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

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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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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