

# Application of multiplex real-time polymerase chain reaction assay for simultaneous quantification of *Escherichia coli* virulence genes in oysters

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**Abstract** Strains of diarrheagenic *Escherichia coli* (DEC) are involved in foodborne disease outbreaks worldwide, especially the enterohemorrhagic *E. coli* O157:H7. This study describes two multiplex quantitative real time PCR (qPCR) assays for simultaneous identification and quantification of genes related to virulence of DEC; a triplex reaction for detection and quantification of *stxA1*, *stxA2*, and *eaeA* genes, and a duplex reaction for detection and quantification of *eaeA* and *virA* genes. The technique was applied in raw oyster samples for direct quantification of DEC, thereby evaluating the applicability of this methodology for microbiological quality assessment of food. Using custom designed primers and specific MGB probes, a *triplex* qPCR assay was performed to quantify *stxA1*, *stxA2*, and *eaeA*, and a *duplex* reaction was performed to quantify *virA* and *eaeA* genes. The assays showed high sensitivity, with the detection limit varying between 5 and 17 copies of the genes. The coefficient of determination ( $R^2$ ) of the standard curves was 0.99. The coefficient of variation was < 1% indicated high intra- and inter-assay reproducibilities. The application of this methodology in oyster samples from tropical environment provided direct quantitative data that determined the presence of the genes *stxA1* (32.1%), *eaeA* (28.6%), *stxA2* (3.6%), and *virA*

(3.6%). This would prove critical for immediate intervention of control strategies, particularly in oysters that are often ingested as raw food.

**Keywords** qPCR · Diarrheagenic *E. coli* · Food safety

## Introduction

*Escherichia coli* is a bacterium that is part of the gut microbiota of humans and animals, but some pathogenic strains can cause intestinal infections (diarrheagenic *E. coli* [DEC]) or extra-intestinal infections, such as meningitis and urinary infections (extraintestinal pathogenic *E. coli* [ExPEC]) (Croxen et al. 2013; De Moura et al. 2012; Nataro and Kaper 1998). The difference between indigenous and pathogenic strains is the presence of virulence factors in the latter.

DEC represents one of the 10 major causes of foodborne disease (FBD) in the world (World Health Organization 2015). The presence of DEC in seafood is directly related to poor water quality and unfavorable hygiene conditions during processing. This is even more evident in bivalve mollusks. Since these are filter organisms, the microbiological quality is directly linked to the water quality of the cultivation area, as demonstrated by several studies (Doi et al. 2015; González et al. 2009; Mignani et al. 2013; Pereira et al. 2006). As oysters are often eaten raw, the risk of FBD outbreak is high. Taminiau et al. (2014) claim that the number of cases of FBD related to seafood has increased.

The identification of *E. coli* by traditional culture methods alone is not sufficient to differentiate indigenous strains from those of DEC, hence more specific approaches are necessary. Laboratories of food microbiology analysis

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have begun to increasingly depend on sensitive and specific molecular techniques that enable earlier detection of virulence factors and quantification of pathogens (Elizaquível et al. 2014; Rodríguez-Lázaro et al. 2013). Faster and more effective diagnosis facilitates a more specific treatment, leading to a better prognosis of the disease (Navarro et al. 2015). In addition to fast results, quantitative data may be useful to carry out quantitative microbial risk assessment (QMRA) thereby allowing early intervention of control strategies (Malorny et al. 2008).

Bustin et al. (2009) reported the possibility of standardization of multiplex qPCR assays, allowing the simultaneous detection of more than one pathogen. It is important to choose target genes that are conserved and specific to each pathogen (Taminiau et al. 2014).

To increase the sensitivity of detection of virulence genes, a pre-enrichment step can be inserted before molecular detection steps (Derzelle et al. 2011). However, this initial bacterial growth makes it impossible to quantify pathogens in the original sample (Maciel et al. 2011) because it does not necessarily reflect the level of contamination of food (Hidaka et al. 2009). On the other hand, the qPCR methodology does not differentiate between DNA from living or dead cells, and may overestimate the level of contamination of the sample. Hence, qPCR can be applied as screening approach, providing immediate quantitative data that helps to determine the level of contamination in the sample. This is essential because early detection of virulence genes of *E. coli* would be crucial for immediate intervention of control strategies, especially in oysters that are often eaten uncooked.

