Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

A duplex qPCR for the simultaneous detection of *Escherichia coli O157:H7* and *Listeria monocytogenes* using LNA probes

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Significance and Impact of the Study: The study highlights a novel duplex qPCR for *Listeria monocytogenes* and *Escherichia coli* O157:H7 that could be used as an alternative to plate-based ISO or singleplex PCR methods while minimizing the costs. The assay uses rapid DNA extraction methods and locked nucleic acid probes. Sensitivity and specificity are 100 and 98·95% respectively. The potential for quantitative rage of the assay is 10^8–10^1 CFU ml^{-1}.

Keywords
diseases, *Escherichia coli* (all potentially pathogenic types), fish (as food), *Listeria*, PCR (polymerase chain reaction).

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2015/0329: received 17 February 2015, revised 1 April 2015 and accepted 1 April 2015

doi:10.1111/lam.12427

Abstract

In this study, a duplex qPCR assay was developed for the needs of the Irish fish industry to screen for the two major food-borne pathogens of fish, *Listeria monocytogenes* and *Escherichia coli* O157:H7. The assay can claim positive or negative results for two pathogens in one go in only 20 h including 16 h universal pre-enrichment and compared to traditional ISO approved plate culture methods the labour and the cost involved in testing of one sample is reduced to minimum. The highly specific genomic areas targeted for PCR amplification in the assay are the *hly* gene for listeriolysin O (LLO) of *L. monocytogenes* and the *stx* gene for Shiga–like toxin expressed by *E. coli* O157:H7. The detection limit of the assay is consistent with the consumer protection limits of 1 pg genomic DNA or 1 CFU 25 g^{-1} fish meat (with enrichment) allowing the test to be considered as a substitute to standard plate culture methods.

Introduction

The consumer protection authorities worldwide recommend testing each high risk foodstuff for specific food-borne pathogens [EC 882/2004 and EC 2073/2005]. Since the implementation of Hazard Analysis Critical Control Points (HACCP) system (Van Schothorst and Jongeneel 1994), the processed foods production costs have escalated disproportionally. Processed fish is recommended to be tested mainly for *Listeria monocytogenes* but many producers additionally acquire testing for enteroheamorrhagic *Escherichia coli* O157:H7 and/or *Salmonella* species.

The traditional plate culture methods, considered a 'gold standard' in the food industry, are reliable but low-throughput, time and labour consuming and expensive. An alternative to traditional cultivation is polymerase chain reaction (PCR), which has become well established for food testing (Cloke *et al.* 2014). Numerous studies have been published on PCR detection of various food-borne pathogens (Bansal *et al.* 1996; Lei *et al.* 2008). In addition, probe based qPCR assays can be used to detect multiple targets in various food matrices (Prudnikov *et al.* 2003; Russo *et al.* 2014). Modified versions of traditional TaqMan probes with locked nucleic acid (LNA) monomers were used in this study to achieve higher thermal stability and hybridization specificity of the probes (Costa *et al.* 2004).

Currently there are some commercially available singleplex qPCR tests for both *L. monocytogenes* and *E. coli* O157:H7: Biocontrol Systems Inc. Assurance GDS

kits; Pall Life Sciences/Gene Systems GeneDisc kits; DuPont Qualicon BAX System PCR Assay; BioRad iQ-Check kits; and Applied Biosystems Inc. TaqMan® Detection Kits. However, multiplex test selection is very limited. The duplex real time PCR tests available on the market include the validated Pathatrix® dual test for *E. coli* O157 and *Salmonella* spp. from Matrix Micro-Science Inc. and the GeneDisc DUO *E. coli* O157 & *Salmonella* spp. kit from Pall Life Sciences.

