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Detection of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* using duplex real-time PCR assay with melting curve analysis on fresh lettuce

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ABSTRACT - In this study, two duplex real-time PCR approach with melting curve analysis is presented for the detection of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus,* which are important food-borne bacterial pathogens usually present in fresh and/or minimally processed vegetables. Reaction conditions were adjusted for the simultaneous amplification and detection of specific fragments in the β-glucuronidase (*uidA, E. coli*), thermonuclease (*nuc, S. aureus*), hemolycin (*hly, L. monocytogenes*) and tetrathionate reductase (*ttr*, *Salmonella* spp.) genes. Melting curve analysis using a SYBR Green I real-time PCR approach showed characteristic T_m values demonstrating the specific and efficient amplification of the four pathogens; 80.6 ± 0.9°C, 86.9 ± 0.5°C, 80.4 ± 0.6°C and 88.1 ± 0.11°C for *S. aureus, E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp., respectively. For all the pathogens, the two duplex, real-time PCR was equally sensitive to uniplex real-time PCR, using same amounts of purified DNA, and allowed detection of 10 genome equivalents. When our established duplex real-time PCR assay was applied to artificially inoculated fresh lettuce, the detection limit was 10^3 CFU/g for each of these pathogens without enrichment. The results from this study showed that the developed duplex real-time PCR with melting curve analysis is promising as a rapid and cost-effective test method for improving food safety.

Key words: Real-time PCR, Multiplex, Melting curve, Fresh, minimally produced vegetable, Food borne pathogens

Introduction

Consumption of fresh produce has increased in recent years and a large number of minimally processed and fresh-cut produce products are available in supermarkets. Unfortunately, the increase in the consumption has come with increased frequency of outbreaks of illness associated with fresh produce[1,2]. Agricultural irrigation with wastewater that can be raw, treated and/or partially diluted, is a common practice worldwide and constitutes the main source of pathogen contamination[3]. Therefore, fresh or minimally processed vegetables which are often eaten raw or minimally processed, can compromise consumer's health safety. The most common bacterial enteropathogens associated with fruits and vegetables are *Escherichia coli* O157:H7, *L. monocytogenes, Salmonella* spp. and *S. aureus*[4-6]. All four pathogens are among the food-borne bacteria currently observed in a wide range of food products since they are frequently reported as the causative agents in food poisoning. Furthermore, they are the human pathogens that cause the most economically important food-borne diseases throughout the world.

The culture based approaches for detection of pathogenic bacteria, are quite laborious and needed many times. To develop more advanced, sensitive and rapid microbial detection methods for detection of pathogenic bacteria in fresh vegetables is need to complement or replace the conventional culture based procedures for the prompt detection of these pathogens, since they are highly perishable products. Real-time PCR is a technology that may allow the rapid detection of pathogens with high specificity and sensitivity. Amongst the available chemistries for real-time PCR, intercalating dyes, such as SYBR Green I, are most commonly used due to universal applicability and associated low cost. After the amplification, the specificity of the product is established by post PCR melting curve analysis that involves heating of the amplified product in a closed system, and determining the T_m[7,8].

In food science, there are numerous reports on development of SYBR Green I based assay for detection of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* in a wide range of products[9-13]. However, most of these assays except a few involved usage of either uniplex or expensive probe based approaches. Multiplex

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real-time PCR assays by targeting more than one pathogen would not only increase rapidity but also reduce the overall reaction cost of the detection tests[14-19].

The aim of this study was to develop a multiplex real-time PCR assay with melting curve analysis for the simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* in fresh vegetables.

Materials and Methods

Bacterial strains, culture media and growth conditions

The bacterial strains used in this study are *S. aureus* ATCC 6538, *E. coli* O157:H7 NCCP 11091, *L. monocytogenes* ATCC 19111, and *Salmonella* spp. ATCC 14028. All these strains were kept as frozen stock cultures at −80°C and were grown on tryptic soy broth (TSB; Difco, MI, USA), or brain-heart-infusion (BHI) at 37°C for 24 h.

DNA isolation

The genomic DNA used in the real-time PCR assays was extracted using a DNeasy® Blood & Tissue kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. In brief, DNA was isolated from cultured bacteria as follows: The cells were pelleted at 5,000 × g for 10 min, resuspended in 180 μl lysis buffer, and incubated at 37°C for 10 minutes. The lysates were then exposed to protease digestion in the presence of Buffer AL at 56°C for 10 minutes and applied to the column (Qiagen). Each column was sequentially washed with Buffer AW1, Buffer AW2 and finally eluted with 100-200 μl of Buffer AE (Qiagen). Purified genomic DNA was used as a template in real-time PCR assays.