Here, we describe the standardization of two multiplex qPCR assays for the simultaneous quantification of virulence genes from diarrheagenic strains of *Escherichia coli*, without pre-enrichment step. In the first test, *stxA1*, *stxA2*, and *eaeA* genes were used as targets, allowing the identification and quantification of *E. coli* strains that harbor genes involved in the production of Shiga toxin (*stx*), such as Shigatoxigenic *E. coli* and enterohemorrhagic *E. coli* (STEC/EHEC), and the intimin gene (*eae*), which is associated with enteropathogenic *E. coli* (EPEC) strains, but is also present in EHEC strains. The second test targeted *eae* and *vir* genes, allowing the simultaneous amplification of EPEC strains and enteroinvasive *E. coli* (EIEC), respectively. The technique was applied in raw oyster samples to directly quantify DEC and evaluate the applicability of this methodology in the microbiological quality assessment of food.

## Materials and methods

### Pathogenic *E. coli* strains

Strains of enterohemorrhagic *E. coli* (EHEC O157:H7 INCQS 00171 [CDC EDL-933]); enteropathogenic *E. coli* (EPEC, INCQS 00181 [CDC O55]) and enteroinvasive *E. coli* (EIEC INCQS 00170 [CDC EDL-1284]) acquired from National Institute of Quality Control in Health (INCQS), Oswaldo Cruz Institute (Rio de Janeiro, Brazil), were used for amplification of the target genes related to virulence (*stxA1*, *stxA2*, *eaeA* and *virA*—Table 1).

An isolated colony of each microorganism was inoculated in 1.0 mL of tryptic soy broth (TSB) and incubated at 37 °C for 18–24 h. This bacterial suspension was then used for genomic DNA extraction using the Easy DNA Extraction Kit (Invitrogen, Carlsbad, CA, USA). The DNA from each strain was quantified at 260 and 280 nm using the Nano Drop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and was then used in conventional PCR to amplify the target gene of each strain and produce the qPCR standard curve.

### Design of primers and probes for qPCR assays

Primers and MGB probes (Table 1) were designed using software Primer Express, version 3.0 (Life Technologies). Regions of each target gene (*stxA1* [GenBank accession AF461172]; *stxA2* [GenBank accession AY143337]; *eaeA* [GenBank accession AE005595] and *virA* [GenBank accession NC\_004851.1]), amplified by conventional PCR using primers described in literature (Table 1) was used as template to design internal primers and probes for qPCR assays which amplify fragments varying between 56 and 91 bp (Table 1).

### Production of standard curves

To generate a standard curve, conventional PCR was performed, using bacterial DNA as a template in individual reactions for specific primers against target genes for each strain of pathogenic *E. coli* (Table 1). The amplifications were performed in a total volume of 50 µL, containing 1X PCR buffer (Invitrogen), 1.5 mM MgCl<sub>2</sub> (Invitrogen), 200 µM dNTP (Invitrogen), 0.4 µM of each primer (sense and anti-sense), 2 U Taq DNA Polymerase (Invitrogen), and 2 µL of DNA at 50 ng/µL. The PCR reactions were carried out with an initial denaturing step (94 °C for 5 min), 32 cycles (94 °C for 60 s, 57 °C for 60 s, 72 °C for 60 s), and a final extension step (72 °C for 7 min). The PCR products were purified using PureLink™ Quick Gel Extraction & PCR Purification Combo kit (Invitrogen), and

**Table 1** Primers and probes used in PCR and qPCR

Reference strain	Target gene (GenBank accession no.)	Primes 1 (5'-3') <sup>a</sup>	Bp	Reference	Primers 2 (5'-3') and probes <sup>b</sup>	bp
<i>E. coli</i> EHEC (O157:H7) INCQS 00171 (CDC EDL-933)	<i>stxA1</i> : Shiga toxin 1 (VT1) (AF4611172)	F: GAAGAGTCCCGTGGGATTACG R: AGCGATGCAGCTATTAATAA	130	Pollard et al. (1990)	F: TCCGTGGGATTACGCACAA R: CATCAGAATTGCCCCAGAGT P: AAAATATTGTGGGATTCATC (FAM)	62
<i>E. coli</i> EPEC (O55)	<i>stxA2</i> : Shiga toxin 2 (VT2) (AY143337)	F: ACCGTTTTTTCAGATTTT(G/A)CACATA R: TACACAGGAGCAGTTTCAGACAGT	298	Svenungsson et al. (2000)	F: GAACGTTCCGGAATGCAAA R:CCATTAAACGCCAGATATGATGAAA P: CAGTCGTCACCTCACTG (VIC)	61
<i>E. coli</i> EPEC (O55)	<i>eaeA</i> : <i>E. coli</i> attaching and effacing (AE005595)	F: AAAAAAGCGCTGACCCGCACCTAAAT R: CACACGAATAAACTGACTAAAAATG	376	Svenungsson et al. (2000)	F: TGACCCCGCACCTAAATTTGC R: CAGGTCGGAGCGCGTTA P: AAAGCGGGAGTCAAT (NED)	56
<i>E. coli</i> EIEC (O157:H7) EDL-1284	<i>virA</i> : <i>Shigella</i> invasion (NC_004851.1)	F: CTGCATTCTGGCAATCTCTTCACA R: TGATGAGCTAACTTCGTAAGCCCTCC	215	Villalobo and Torres (1998)	F: CCGAAAAACATAGAAAAACAGGCTTAA R: GGGACAACCTGGGTTGATTTTAT P: TTCATGCCTGAACAAC (VIC)	91