In this study we developed a duplex qPCR method for the quick and cheap detection of two common food-borne pathogens, *L. monocytogenes* and *E. coli* O157:H7. While there has been considerable effort into the simultaneous detection of these two organisms by qPCR, no previous literature examined their presence in fatty fish such as raw salmon (*Salmo salar*; Sánchez *et al.* 2012; Garrido *et al.* 2013; Russo *et al.* 2014). Thus, our study is optimized for raw salmon but the qPCR assay can be equally applied to other kinds of food matrices if an appropriate enrichment is used.

Results and discussion

Assay preparation

In recent years the use of culture independent, PCR-based methods have been approved for use in ISO guidelines for the detection of foodborne pathogens (Russo *et al.* 2014). In this study, a duplex qPCR was developed for the detection of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in food which is now considered a method of choice for the detection and quantification of micro-organisms utilizing a minimum enrichment time (Postollec *et al.* 2011).

The assay was developed for the need of food industry where all testing methods need to be fast, robust and reproducible. Most steps of the qPCR assay can be automatized in industrial setting making the turnover time of the samples shorter and the outcome of the test less dependent on operators. Although the sample preparation and cycling time can be reduced to 2 h in robotic set-ups and fast PCR cyclers and 4 h with manual labour and standard PCR machines, the pre-enrichment of the food samples is still the most time consuming step of the assay.

Although the described assay sensitivity is approx. 1 CFU qPCR reaction^{-1}, the bacterium needs sufficient enrichment time to achieve the required cell density to be detected by qPCR. The growth rates of bacteria were studied using both in house testing and data available from literature. According to Tsai & Hodgson (Tsai and Hodgson, 2003) the lag time for *L. monocytogenes* in BHI is <5 h and cells then require ca. 0·9 h to duplicate bringing the minimum needed incubation time to 12·2 h. The other study provided by Whiting & Golden (Whiting and Golden, 2002) with growth rates of 17 strains of *E. coli* O157:H7 described duration of lag phase between 13·7 and 55·6 h, on average 26·2 h, and the duplication time of 0·24 h during the logarithmic growth phase. Results of bacterial growth curves showed the maximum lag phase *for L. monocytogenes* to be 12 h while for *E. coli* O157:H7, it was 15 h, the maximum time of exponential growth was respectively 4·5 and 4 h. Based on these results, a single-step pre-enrichment of 19 h in BHI is enough for bacterial culture with *L. monocytogenes* or *E. coli* O157:H7 to reach the stationary stage of growth. Although the study presented by Whiting & Golden suggests that enrichment should be carried for 58 h to ensure the detection of all strains of *E. coli* O157:H7, the current AFNOR approved industry protocol involves enrichment for only 24 h prior to plating 1 ml samples into selective agar which was sufficient to detect all positive samples in this study.

DNA extraction and avoidance of PCR inhibitors

Bacteria on food matrices are exposed to various stresses and inhibitors that hinder DNA extraction and PCR reactions (Rossen *et al.* 1992; Russo *et al.* 2014). Using an enrichment broth helps recovery of damaged, life bacteria while diluting or neutralizing inhibitors prior to qPCR cycling. For fatty foods, the middle 'clean' phase under the top fat layer in incubated enrichment broths contain as many bacteria as the food residues on the bottom but the concentration of PCR inhibitors is diluted in it (Ericsson and Stalhandske 1997). It is recommended to take the 1 ml sample from the middle 'clear' phase to avoid both the PCR inhibitors and the food residues that can block the pipette. The described Chelex®100 DNA extraction method was chosen not only because of the ease and speed of use but also because Chelex, both sodium and iron forms, protects DNA by chelating the metal ions that catalyse the DNA digestion as well as inactivate the polymerase inhibitors that affect cycling performance (Walsh *et al.* 1991). The quantity and quantity of each DNA extraction was assessed using an Implem NanoPhotometer (Munich, Germany) at an absorbance of 260 nm and λA260/A280 respectively. DNA recovery ranged from 40 to 100 ng μl^{-1} with an average purity (λA260/A280) of 1·72 $\pm$ 0·06.