Oligonucleotides

Primers used in this study are listed in Table 1. They are targeted to genes encoding for the thermonuclease (*nuc*) in *S. aureus*, the β-glucuronidase (*uid*A) in *E. coli* O157:H7, the hemolycin (*hly*) in *L. monocytogenes,* and the tetrathionate reductase in *Salmonella* spp (*ttr*). Oligonucleotides were synthesized by Bioneer (Daejeon, Korea).

Duplex Real-Time PCR assay and melting-curve analysis

A real-time PCR assay was performed using the SYBR® Green Realtime PCR Master Mix (Toyobo, Tokyo, Japan). Each reaction contained 10 μl of SYBR® Green Realtime PCR Master Mix, 0.4 μM of each primer, and 1 μl (50 ng) of template made up to 20 μl with filter sterilized water. Real-time PCR amplification was performed in a ABI 7500 (Applied Biosystems, Foster City, CA, USA) using the following conditions: 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 62°C and 30 s at 72°C. The reaction carried out without DNA sample was used as negative control. Reactions were done in triplicate. PCR results were given as the increase in the fluorescence signal of the reporter dye-detected and visualized by the GeneAmp® 5700 SDS software (Applied Biosystems). C_T values (threshold cycle) represent the PCR cycle in which fluorescence first increased, over a defined threshold, for each amplification plot. For melting curve analysis after PCR amplification, the thermal protocol for dissociation is defined as 10 sec at 95°C, 30 sec at 60°C and 20 min slow ramp between 60 and 95°C. The data for the dissociation curve is captured during this slow ramp.

Sensitivity of the duplex real-time PCR reaction

In order to establish detection limits of the duplex Real-Time PCR, amplifications were carried out for each pathogen using both uniplex and the duplex reaction. To this end, 10-fold serial dilutions of the reference type strains *S. aureus* ATCC 6538, *E. coli* O157:H7 NCCP 11091, *L. monocytogenes* ATCC 19111, and *Salmonella* spp. ATCC 14028 were done covering a range from 1 to 10^4 genome equivalents, which were calculated assuming that 1 ng DNA of *S. aureus*, *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. equals 6×10^5, 1.7×10^5, 3×10^5, and 2×10^5 times more than that of the entire genome of each strain, respectively.

Artificially inoculated food assays

Sensitivity of the duplex real-time PCR reaction was analysed on cleaned and packed lettuce collected in a local supermarket. The samples were artificially inoculated with

Table 1. Primers used in this study

Organism	Targeted gene	Sequence (5'-3')	Tm (°C)	Reference
E. coli O157:H7	β-glucuronidase, *uidA*	CAGTCTGGATCGCGAAAACTG ACCAGACGTTGCCCACATAATT	64	20
S. aureus	thermonuclease, *nuc*	CGCTACTAGTTGCTTAGTGTTAACTTTAGTTG TGCACTATATACTGTTGGATCTTCAGAA	80	21
L. monocytogenes	hemolycin, *hly*	ACTTCGGCGCAATCAGTGA TTGCAACTGCTCTTTAGTAACAGCTT	60	5
Salmonella spp.	tetrathionate reductase, *ttr*	TTTATACCGGCCGCGAAGT CCTGTAAGGCGCTAAGAAACATC	87	30

different cell concentrations of the four pathogens as an approach to simulate their probable occurrence in fresh vegetables. Inoculation assays were prepared as follows: 25 g of each sample were cut aseptically into small pieces and were added to 225 ml of Buffered Peptone Water (Difco), in a sterile plastic bag with lateral filter (BagPage S 400, BagSystem, Interscience, Saint-Nom la Bretèche Arpents, France) and homogenised in a stomacher (Pulsifier, Microgen Bioproducts Ltd, UK) for 1 min. The resulting mixture was taken from the filter side and distributed in aliquots of 10 ml. They were inoculated with 100 μl of 10-fold serial dilutions of 18 h culture of each pathogen (at the same time) in sterile saline solution (0.85% NaCl), covering a range from 10^2 to 10^7 CFU/g (determined by plate count) for each pathogen. A non-inoculated negative control was included in each experiment.

One millilitre aliquot of each inoculated sample was centrifuged at 16,000 × g for 5 min. Pellets were washed in 0.5 ml of TE (10 μM Tris-HCl; 1 uM EDTA, pH 8.0) and DNeasy® Tissue kit (Quiagen), and DNA was eluted in 100 μl bi-distilled water and 5 μl were used as template for amplification.