<sup>a</sup>Used in the conventional PCR to amplify the target gene. The PCR product was used to build the standard curve of the qPCR

<sup>b</sup>Designed in this study through Primer Express® Software For Real-Time PCR, version 3.0 (Applied Biosystems™)

the intactness of prepared DNA was estimated by absorbance measured at 260 and 280 nm using the NanoDrop 2000 (Thermo Scientific) spectrophotometer. The purified PCR products were used to generate the standard curve in qPCR.

The purified products of target gene amplifications for each bacterial strain were diluted to 50 ng/ $\mu$ L and the gene copy numbers were determined by the formula:

$$\text{Gene copy number} = \text{Amount of DNA } (\mu\text{g}) \times 6.022 \times 10^{23} / \text{DNA fragment (bp)} \times 10^6 \times 650.$$

The standard curves used in qPCR were constructed using serial dilutions of  $10^5$ – $10^1$  copies of target genes from each strain, viz: *stxA1* ( $2.8 \times 10^5$  to  $2.8 \times 10^1$ ); *stxA2* ( $1.24 \times 10^5$  to  $1.24 \times 10^1$ ); *eaeA* ( $5.0 \times 10^4$  to  $5.0 \times 10^0$ ) and *virA* ( $1.72 \times 10^5$  to  $1.72 \times 10^1$ ).

### Multiplex qPCR for simultaneous quantification of *E. coli* virulence genes

Initial qPCR was performed using SYBR Green (Power SYBR<sup>®</sup> Green, Applied Biosystems, Warrington, UK), to standardize the amount of primers required for the highest reaction efficiency, through melting curve analysis. The reactions were performed using the AB 7500 Fast (Life Technologies, Carlsbad, California, USA), in a total volume of 20  $\mu$ L containing 0.5  $\mu$ L of each primer (at 5  $\mu$ M), 10  $\mu$ L of SYBR<sup>®</sup> Select Master Mix (Applied Biosystems), 2  $\mu$ L of DNA at 50 ng/ $\mu$ L. The reactions were carried out with an initial denaturing stage (95 °C for 20 s) followed by 40 amplification cycles (95 °C for 3 s, 60 °C for 30 s). Dissociation curves were obtained in one cycle of 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s, 60 °C for 15 s.

Once the amount of primers were defined, multiplex qPCR reactions were carried out through the TaqMan system (Applied Biosystems), using TaqMan<sup>®</sup> MGB probe labeled with FAM, VIC or NED fluorophores for each target gene, as described in Table 1. Two multiplex reactions were standardized; a triplex reaction for detection and quantification of *stxA1*, *stxA2*, and *eaeA* genes, and a duplex reaction for detection and quantification of *eaeA* and *virA* genes. The amplifications were performed in a total volume of 20  $\mu$ L containing 0.5  $\mu$ L of each primer at 5  $\mu$ M, 0.5  $\mu$ L of each probe at 5  $\mu$ M, 10  $\mu$ L of TaqMan<sup>®</sup> Fast Advanced Master Mix (Applied Biosystems) and 2  $\mu$ L of DNA at 50 ng/ $\mu$ L with sterile DNase and RNase-free water to make up to the final volume. The reaction was carried out with an initial cycle (95 °C for 20 s) and 40 cycles (95 °C for 30 s, 60 °C for 30 s), in the AB 7500 fast (Life Technologies). Standard curves were analyzed to

verify slopes close to  $-3.32$ , which represents 100% efficiency.

### Direct quantification of DEC in oyster samples by multiplex qPCR

The multiplex qPCR technique was applied directly on samples of raw oysters intended for consumption without a pre-enrichment step. Twenty-eight samples composed of a pool of 40 oysters (totaling 1120 oysters) were randomized collected in four different regions: two regions of cultivation (coordinates 13°40'02"S; 39°09'06"W; and 13°40'02"S; 39°09'06"W, located in the Camamu Bay, in the cities of Camamu and Maraú, Northeast Brazil); and in two extractive estuary regions (coordinates 14°50'57"S; 39°03'14"W; and 14°49'63"S; 39°03'49"W, in the Cachoeira River estuary in the city of Ilhéus, Northeast Brazil). Seven collections in each region were made during 1 year. All oysters were opened with a sterile knife, and the organic material was collected in a sterile beaker and homogenized using an NT136 tissue homogenizer (Novatécnica, Piracicaba/SP, Brazil), and 1.0 g of each sample was then used for DNA extraction using the Easy DNA extraction Kit (Invitrogen). For qPCR reaction, 50 ng DNA/ $\mu$ L was used as a template, according to the standard protocol described above. To check the absence of PCR inhibition that could produce false negative results, the Starter Kit (Invitrogen, Carlsbad, CA, USA) was added to the multiplex reaction as internal exogenous control, following the manufacturer's standards. Samples which presents fluorescence signs below to the limit of detection of the technique was considered as false-positive and was not considered in the analysis. DNA from autoclaved oyster (uncontaminated sample) was used as negative control.