In silico testing

The specificity of the qPCR assay was tested by comparing the designed LNA probes and primer sequences (Table 1) to available GenBank data and with the use of BLASTN for

Table 1 Primer and probe sequences used for qPCR identification of food-borne pathogens. The position of the probe labels is given in ( ). Capital nucleotides of locked nucleic acid (LNA) probe indicates the position of locked nucleic acids

Micro-organisms	Primers and Probes	Probe label	Sequence (5′ – 3′)	Gene	Accession number	Amplicon size
Listeria monocytogenes	Lm515		GCACCTTTGTAGTATTGTAAATTC	*hly*	EU372057	110 bp
	Lm625		TAACCAATGGGATCCACAAG			
	LmLNA543	(5′)Rox - BHQ2(3′)	tggTccCgtTctCactg			
Escherichia coli O157:H7	Ec967		TAAGCATGAAGAAGATGTTTATGG	*stx*	AB048837	109 bp
	Ec1076		ATCCTCATTATACTTGGAAAACTC			
	EcLNA1042	(5′)Hex - BHQ1(3′)	ttaGcaCaaTccGccg			

matches to possible mis-match sequences. Strains were chosen from both closely related and nonrelated families as well as basing on similar toxins emitted by the bacteria. No mis-match sequences or identical sequences other than those targeted (100% of query cover and max identity) were observed. Annealing sites were free of significant secondary structures. *Shigella dysenteriae* is known for producing a Shiga toxin very similar to the verotoxin excreted by *E. coli* O157:H7 (Johnson and Lior, 1988). However, the DNA sequence similarity of the toxin encoding genes is not great enough to cause false positive results.

Assay performance

Assay specificity

The specificity of the PCR primers was tested using a SYBR-Green melting (dissociation) curve. A narrow, symmetrical target peak devoid of any other anomalies, such as shoulders, humps, or splits was observed when using the target organisms in a singleplex assay. To test the specificity of the probes and the primers together, template DNA isolated from target and non-target organisms was used (Table 2). DNA was extracted from 10^8 bacteria cells. The probes and primers gave positive signals for their target organisms (either *L. monocytogenes* or *E. coli* O157:H7) in single and duplex qPCR reactions. No signal was detected for nontarget organisms.

Assay sensitivity and spiked food matrices

Traditional plate culture from 25 g pieces of salmon using ALOA Chromogenic Agar for *L. monocytogenes* and sorbitol MacConkey agar plates for *E. coli* O157:H7 showed that samples used for the spiking assay were free of these contaminants.

Results show that the duplex qPCR assay gave 100% sensitivity down to 1 pg of genomic DNA (without

Table 2 Bacterial strains for the evaluation of specificity of the qPCR in this study

Strain		Code	Strain		Code
1	*Listeria monocytogenes* ser. 4b	NCTC 11994	22	*Escherichia coli* O157:H7	NCTC 12079
2	*L. monocytogenes* strain Petite Scott A	ATCC 49594	23	*Bacillus cereus*	DSM 31
3	*L. monocytogenes* ser. 1/2b	DSM 19094	24	*Bacillus subtilis*	DSM 10
4	*L. monocytogenes* ser. 1/2a (type strain)	DSM 20600	25	*Citrobacter freundii*	DSM 30039
5	*L. inocua*	DSM 20649	26	*Cronobacter sakazakii*	DSM 4485
6	*L. welshimeri*	DSM 20650	27	*Enterococcus faecalis*	DSM 20478
7	*L. seeligeri*	DSM 20751	28	*Escherichia coli*	DSM 30083
8	*L. ivanovii* subsp. *londoniensis*	DSM 12491	29	*Klebsielle pneumoniae*	DSM 30104
9	*L. ivanovii* subsp. *ivanovii*	DSM 20750	30	*Micrococcus luteus*	DSM 20030
10	*L. grayi*	DSM 20601	31	*Proprionibacterium acnes*	DSM 1897
11	*L. murrayi*	DSM 20596	32	*Proteus hauseri*	DSM 30118
12	*Salmonella* arizonae	DSM 9386	33	*Pseudomonas aeruginosa*	DSM 50071
13	*Salm.* bongori	DSM 13772	34	*Shigella boydii*	DSM 7532
14	*Salm.* diarizonae	DSM 14847	35	*Shigella flexneri*	DSM 4782
15	*Salm.* enterica ser. enteritidis	DSM 17420	36	*Shigella sonnei*	DSM 5570
16	*Salm.* enterica ser. gallianrum	DSM 4883	37	*Shigella dysenteriae*	NCTC 2670
17	*Salm.* enterica ser. typhi	NCTC 8393	38	*Staphylococcus aureus*	DSM 20231
18	*Salm.* enterica ser. typhimurium	ATCC 1428	39	*Staphylococcus epiderminis*	DSM 20044
19	*Salm.* houtenae	DSM 9221	40	*Streptococcus bovis*	DSM 20480
20	*Salm.* indica	DSM 14848	41	*Vibrio parahaemolyticus*	DSM 10027
21	*Salm.* salamae	DSM 9220	42	*Yersinia enterocolitica*	DSM 4780