Results and Discussion

Set up of the duplex real-time PCR assay

Optimized reaction conditions for real-time PCR were obtained by using 100 nM of *E. coli* O157:H7 and *Salmonella* spp., 150 nM of *L. monocytogenes*, and 300 nM of *S. aureus* specific primers. Specificity of primers used for four pathogens had previously been tested[20-23]. Melting-curve analysis of amplicons corresponding to *S. aureus* ATCC 6538, *E. coli* O157:H7 NCCP 11091, *L. monocytogenes* ATCC 19111, and *Salmonella* spp. ATCC 14028 showed T_m values of 81.1 ± 0.6, 87.2 ± 0.4, 81.2 ± 0.8 and 88.2 ± 0.13°C, respectively. In this study, to develop efficient multiplex PCR, reaction conditions were optimized using the SYBR GreenI. The real-time PCR methods are the simplest and least expensive method and enables products to be identified by their different T_m, by analysing the melting curve of the amplicon post-PCR besides detection of target DNA during amplification[24]. The presence of a single peak indicates the specificity of the reaction and when more than one amplicon is obtained, they can be distinguished by differences of at least 1°C in T_m[25,26]. Both of duplex real-time PCR developed in this study yielded only two peaks showing clearly different Tm values for each pathogen without nonspecific amplification products and primer dimers: 80.6 ± 0.9 and 86.9 ± 0.5°C for *S. aureus* and *E. coli* O157:H7, and 80.4 ± 0.6 and 88.1 ± 0.11°C for *L. monocytogenes*, and *Salmonella* spp. (Fig. 1).

Sensitivity of the duplex real-time PCR compared to uniplex real-time PCR assay

Tenfold dilutions of DNA from the four pathogens, covering five log orders, were used for duplex and uniplex real-time PCR assays in order to evaluate the efficiency of the duplex real-time PCR compared to the uniplex real-time PCR. The C_T values are shown in Table 2. Compared to uniplex real-time PCR, the C_T values obtained by duplex real-time PCR for *S. aureus* and *Salmonella* spp. were similar when using high DNA concentrations. For all pathogens, C_T values from the duplex real-time PCR were slightly higher than those obtained with the uniplex. In the duplex reaction, PCR has a limited quantity of enzyme and nucleotides, and

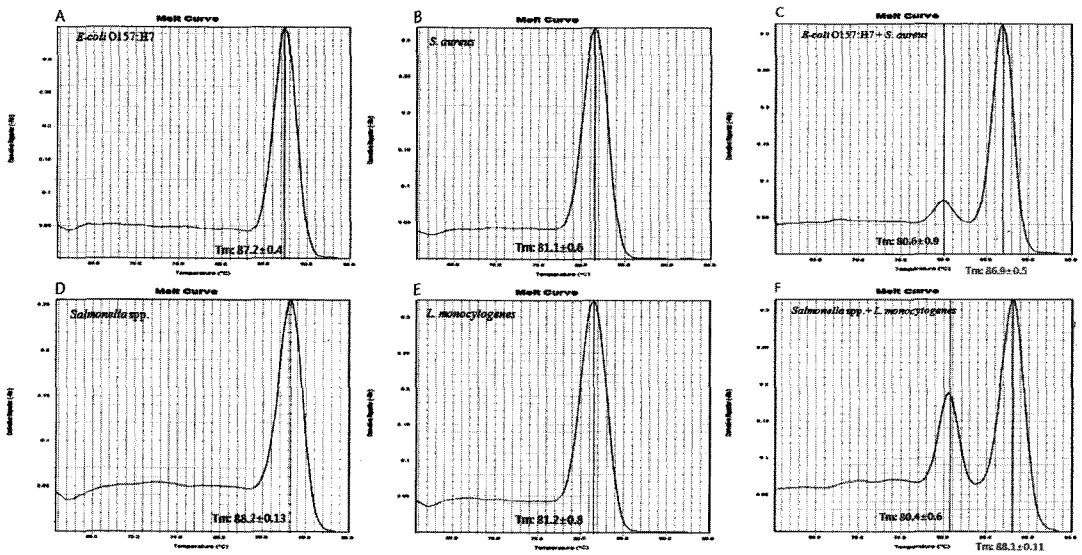

Fig. 1. Melting-curve analysis corresponding to the amplicons generated by SYBR Green uniplex (A, B, D and E) and duplex(C and F) real-time PCR showing the peaks and T_m values of *E. coli* O157:H7, *Staphylococcus aureus, Salmonella* spp and *Listeria monocytogenes.*