### Sensitivity and specificity of qPCR reactions

The limit of detection of each gene was determined through serial 1:2 dilutions: *stxA1* (140; 70; 35; 17.5 gene copy numbers); *stxA2* (62; 31; 15.5; 7.75 gene copy numbers); *eaeA* (49; 24.5; 12.25; 6.13 gene copy numbers), and *virA* (86; 43; 21.5; 10.75 gene copy numbers). The dilutions were added to DNA extracted from an autoclaved oyster sample. To confirm the specificity of the primers and probes used in qPCR, the sequences of target genes were analyzed using Basic Local Alignment Search Tool (BLASTn) (<http://blast.ncbi.nlm.gov/Blast.cgi>) to check for similarities with sequences available in the database. In addition, a conventional PCR reaction was performed using each primer pair to amplify DNA extracted from the different bacterial strains used in this study.

## Rapid culture method

The results of multiplex qPCR were confirmed by those of the culture method Compact Dry® (HyServe, GmbH & Co. KG, Uffing Germany), using the rapid identification kit for enumerates *E. coli* (Compact Dry EC). One hundred grams of oysters homogenized in 100 mL of peptone water (Acumedia - Neogen do Brasil, Indaiatuba, SP, Brazil) were used in this analysis and serial dilutions from  $10^{-1}$  to  $10^{-5}$  were performed. Then, 1 mL of each dilution was poured on Compact Dry EC plates and incubated at 37 °C for 18–24 h. All analyses were performed in duplicate and according to the manufacturer's instructions. After incubation, 3–5 colonies were selected to be confirmed via conventional PCR for the presence of virulence gene, as described above.

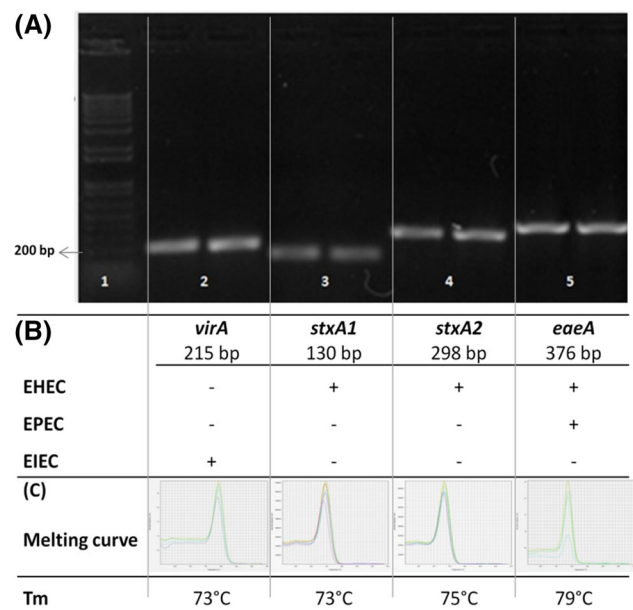
## Statistical analysis

The average of the cycle threshold ( $C_T$ ), the standard deviation ( $C_T$  SD), and the coefficient of variation (CV) were calculated in five different runs, including three replicates of each target gene, using known concentrations of  $10^5$ – $10^1$  copies. These data were used to evaluate the intra- and inter-assay reproducibility. The confidence interval for the proportion of positive oysters for *E. coli* virulence genes was calculated with 95% confidence level.

## Results and discussion

The primers for *stxA1* and *stxA2* genes were specific to the strain O157: H7 (EHEC); *virA* was specific to the EIEC strain; and *eaeA*-specific primers amplified DNA from EPEC and EHEC strains (Fig. 1). BLASTn confirmed that all sequences amplified by the primers described in this study showed 100% similarity to specific regions of *stxA1*, *stxA2*, *eaeA*, and *virA* genes, according to GeneBank access numbers AF461172, AY143337, AE005595 and NC\_004851.1 NC, respectively. SYBR Green-based dissociation curve analysis showed a melting temperature ( $T_m$ ) of 73 °C for *stxA1*, 75 °C for *stxA2*, 79 °C for *eaeA*, and 73 °C for *virA* and a single peak was observed in each case. Primer dimers were not detected (Fig. 1).