enrichment) and 1 CFU ml^{-1} of target organisms in 25 g of spiked salmon samples using enrichment broth (Table 3). Duplex LNA-qPCR assays using 10 fold dilutions from 10 ng to 1 pg of total DNA gave mean C_t values of 17·1–36·9 (σ range: 0·07–0·71) for *L. monocytogenes* and 18·4–38·3 (σ range: 0·00–0·49) for *E. coli* O157:H7 (Table 4). While detection was possible with only 0·05 pg of total DNA, a lot of false negatives were reported at that concentration. Thus, considering the total DNA $mass^{-1}$ of a single *L. monocytogenes* genome to be $\approx 2{\cdot}94 \times 10^{-15}$ g, a total of 170 CFU are required to give a positive signal without prior enrichment (Russo *et al.* 2014). Correspondingly, with the total DNA/mass of a single *E. coli* O157:H7 genome being $\approx 6{\cdot}13 \times 10^{-15}$ g, a total of 81 CFU are required to give a positive signal without prior enrichment (Russo *et al.* 2014). The potential for quantitative use of our assays was demonstrated over a range of 10^8–10^1 CFU ml^{-1} with prior enrichment using BHI (37°C, 24 h). Standard curves of duplex LNA-qPCRs with trend line equation and the corresponding square regression coefficient (R^2) using 10-fold serially diluted genomic DNA of the target organisms are shown in Fig. 1.

Table 3 Results of a duplex qPCR using different amounts of genomic DNA and known numbers of target bacterial cells on 25 g salmon fillet (CFU 25 g^{-1}) using enrichment in BHI, 37°C for 24 h. Target organisms: *Listeria monocytogenes* (ATCC 49594; LM) and *Escherichia coli* O157:H7 (NCTC 12079; EC)

Assay sensitivity			Sensitivity in spiked salmon		
[DNA] of each target	LM	EC	CFU/ml of each target	LM	EC
50 ng	+	+	10^8	+	+
10 ng	+	+	10^7	+	+
5 ng	+	+	10^6	+	+
1 ng	+	+	10^5	+	+
0·5 ng	+	+	10^4	+	+
100 pg	+	+	10^3	+	+
10 pg	+	+	10^2	+	+
1 pg	+	+	10^1	+	+
0·05 pg	+/– (82%)	+/– (87%)	1	+	+

+ = positive, – = negative, +/– = some samples negative (% detection).

