expressed as RSDr should be less than 25% over the whole dynamic range of the PCR. The results presented in this study are in agreement with the ENGL (ENGL, 2015) guidelines statement indicating a suitable degree of accuracy of the assay and evidence that the method is repeatable. In all the 30 oyster samples tested, the difference in CFU/g counts between the qPCR and the culture method was within 1 order of magnitude (≤0.5 log CFU/g) and in 83% (n = 25) the difference was less than 0.3 log (Table 6).

In 83% (n = 25) of the 30 oyster samples, the qPCR method showed higher CFU/g counts than culture method. The *E. coli* counts obtained through qPCR method had good correlation (R^2 = 0.98) with the culture method within a range of 10^2–10^6 CFU/g (Fig. 3). From these results, we considered this qPCR method to be applicable for enumerating *E. coli* in oyster samples.

Table 6
Evaluation of qPCR method in inoculated samples and comparison with reference method.

		E. coli Log CFU/g						Mean	SD	% RSD_r
10^2	qPCR method	2.3	2.1	2.2	2.1	2.0	2.2	2.2	0.10	4.58
	Reference method	2.5	2.0	2.2	2.1	2.2	2.0			
	Difference[a]	0.2[b]	0.1	0.0	0.1	0.2[b]	0.1			
10^3	qPCR method	3.3	3.4	3.3	3.4	3.5	3.7	3.4	0.17	4.92
	Reference method	3.2	3.1	3.1	3.0	3.0	3.5			
	Difference[a]	0.1	0.3	0.2	0.4	0.5	0.3			
10^4	qPCR method	4.5	4.4	4.0	4.3	4.9	4.6	4.5	0.30	6.81
	Reference method	4.1	4.5	4.0	4.0	4.4	4.3			
	Difference[a]	0.4	0.1[b]	0.0	0.3	0.5	0.3			
10^5	qPCR method	5.1	5.4	5.2	5.4	5.3	5.5	5.3	0.14	2.60
	Reference method	5.4	5.4	5.0	5.0	5.0	5.5			
	Difference[a]	0.3[b]	0.0	0.1	0.4	0.3	0.0			
10^6	qPCR method	5.9	6.2	6.3	6.2	6.5	6.6	6.3	0.25	4.03
	Reference method	6.2	6.0	6.0	6.1	6.4	6.4			
	Difference[a]	0.3[b]	0.2	0.3	0.1	0.1	0.2			

[a] Difference in Log between the qPCR and reference method *E. coli* counts.
[b] qPCR method *E. coli* count is lower than the reference method.

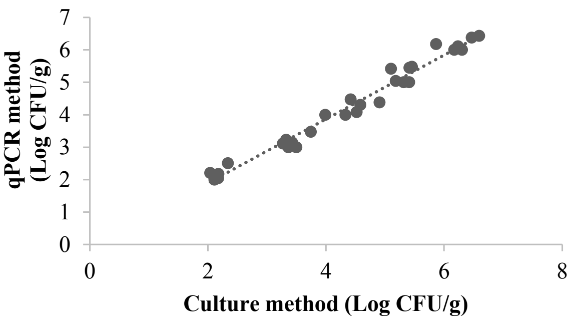

Fig. 3. Linear regression comparing *E. coli* CFU/g counts estimated by qPCR method and culture method in oyster samples.

Considering the results with the 60 natural oyster samples, the difference in CFU/g counts between the qPCR and the culture method was less than 1 log in 62% (n = 37) of the samples and a good correlation was found ($R^2 = 0.82$) between the two methods in 55% (n = 33) of the samples.

Among the 60 samples, in 62% (n = 37), the qPCR showed higher CFU/g counts than the culture methods; this fact might be due to the TBX agar. The culture method used in the present study is the ISO reference method for enumeration of β-glucuronidase-positive *E. coli*. Strains of *E. coli* which do not grow at 44 °C and, in particular, those that are β-glucuronidase negative, such as *E. coli* O157, will not be detected by this method (ISO, 2001), which leads, inevitably, to a higher counting using qPCR method and demonstrates an advantage in relation to the culture method.

Higher bacterial counts using qPCR method compared to culture method were also observed in previous studies with quantification of *Enterobacteriaceae* and *Listeria monocytogenes* in food samples (Berrada et al., 2006; Takahashi et al., 2017). This could be due to some limitations of the PCR method, which amplifies DNA from both live and dead cells, since DNA can remain intact after cell death and may persist for a few days to 3 weeks after cell death. Therefore, the presence of living microorganisms may be overestimated or false positive results may occur in using this technique (Nocker et al., 2007).

It should be noted that in 8% (n = 5) of the samples, the counts by culture method were higher than qPCR method and in 13% (n = 8) of the samples, the Cq value of qPCR were > 40, but by culture method the counts were between 1.7 log and 2.2 log CFU/g. This may be due by the presence of impurities in the DNA that have inhibited the amplification or because the *E. coli* counts were lower than the LOD of the qPCR method. In these cases, a culture enrichment prior to qPCR would be needed, as previously reported by Takahashi et al. (2009). Finally, the qPCR results showed higher *E. coli* counts compared to the culture method, which encourages future studies focusing on the DNA-binding reagents in order to eliminate DNA from dead bacterial cells.

4. Conclusions

In conclusion, the present study described a suitable and sensitive qPCR method for quantification of *E. coli* in oysters in less time than the culture method. This is the first report of a method exclusively developed for direct quantification of *E. coli* in oyster which is extremely important considering *E. coli* counts are used for microbiology control of oysters and also for classifying the harvesting areas in many countries. The designed primers and probe set enable selective and sensitive detection of *E. coli* even in the presence of DNA from oyster sample. The efficiency, good correlation and selectivity results found are in agreement with the recommendations by the literature. We can conclude that the results with artificially inoculated samples were more suitable than with natural samples. Further studies will be needed in order to ensure the selective quantification of viable cells.

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