The qPCR assay showed high sensitivity, presenting a detection limit of 17 copies for the *stxA1* gene, 6 copies for *stxA2*, 5 copies for *eaeA*, and 8 copies for *virA* genes. This information is important because it makes it possible to estimate the infective dose of each strain during outbreaks, besides generating quantitative data that facilitates microbiological risk assessment. The intra- and inter-assay variations were statistically low; the intra-assay coefficient of variation (CV) was < 2% while the inter-assay CV



**Fig. 1** Amplification of *E. coli* target gene fragments. **a** Agarose Gel showing the amplification products: [1] molecular weight Marker 1 Kb Plus DNA Ladder (Invitrogen™); [2] *virA*; [3] *stxA1*; [4] *stxA2*; [5] *eaeA*. **b** PCR results using strains of Enterohemorrhagic *E. coli* (EHEC—O157: H7—INCQS 00171/CDC EDL-933); Enteroinvasive *E. coli* (EIEC—INCQS 00170/CDC EDL-1284); Enteropathogenic *E. coli* (EPEC—INCQS 00181/CDC O55). **c** Melting curves through SYBR Green qPCR showing unique peak during the amplification (no primer dimers was detected)

was < 1% (Table 2), proving the assay to be a highly reproducible technique. The standard curve slopes were  $-3.588$ ,  $-3.825$ ,  $-3.255$ , and  $-3.469$ , for the quantification of *stxA1*, *stxA2*, *eaeA*, and *virA*, respectively, which represents efficiencies varying between 82 and 102%, depending on the amplified target gene (Fig. 2). These slope values were close to the ideal value of  $-3.32$  representing 100% efficiency. The amplification efficiency assesses whether the primer pair amplifies the target gene exponentially at each cycle (Raymaekers et al. 2009). The curves showed coefficients of determination ( $R^2$ ) of 0.997 for *stxA1*, 0.998 for *stxA2*, 1.00 for *eaeA*, and 0.999 for *virA* (Fig. 2).

The culture method followed by the detection of virulence genes through PCR detected the presence of *eaeA* in 10.7% of samples and *stxA2* in 3.6% of samples. No isolate derived from microbiological culture was positive for the presence of *stxA1* and *virA* genes. However, the multiplex qPCR technique presented greater sensitivity and could detect and quantify virulence genes in a proportion of 16.64%. According to this technique, the 95% confidence interval for the proportion of 16.96% positive oysters for *E. coli* virulence genes varied between 8.92 and 19.64%, where 8.92% represents the inferior limit and 19.64% the superior limit in a significance level of 0.05. The *eaeA* gene

**Table 2** Inter and intra-assay reproducibility of qPCR

Gene copy number	Intra-assay reproducibility		Inter-assay reproducibility	
	Ct (average)	CV (%)	Ct (average)	CV (%)
<i>stxA1</i> (detection limit: 17 gene copies)				
$2.8 \times 10^5$	22.87	0.17	21.75	0.28
$2.8 \times 10^4$	26.25	0.04	25.03	0.20
$2.8 \times 10^3$	29.71	0.10	27.84	0.25
$2.8 \times 10^2$	32.68	0.91	31.04	0.16
$2.8 \times 10^1$	33.65	0.27	33.91	0.27
<i>stxA2</i> (detection limit: 6 gene copies)				
$1.24 \times 10^5$	22.50	0.58	20.06	0.30
$1.24 \times 10^4$	26.57	0.15	25.49	0.20
$1.24 \times 10^3$	29.97	0.07	28.78	0.07
$1.24 \times 10^2$	31.44	0.45	30.43	0.16
$1.24 \times 10^1$	34.98	0.91	33.42	0.30
<i>eaeA</i> (detection limit: 5 gene copies)				
$5.0 \times 10^4$	20.02	0.90	22.22	0.38
$5.0 \times 10^3$	23.72	1.48	25.70	0.32
$5.0 \times 10^2$	26.94	0.45	29.07	0.23
$5.0 \times 10^1$	30.06	0.13	33.01	0.18
$5.0 \times 10^0$	33.68	1.16	33.71	0.15
<i>virA</i> (detection limit: 8 gene copies)				
$1.72 \times 10^5$	23.72	0.08	23.65	0.51
$1.72 \times 10^4$	31.06	0.35	27.53	0.22
$1.72 \times 10^3$	34.91	0.34	31.93	0.31
$1.72 \times 10^2$	37.59	0.32	21.54	0.56
$1.72 \times 10^1$	37.91	0.16	30.46	0.40