Table 4 Artificial mix prepared with different amounts of bacterial DNA to test the sensitivity of duplex locked nucleic acid qPCR. Target organisms: *Listeria monocytogenes* (ATCC 49594) and *Escherichia coli* O157:H7 (NCTC 12079)

	L. monocytogenes		*E. coli* O157:H7	
[DNA]	Average C_t values	σ	Average C_t values	σ
10 ng	17·1	0·07	18·4	0·07
1 ng	23·4	0·71	23·7	0·35
100 pg	28·1	0·07	28·9	0·00
10 pg	33·5	0·92	32·2	0·28
1 pg	36·9	0·28	38·3	0·49

Intra and inter-assay reproducibility

To obtain values for the intra- and inter-assay variation of each LNA assay, 10 ng (approx. $3{\cdot}40 \times 10^7$ CFU *L.* monocytogenes and $1{\cdot}63 \times 10^7$ CFU *E. coli* O157:H7) of purified genomic DNA was analysed in triplicate. The coefficients of variation, expressed in % as RSD ($(\sigma/\bar{x}) \times 100$) of the C_t values ranged from 0·2 to 3·03% for intra-assay experiments and from 0·4 to 4·82% for inter-assay experiments.

Detection probability

The detection probability for *L. monocytogenes* and *E. coli* O157:H7 at concentrations between 10^6 to 10^3 CFU ml^{-1} was 100%. At 10^2 CFU ml^{-1}, the detection probability for *E. coli* O157:H7 was 80% while for *L. monocytogenes*, it was 60%. Detection of 10^1 CFU ml^{-1} or less was not possible (detection probability 0%) without prior enrichment for *L. monocytogenes* while for *E. coli* O157:H7, the detection probability at 10^1 CFU ml^{-1} was 20% before dropping to 0% at 10^0 CFU ml^{-1}. This corresponded well with theoretical results, where 170 and 81 CFU (~0·5 pg) were required for a positive signal without prior enrichment for *L. monocytogenes* and *E. coli* O157:H7 respectively.

Assay performance using QC samples

After the specificity and sensitivity of the test showed to be sufficient for industry use, the duplex qPCR assay was evaluated against traditional plate culture methods used by local food microbiology laboratory (Marine Harvest Ltd, Letterkenny, Ireland). The laboratory uses the ISO 11290-2:1998 methods for testing ready fish products and production line sterility for presence of *L. monocytogenes* and sorbitol MacConkey agar for *E. coli* O157:H7.

A total of 101 samples were analysed. Results of the two methods were comparable, with the qPCR assay detecting 8 (7·9%) *L. monocytogenes* positive samples while ALOA Chromogenic Agar plates detected 7 (6·9%) positive samples. The sample corresponding to our additional positive sample tested positive for *Listeria spp*. but not for *L. monocytogenes* using ALOA plates while our qPCR assay showed presence of *L. monocytogenes*. ALOA plates had been fully grown and there could have been *L. monocytogenes* among the *Listeria spp*. as the halo around the colony was impossible to recognize due to big

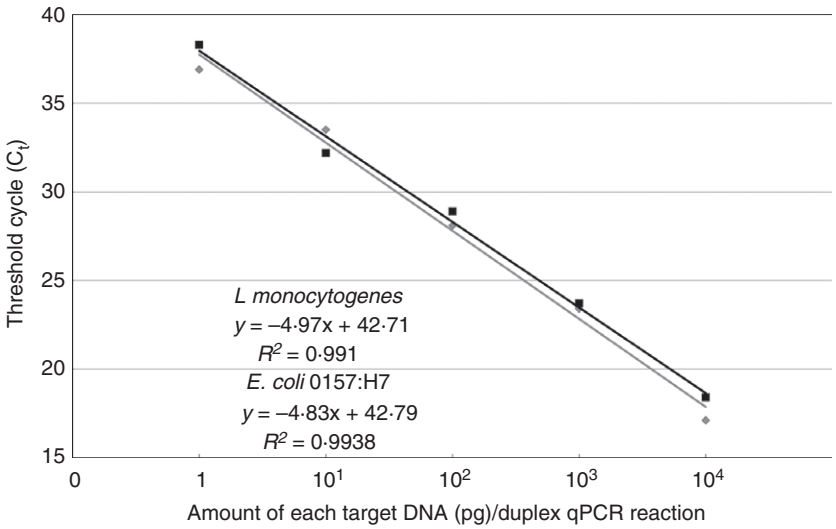

Figure 1 Standard curve and amplification plot of the 110 bp *hly* gene fragment (*Listeria monocytogenes*) and the 109 bp *stx* gene fragment (*Escherichia coli* O157:H7) of serially diluted, purified DNA. ◆ *L. monocytogenes* (ATCC 49594); ■ *E. coli* (NCTC 12079).