Table 2. Comparison of C_T values obtained by real-time PCR uniplex (U) and duplex (D) assays using as template purified DNA of *E. coli* O157:H7, *S. aureus*, *Salmonella* spp. and *L. monocytogenes* from 10^4 to 1 genome equivalents/reaction

		Genome equivalents/reacion[a]				
		10^4	10^3	10^2	10	1
E-coli O157:H7	U	21.89 ± 0.87[b]	25.02 ± 0.65	30.82 ± 0.78	34.23 ± 0.61	37.9 ± 0.7
	D	22.11 ± 0.21	25.89 ± 0.47	31.23 ± 0.4	35.84 ± 0.32	38.3 ± 0.5
S. aureus	U	25.32 ± 0.78	28.43 ± 0.64	34.02 ± 0.47	36.81 ± 0.63	39.6 ± 0.2
	D	25.43 ± 0.56	28.66 ± 0.46	35.14 ± 0.34	38.02 ± 0.2	40 ± 0
Salmonella spp.	U	22.41 ± 0.7	27.32 ± 0.61	31.36 ± 0.82	35.11 ± 0.72	38.1 ± 0.7
	D	22.68 ± 0.41	27.55 ± 0.34	32.41 ± 0.48	36.78 ± 0.55	39.4 ± 0.3
L. monocytogenes	U	23.57 ± 0.81	26.52 ± 0.71	32.96 ± 2.1	35.98 ± 0.84	39.2 ± 0.4
	D	24.08 ± 0.13	27.77 ± 0.33	33.71 ± 1.1	37.22 ± 0.23	40 ± 0

[a]Genome equivalents calculated assuming that 1 ng of DNA of *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. equals 1.7×10^5, 6×10^5, 3×10^5, and 2×10^5 times the entire genome of each strain, respectively.
[b]C_T values (mean ± SD) corresponding to three replicates.
[c]C_T values ≥ 40, no amplification

Table 3. Results obtained from real-time PCR uniplex (U) and duplex (D) assays for detection of *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. from artificially inoculated vegetables

Inoculation level (CFU g^{-1})[a]	*E-coli O157:H7*		*S. aureus*		*Salmonella* spp.		*L. monocytogenes*	
	U	D	U	D	U	D	U	D
10^6	21.67 ± 1.22[b]	22.46 ± 0.34	27.73 ± 1.93	29.06 ± 0.74	22.13 ± 1.84	23.25 ± 0.52	24.72 ± 1.69	25.55 ± 1.34
10^5	24.11 ± 2.11	25.23 ± 0.77	32.45 ± 1.27	34.39 ± 0.63	26.26 ± 1.45	27.55 ± 0.62	28.29 ± 1.21	29.14 ± 0.7
10^4	28.43 ± 1.32	30.25 ± 0.89	34.57 ± 1.66	37.24 ± 1.21	29.77 ± 2.06	31.17 ± 1.1	30.73 ± 1.49	33.05 ± 0.85
10^3	32.66 ± 0.98	35.13 ± 0.61	37.22 ± 1.57	39.45 ± 0.35	34.42 ± 1.67	36.83 ± 0.74	35.68 ± 1.24	38.69 ± 0.63
10^2	36.39 ± 1.05	38.75 ± 0.49	39.23 ± 1.12	40 ± 0	37.43 ± 1.23	39.32 ± 0.34	38.76 ± 1.03	40 ± 0

[a]It corresponds to 8.6×10^6, 7.8×10^5, 8.2×10^4, 7.6×10^3 and 8.4×10^2 in *E. coli* O157:H7; 2.6×10^6, 2.9×10^5, 3.1×10^4, 2.6×10^3 and 3.2×10^2 in *S. aureus*; 4.8×10^6, 4.1×10^5, 4.3×10^4, 4.7×10^3 and 4.2×10^2 in *L. monocytogenes*; 6.8×10^6, 6.3×10^5, 5.9×10^4, 6.6×10^3 and 6.4×10^2 in *Salmonella* spp.
[b]C_T values (mean ± SD) corresponding to three replicates.
[c]C_T values ≥ 40, no amplification

all products compete for the same pool supplies[27]. In *S. aureus* and *L. monocytogenes*, the lowest amount of DNA tested, corresponding to 1 genome equivalent/reaction, rendered a positive result when analyzed in a uniplex but not when used for duplex real-time PCR. These results indicate a small decrease in sensitivity when using the two sets of primers, at the lowest concentration tested. Nevertheless, our results demonstrate that amplification of the two loci is efficient in the presence of the other competing primers and the detection limit was kept 10 genome equivalents per reaction for all of them.