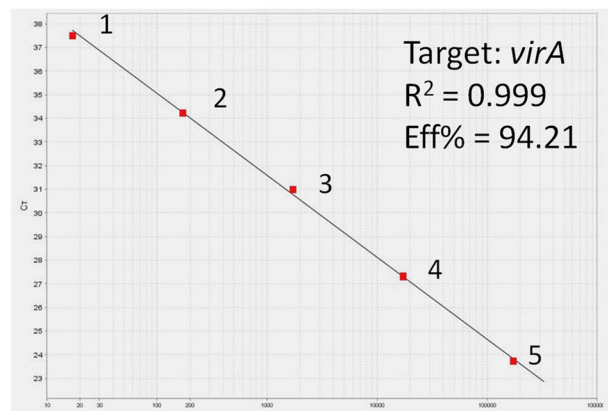
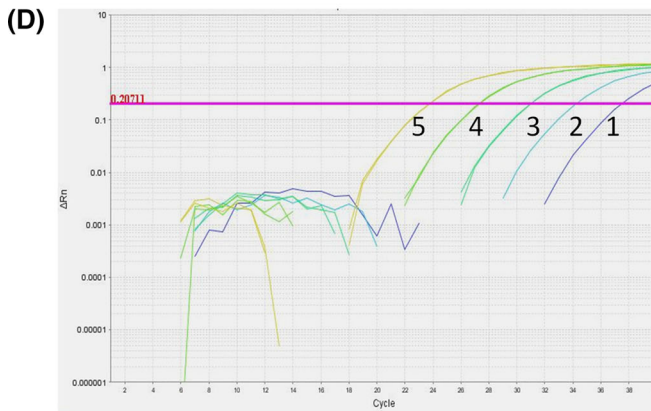
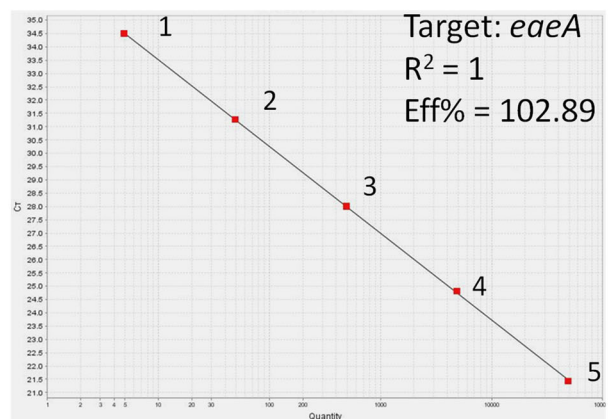
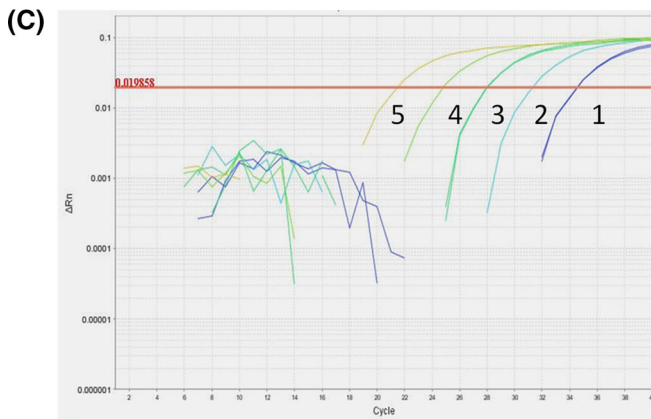
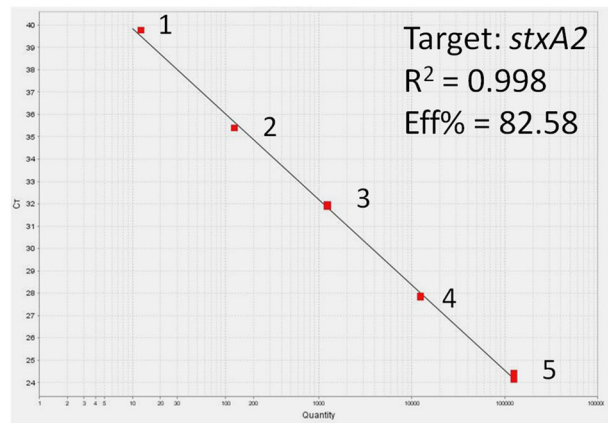
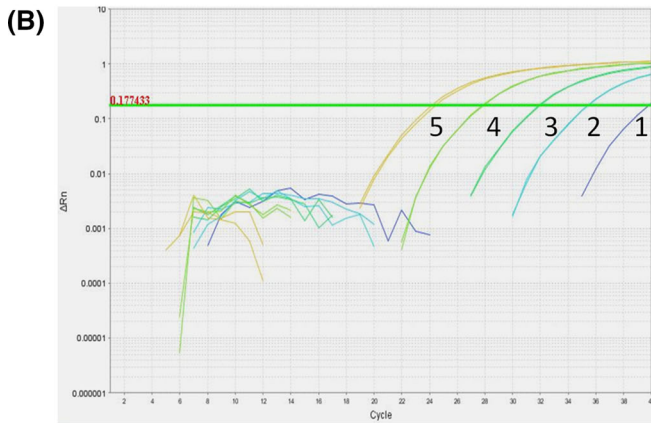
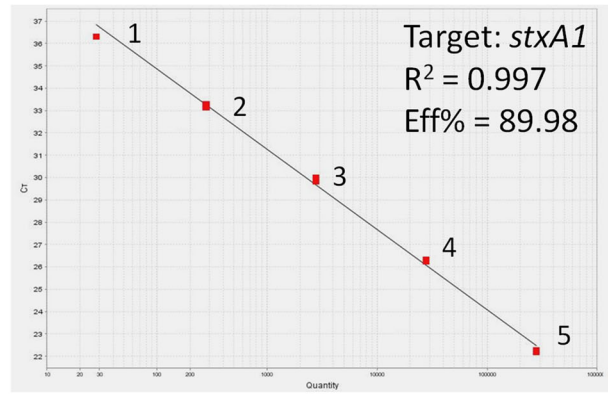
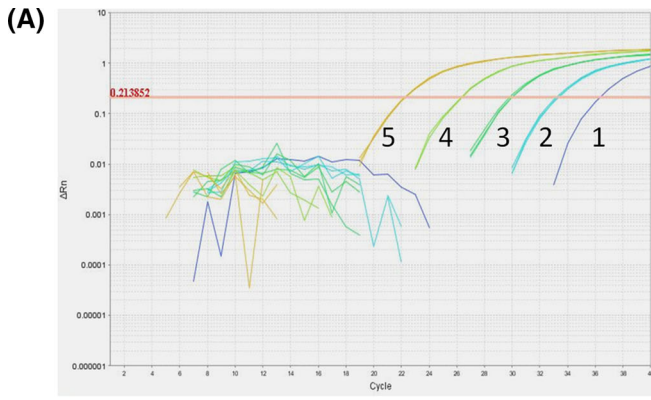
Ct cycle threshold, CV coefficient of variation