amount of colonies on the plate. However, this sample was still considered to be a false positive. None of the samples tested positive for *E. coli* O157:H7 with either method. Therefore, using the following equations (Kotzekidou 2013):

Accuracy (%) = 100 × (TP + TN)/(TP + TN + FP + FN),
Sensitivity (%) = 100 × TP/(TP + FN),
Specificity (%) = 100 × TN/(TN + FP),
Predicted value of a positive test (%) = 100 × TP/(TP + FP), and
Predicted value of a negative test (%) = 100 × TN/(TN + FN),

(TP: True positive (positive test with culture confirmation), FN: False negative (negative test with culture confirmation), TN: True negative (negative test with culture confirmation), FP: False positive (positive test without culture confirmation)) one can conclude the following for *L. monocytogenes*:

Assay accuracy = 99·02%
Assay sensitivity = 100%
Assay specificity = 98·95%
Predicted value of positive test = 87·5%
Predicted value of negative test = 100%.

Results showed good reproducibility when compared to standard methods and was equivalent to methods from literature using qPCR for other food matices (Sánchez *et al.* 2012; Garrido *et al.* 2013; Russo *et al.* 2014).

In conclusion, the developed duplex qPCR assay enables the rapid detection of two food-borne pathogens in one reaction. The assay consists of one universal enrichment step prior to DNA extraction, which was optimized for high-throughput use and low cost and labour input. The developed assay has proved equivalent to current ISO methods available for the detection of two significant pathogens in salmon fillets due to its high sensitivity, specificity and accuracy. Due to its suitability for high-throughput approaches, it may, with further testing, prove appropriate for accurate and rapid diagnosis of foodborne outbreaks of *L. monocytogenes* and *E. coli* O157:H7 and have the potential to be used in routine diagnostic laboratories.

Materials and methods

Bacterial strains and culture methods

The bacterial strains used in this study are listed in Table 2. All the strains were obtained from DSMZ (Braunschweig, Germany), NCTC (Collindale, UK) and ATCC (LGC Standards, Teddington, UK). To maintain the purity of the positive control bacteria, all were cultured on selective agars: *Listeria* spp. on ALOA agar (AES Chemunex, Essex, UK) according to ISO 11290-2:1998, and *Escherichia coli* O157:H7 on Sorbitol MacConkey agar (all from Oxoid Ltd, Basingstoke, UK). The negative control strains were cultured on Nutrient Agar (Oxoid Ltd).

Bacterial growth curves

In order to establish the optimal enrichment time for samples the bacterial growth curves were created using light spectroscopy analysis during 24 h cultivation. *Listeria monocytogenes* (ATCC 49594) and *E. coli* O157:H7 (NCTC 12079) stock cultures were grown in BHI broth (Oxoid Ltd) over night at 37°C. Number of cells in each culture was counted (in three repeats) using a Neubauer improved counting chamber (Sigma-Aldrich, Wicklow,

Ireland) and dilutions of stocks were made to achieve concentrations of bacteria from 1 to 10^7 CFU 100 μl. Triplicates of each dilution and strain were aliquoted to flat bottom 96-well plate together with triplicate control samples with no bacteria. The plate was incubated in a plate reader (BMG Labtech SpectroStar Omega, Ortenberg, Germany) for 24 h at 37°C with readings set for every 30 min at 580, 600, 620 and 650 nm simultaneously. The plate was shaken for 1 min at 100 rev min^{-1} before every reading. Growth curves were created using reader specific OMEGA software (BMG Labtech SpectroStar Omega, Orten-berg, Germany) and analysed manually. To confirm the initial bacteria counts, triplicates of each 10^0, 10^1 and 10^2 dilutions were grown on selective agar plates, ALOA (AES Chemunex) and Sorbitol McConkey (Oxoid Ltd) and the number of colonies was counted after overnight incubation at 37°C.