Duplex real-time PCR assays in artificially inoculated food

In order to assess detection sensitivity of the duplex real-time PCR for the application to vegetable samples, cells of all four pathogens were inoculated in fresh lettuces and a non-inoculated sample of lettuce was included as negative control. After DNA extraction by the DNeasy Tissue kit (Quiagen), they were used for real-time PCR amplification, in triplicate. Amplification products corresponding to the four pathogens were obtained in the reactions used as external amplification control thus confirming the absence of PCR inhibitors. Detection limit, estimated as the highest dilution from which amplification is obtained and derived from inoculation experiments, was established at 10^2 CFU/g for all four pathogens by uniplex real-time PCR, although C_T values over 39 were obtained in *S. aureus* (Table 3). By duplex real-time PCR, the limit of detection in *S. aureus* and *L. monocytogenes* deteriorated one log order, being 10^3 CFU/g. For all pathogens, C_T values from the uniplex real-time PCR were lower than those obtained with the duplex. Results obtained from artificially inoculated lettuce with equal amount of the four pathogens, using duplex real-time PCR, proved that it was

able to detect 1.1, 5.0, 3.8 and 2.1 CFU per reaction of *E. coli* O157:H7, *S. aureus*, *L. monocytogenes* and *Salmonella* spp., respectively, corresponding to 10^3 CFU/g each, which is agree with the detection level recognised for PCR in food[22,28]. It also improved the detection limits obtained by conventional multiplex PCR, without enrichment, for *Salmonella* spp. and *S. aureus* in one and two log orders, respectively[17].

The 10^3 CFU/g detection scored by the duplex SYBR Green real-time PCR used in this study, is at the same level that other uniplex TaqMan real-time PCR assays applied to food, without prior enrichment. Fu et al.[29] obtained a sensitivity of 1.3×10^4 cells/g for *E. coli* O157:H7 in ground beef, combining immunomagnetic separation and real-time PCR. Alarcón et al.[21] reported a sensitivity of 4.9×10^3 CFU/g for *S. aureus* from artificially inoculated beef samples after DNA extraction. Lower detection levels such as 10 CFU/g have been only achieved after 18 hours of enrichment against *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. in milk[30], and *Salmonella* spp. in meat[31,32].

However, the current criteria for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. except for *S. aureus* require absence in 25 g of food sample which cannot be guaranteed by direct detection as described in this study. These drawbacks of the real-time PCR can be prevented by the application of an enrichment step previous to the real-time PCR assay that would improve the detection level allowing to fulfill the criteria of absence required for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp.. In addition, the enrichment step would also allow the living cells to grow and increase its relative population with respect to dead cells, improving the accuracy of the assay. Thus, the developed duplex real-time PCR with melting curve analysis developed is a promising technique for improving food safety since it is a rapid and cost-effective procedure that allows a high-throughput of samples. Combined with an enrichment step it would become a very valuable tool for the food industry.

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요 약

본 연구에서는 신선편의 채소와 과일에서 주로 검출되는 대장균(*E. coli* O157:H7), 리스테리아(*L. monocytogenes*), 살모넬라(*Salmonella* spp.), 황색포도상구균(*S. aureus*)을 검출하고 동정할 수 있는 real time PCR 방법을 melting curve 분석을 활용하여 single tube 반응으로 두 종의 식중독균을 동시 검출하는 방법을 개발하고자 하였다. 대장균의 β-glucuronidase (*uidA*), 황색포도상구균의 thermonuclease (*nuc*), 리스테리아의 hemolycin (*hly*), 살모넬라의 tetrathionate reductase (*ttr*) 를 특이적으로 검출할 수 있는 4종의 프라이머 세트에 대한 real-time PCR의 melting curve 분석을 통하여 황색포도상구균과 대장균 동시분석 시 T_m 값이 80.6±0.9°C와 86.9±0.5°C, 리스테리아와 살모넬라 동시분석 시 Tm 값이 80.4±0.6°C와 88.1±0.11°C 로 확인하였고, 그 결과 정제되어진 각 식중독균의 genomic DNA를 주형으로 한 duplex real-time PCR 방법이 uniplex real-time PCR 방법과 마찬가지로 10 genome equivalents 까지 검출할 수 있는 민감도를 나타내었다. 또한, 양배추에 네 종의 식중독균을 접종하고, 증균배양 없이 DNA를 추출하여 duplex real-time PCR 을 수행한 결과 모든 식중독균에서 10^3 CFU/g의 검출한계를 나타내었다. 결과적으로 개발된 melting curve 분석을 이용한 duplex real-time PCR 방법은 식품안전 증진을 위한 시간, 노동력, 비용 절감에 있어서 유효한 방법이 될 것으로 판단된다.

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