was detected in 28.6% of samples (with an average of  $1.87 \log_{10}$  copies of *eaeA*  $g^{-1}$  oyster), *stxA1* in 32.1% of samples (with an average of  $2.62 \log_{10}$  copies of *stxA1*  $g^{-1}$  oyster), and *stxA2* and *virA* in 3.6% of samples (with an average of  $2.34 \log_{10}$  copies of *stxA1*  $g^{-1}$  oyster and  $2.05 \log_{10}$  copies of *vir*  $g^{-1}$  oyster) (Table 3). In one sample, it was possible to simultaneously detect the genes *eaeA* (50 copies  $g^{-1}$ ) and *stxA1* (390 copies  $g^{-1}$ ). In this case, the total count of *E. coli* was  $2.92 \log_{10}$  cfu  $g^{-1}$ . Another sample was positive for both *eaeA* gene (220 copies  $g^{-1}$ ) and *stxA2* (220 copies  $g^{-1}$ ), and presented a total count of *E. coli* of  $2.82 \log_{10}$  cfu  $g^{-1}$ .

In total, 57.9% of positive oyster samples were from extractive estuary regions, while 42.1% were from cultivation areas. The presence of *eaeA* gene is related to EPEC and EHEC strains. This gene can also be detected in *Citrobacter rodentium*, responsible for causing transmissible murine clonic hyperplasia (Schauer et al. 1995) and *Hafnia alvei* (Donnenberg et al. 1993; Nataro and Kaper, 1998), which has lower epidemiological relevance in diarrheic conditions (Giinthard and Pennekamp, 1996). The presence of *stx1* and *stx2* genes is related to EHEC/STEC

strains, which are responsible for causing bloody diarrhea and hemorrhagic colitis (Nataro and Kaper, 1998). *stx1* is 99% identical to the Shiga toxin gene produced by *Shigella*. *stx2* is 55% identical to *stx1* and is produced by approximately 97% of EHEC strains, with or without simultaneous production of *stx1* (Garcia and Le Bouguéne 1998; Gouali and Weil 2013). The detection and quantification of these genes are particularly important, since the infecting dose of EHEC is low, needing only a few cells to mount a diarrheic condition (Nataro and Kaper 1998). Therefore, one must take a molecular approach that generates quantitative data, such as qPCR, because it makes possible the detection of very few copies of target genes. In this study, we were able to detect a minimum of 6–17 copies, depending on the target gene, thereby proving that the qPCR assay is a very sensitive method.