Sample preparation from pure bacterial cultures

The DNA extracted from pure bacterial cultures was used in order to evaluate the specificity of the qPCR test and to estimate the sensitivity of the qPCR method.

Bacterial colonies from agar plates were picked into 1 ml of sterile PBS solution (Sigma–Aldrich, Steinheim, Germany). One ml of diluted sample was centrifuged at 10 000 ***g*** for 10 min. The bacterial pellet was used for further DNA extractions.

Genomic DNA extraction and purification

The Chelex®100 DNA extraction method was optimized for this assay based on that presented originally by Walsh in 1991 (Walsh *et al.* 1991). The bacterial pellets were suspended into 150 μl of 5% Chelex®100 (Na^{+1}) Molecular Biology Grade Resin (Bio-Rad, Hercules, CA) solution and boiled for 10 min. Solution was centrifuged for 2 min at 6000 ***g*** and the supernatant with DNA was collected to fresh tubes. Aliquots (2·5 μl) of the extracts were used as templates for the PCR amplification. The quantity and purity of the DNA extract were determined spectrophotometrically (NanoPhotometer, Implen, Munich, Germany) at an absorbance of 260 nm and A260/A280 respectively.

qPCR primers and probe design

Oligonucleotide primers, ranging from 20– to 24–mers, and LNA probes were designed using Beacon Designer® 7 (Premier Biosoft, Palo Alto, CA). The highly specific genomic regions chosen for selective identification of bacteria included Listeriolysin O encoding *hly* gene from the *L. monocytogenes* (EU372057) genome and the Shiga toxin 2 encoding *stx* gene from *E. coli* O157:H7 (AB048837) genome. As the *hly* gene is unique to *L. monocytogenes*, it is most commonly used for detection using PCR or qPCR (Guan *et al.* 2013). Supplemental *in silico* analysis of amplicon specificity was carried out by using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/). Target secondary structures and primer/template accessibility were assessed by using the MFOLD web server (http://mfold.rna.albany.edu/?q=mfold) using correction for ionic conditions of 50 nmol l^{-1} Na^+ and 6 mmol l^{-1} Mg^{2+}. The primers and probes used in this study are listed in Table 1.

qPCR

For all qPCR assays, positive and negative controls were added using 2·5 μl of purified DNA from a 10^3 CFU ml^{-1} dilution of *L. monocytogenes* (DSM 20600) and *E. coli* O157:H7 (NCTC 12079) or nuclease free molecular grade water respectively.

LNA probe assay

Quantitative PCR amplifications were performed in a final volume of 20 μl using iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA). The final (1×) qPCR mix contained 6 mmol l^{-1} $MgCl_2$, 0·7 μmol l^{-1} *E. coli* O157:H7 and *L. monocytogenes* specific primers and 0·3 μmol l^{-1} of each LNA probe. 2·5 μl of template DNA from 1 ml of homogenized sample was added to each reaction, except for the negative PCR control, in which the DNA was replaced by nuclease free molecular grade water. DNA from target and nontarget reference strains correlating to the duplex assay was used. A series of dilutions of the pure culture genomic DNA (from 50 ng down to 0·05 pg) were tested determine the minimum amount of template DNA detected by the assay (qPCR sensitivity). The reactions were carried out in 96-well plates sealed with heat bonding film. PCR amplification of duplicate samples was achieved on a Bio-Rad iCycler iQ (Bio–Rad Laboratories). A thermal profile of 95°C for 10 min (to activate the Taq polymerase), 55 cycles at 95°C for 15 s and 56°C for 60 s followed by 72°C for 5 min was the same for each reaction. A well picture was taken at the end of every cycle (95%). The per–well baseline cycles were determined automatically while the threshold was set manually at 100.