**Fig. 2** Amplification curves (left); standard curves (right) of serial dilutions of *E. coli* gene through TaqMan qPCR. Copy numbers of each gene: a *stxA1* ( $2.8 \times 10^5$  to  $2.8 \times 10^1$ ); B. *stxA2* ( $1.24 \times 10^5$  to  $1.24 \times 10^1$ ); C. *eaeA* ( $5.0 \times 10^4$  to  $5.0 \times 10^0$ ); D. *virA* ( $1.72 \times 10^5$  to  $1.72 \times 10^1$ )



**Table 3** Percentage of positive oyster samples for *Escherichia coli* virulence genes by multiplex real-time polymerase chain reaction assay and culture method

Method	<i>E. coli</i> virulence genes (% positive oyster samples)			
	<i>stxA1</i>	<i>stxA2</i>	<i>eaeA</i>	<i>virA</i>
Multiplex qPCR	32.1% (2.62 <i>Log</i> <sub>10</sub> ± 0.78)	3.6% (2.34 <i>Log</i> <sub>10</sub> ± 0.00)	28.6% (1.87 <i>Log</i> <sub>10</sub> ± 1.35)	3.6% (2.05 <i>Log</i> <sub>10</sub> ± 0.00)
Culture method followed by PCR	ND	3.6%	10.7%	ND
Total <i>E. coli</i> counting by culture method <sup>a</sup>	4.72 <i>Log</i> <sub>10</sub>	2.82 <i>Log</i> <sub>10</sub> <sup>b</sup>	2.49 <i>Log</i> <sub>10</sub> <sup>c</sup>	3.13 <i>Log</i> <sub>10</sub>

ND not detected

Numbers in italic mean the average of gene copy number per gram ± standard deviation

<sup>a</sup>Average of cfu g<sup>-1</sup>

<sup>b</sup>One sample was negative by culture method and positive by qPCR

<sup>c</sup>Two samples was negative by culture method and positive by qPCR

Villalobo and Torres (1998) affirm that the use of *virA* gene in molecular diagnosis is highly sensitive and specific, capable of detecting strains of EIEC and *Shigella*, both of which have a secretion system type III mediated by the plasmid Vir, through which they can inject proteins across the cell membrane of the host cell, thus facilitating bacterial invasion (Page and Liles 2013). These bacteria are responsible for causing fever and bacillary dysentery (profuse bloody diarrhea with presence of mucus) (Lampel 2014). In addition to a similar mechanism of action, EIEC has a genetic similarity with *Shigella*, due to which there are chances of underreporting of EIEC infections (Croxen et al. 2013; Van Den Beld and Reubsaet 2012). Thus, the use of both culture-based and molecular methods is necessary for accurate epidemiological study. Even so, because qPCR is more sensitive, it may be applied directly to the sample with an aim of immediate detection and quantification of EIEC, since the infecting dose of this pathogen is also low (Maurelli 2013).

Usually, studies about qPCR standardization to evaluate the microbiological quality of foods, concerning to *E. coli* virulence genes, applies a pre-enrichment process before the qPCR analysis. Derzelle et al. (2011) detected *stx* gene in 100% of artificial contaminated milk and ground beef, with a limit of detection of 4 cfu of STEC per 25 g. In order to investigate the prevalence of pathogenic *E. coli* in animal feces, taking into account the possible risks of food contamination, several authors standardized multiplex qPCR trials to detect *E. coli* virulence genes. Sharma and Dean-Nystrom (2003) inoculated strains of *E. coli* in cattle and pigs and then verified the presence *stx1*, *stx2* and *eae* genes in feces and intestinal tissue samples using multiplex qPCR assay, detecting up to 2 ufc and 20 ufc/g of feces, using the *stx* and *eae* probes, respectively. Luedke et al. (2014) standardized a multiplex qPCR to detect *stx*, *eae* and *ecf1* genes, detecting 1.25 × 10<sup>3</sup> ufc/g feces.

According to all these studies, multiplex qPCR to detect *E. coli* virulence genes is viable and a sensitive technique, especially if a pre-enrichment step is applied. Nevertheless, this initial bacterial growth makes it impossible to quantify pathogens in the original sample (Maciel et al. 2011) because it does not necessarily reflect the level of contamination of food (Hidaka et al. 2009). In our work, the minimal gene amount detected in naturally contaminated oysters, without the pre-enrichment step, was 45 copies of *eaeA* gene/g oyster, using as the limit of detection of 5 copies for this gene. The maximal amount was 889 copies of *stxA1* gene/g oyster. Thus, application of qPCR directly to samples of raw oysters can be used in order to quantify *E. coli* virulence genes, which reflects the microbiological quality of their habitats because they are filter feeders. In addition, it allows the early intervention of control strategies, since oysters are normally consumed raw, and the risk of FBD is high.

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