Detection probability

To estimate the probability of detecting *L. monocytogenes* and *E. coli* O157:H7 in a suspension of known concentration, *L. monocytogenes* (DSM 20600) and *E. coli* O157:H7 (NCTC 12079) were grown for 16 h at 37°C

(~10^8 CFU ml^{-1}). Cultures were serially diluted 10-fold in 0·9% (w/v) NaCl to 10^6 to 1 CFU ml^{-1}. Number of cells in each dilution was counted (in three repeats) using a Neubauer improved counting chamber (Sigma-Aldrich) and adjusted as necessary. Counts for dilutions $<10^3$ CFU ml^{-1} were further confirmed using ALOA Chromogenic Agar (AES Chemunex) for *L. monocytogenes* and sorbitol MacConkey agar plates (Oxoid Ltd) for *E. coli* O157:H7. Aliquots (1 ml) of each homogenized dilution were used for DNA extraction as described above. Two and a half microliters of each DNA (of each organism) was added to five separate PCR tubes using 10 replicates. Each qPCR gave a positive or negative result at the concentration tested. The detection probability of the qPCR assay was obtained by plotting the relative number of positive qPCRs observed against the cell dilution used.

Spiked food matrices

Salmon fillets were obtained from local food microbiology laboratories (Marine Harvest Ltd, Fanad and United Fish Industries, Killybegs, Ireland). To check for contamination of *E. coli* O157:H7 and/or *L. monocytogenes*, 25 g samples were analysed using ALOA Chromogenic Agar (AES Chemunex) for *L. monocytogenes* and sorbitol MacConkey agar plates (Oxoid Ltd) for *E. coli* O157:H7.

Twenty-five g of skinned fish from salmon fillets were artificially contaminated on the surface with 1 ml BHI containing 10^8 CFU ml^{-1} to approx. 1 CFU ml^{-1} of available pure cultures as determined by Neubauer improved counting chambers (Sigma, Wexford, Ireland). Un-inoculated salmon samples (25 g) and sterilized BHI (1 ml) were included as negative assay controls. All samples were run in triplicates. After 20 min of drying, the fish was homogenized in a double stomacher bag with 100 ml of brain heart infusion broth (BHI; Oxoid Ltd). The final volume of BHI was adjusted to 250 ml and incubated at 37°C for 24 h. Aliquots (1 ml) of each fish enrichment were centrifuged at 14 000 ***g*** for 10 min. The pellet was used for further DNA extractions as described above.

Assay performance using QC samples

In order to evaluate the qPCR assay, 101 samples were taken from local fish processing plants quality control laboratory (Marine Harvest, Letterkenny, Ireland) and tested with the studied qPCR assay. Samples included both swabs of the production line surfaces and ready to eat meat samples. The enrichment was performed as for plate cultures in Half Fraser Broth (Oxoid Ltd) over night at 35°C and parallel to plating the samples, duplicates of 1 ml enrichment, were taken in microcentrifuge tubes into the study laboratory for sample processing. DNA extraction and qPCR assay were performed as described earlier. Results of the qPCR test were compared with the results of AFNOR validated ALOA Chromogenic Agar (AES Chemunex) for *L. monocytogenes* and sorbitol MacConkey agar plates (Oxoid Ltd) for *E. coli* O157:H7.

Acknowledgements

CAMBio – Applied Research Centre is funded by Enterprise Ireland's Applied Research Enhancement Programme under the National Development Plan 2007 – 2013. The authors thank Marine Harvest Ireland (Letterkenny, Ireland) for providing the test samples.

Conflicts of Interest

No conflict of interest declared.

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