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A new single-tube platform of melting temperature curve analysis based on multiplex real-time PCR using EvaGreen for simultaneous screening detection of Shiga toxin-producing *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* in food

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* spp. and *Listeria monocytogenes* are continuously reported as causative agents of great concern regarding food safety and widespread contamination in many food varieties. Therefore, their simultaneous detection may be advantageous in terms of cost, time and labor savings and simplicity. This study developed a new, simple platform of multiplex real-time polymerase chain reaction (mRT-PCR) for specific, sensitive and rapid detection of STEC, *Salmonella* spp. and *L. monocytogenes* in food. The single-tube mRT-PCR format was developed by combining an 18 h enrichment step in simultaneous enrichment broth, boiling based on DNA extraction assay and a mRT-PCR detection system based on melting curve analysis using a fluorescent dye (EvaGreen) for detection of the presence or absence of the three target bacterial pathogens in food samples. Three specific peaks were clearly detected with average melting temperatures of 84.52 ± 0.90 °C, 87.51 ± 0.54 °C and 79.32 °C ± 0.48 °C for STEC, *Salmonella* spp. and *L. monocytogenes*, respectively. The sensitivity and specificity of these newly developed mRT-PCR platforms were further investigated using artificially and naturally contaminated food samples. The relative sensitivity, relative specificity and relative accuracy were all 100%, with a detection limit of 1 cfu for each target pathogen in 25 g of food sample. The developed platform of EvaGreen-based single-tube mRT-PCR for detection of the three target pathogenic bacteria in food samples provided results of absence or presence within 20 h. The newly developed mRT-PCR platform in this study offers a promising approach for simple, rapid, sensitive, specific and accurate detection of the three target bacterial pathogens in food.

1. Introduction

Foodborne pathogens pose a significant threat to human public health, leading to a substantial economic burden both in developed and less developed countries. Among the foodborne pathogenic bacteria currently observed in a wide range of foods and food products, Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* spp. and *Listeria monocytogenes* are frequently reported as the main three causative agents in food poisoning. STEC is responsible for haemorrhagic colitis and haemolytic uremic syndrome in humans (Valderrama, Dudley, Doores, & Cutter, 2016). Among members of the STEC group, *E. coli* O157 is primarily the major agent for the most severe cases of these diseases (Kaper, Nataro, & Mobley, 2004). However, the prevalence of the big six non-O157-STEC-O26, O45, O103, O111, O121, and O145 infection has been reported increasingly worldwide (Wickham et al., 2006). *Salmonella* spp. are the leading bacterial cause of salmonellosis in humans. *L. monocytogenes* can be widely found in different foodstuffs and the natural environment. *L. monocytogenes* is associated with severe listeriosis in a high-risk group of consumers (Mook, Patel, & Gillespie, 2011). Therefore, the rapid and specific detection of these bacterial pathogens is of great importance to prevent or reduce food safety problems.

The current routine detection of STEC, *Salmonella* spp. and *L. monocytogenes* in foods is still based on standard reference methods. However, these methods are laborious, time-consuming and costly. Additionally, the low-throughput of these conventional methods does

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not allow rapid screening of large numbers of food samples for the presence of one or more pathogens (Abubakar et al., 2007). Therefore, to overcome such problems, sensitive, specific and rapid screening methods for primary detection of the pathogens specifically present in foods are essential. Molecular-based methods with different platforms, especially polymerase chain reaction (PCR), have increasingly gained great attention for the detection of pathogens in food products due to their simplicity, rapidity, accuracy and lower cost compared to the standard reference methods.

Standard and multiplex PCR approaches have some significant drawbacks such as being time-consuming for the analysis of post-PCR results and the risk of sample contamination. On the other hand, real-time PCR approaches provide similar or more sensitive results than standard PCR while the methods also do not require post-PCR analysis (Forghani, Wei, & Oh, 2016). The detection of pathogens using mRT-PCR, commonly utilizes a fluorogenic dye-base with melting temperature (T_m) curve analysis and multicolor-fluorophore-labeled probes. Fluorogenic dyes, such as SYBR Green, SYTO and EvaGreen, are relatively more cost beneficial and easier to use than probes. However, mRT-PCR still has a limited detection of sensitivity regarding the presence of the fewest viable target microbial cells in food samples. Furthermore, one significant disadvantage of mRT-PCR is that it is not able to distinguish a false positive result produced by DNA from dead or viable target bacterial cells. An enrichment step in an appropriate medium is still needed to overcome the problems of low target microbial cell numbers and to limit the risk of detecting dead cells as well as to resuscitate physiologically stressed or injured cells (Badosa, Chico, Pla, Pares, & Montesinos, 2009).

Several studies have previously reported the application of multiplex PCR and mRT-PCR systems to specifically detect two or more target bacterial pathogens in a single-tube reaction. Son et al. (2014), Paddock, Shi, Bai, and Nagaraja (2011) and Fratamico and DebRoy (2010) reported the development of a multiplex PCR assay for detection of *Escherichia coli* O157:H7 and STEC in different foods and cattle feces. A multiplex PCR detection method for milk based on novel primers specific for *L. monocytogenes* 1/2a serotype was reported (Sheng et al., 2018). Kim, Park, Lee, and Ricke (2017) developed rapid and simple method by combining FTA™ card DNA extraction with two set multiplex PCR for simultaneous detection of non-O157 STEC strains in ground beef. Hasap, Thanakiatkrai, Linacre, and Kitpipit (2017) developed Heptaplex-direct PCR assay for simultaneous detection of *Shigella* spp., STEC, *Streptococcus pyogenes*, *Campylobacter jejuni*, *S.* Typhi, *L. monocytogenes*, and *Staphylococcus aureus* in food samples randomly chosen from local market and supermarket. mRT-PCR methods have been developed for the simultaneous detection of *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* in various types of foods (Carloni, Rotundo, Brandi, & Amagliani, 2018; Suo, He, Tu, & Shi, 2010; Omiccioli, Amagliani, Brandi, & Magnani, 2009). Garrido-Maestu, Azinheiro, Carvalho, and Prado (2018) reported a development of rapid and sensitive detection of viable *L. monocytogenes* in various food products by a filtration-based protocol and mRT-PCR. Maciel, de Mello, Lopes, Boehs, and Albuquerque (2018) applied of mRT-PCR assay for simultaneous quantification of *E. coli* virulence genes in oysters. In addition, mRT-PCR for simultaneous identification of *Brucella melitensis*, *Cronobacter sakazakii* and *L. monocytogenes* in raw milk and cheese was developed (Tutar, Akinci, & Akyol, 2018). Agrimonti, Sanangelantoni, and Marmiroli (2018) developed the melting curve analysis following duplex real time PCR in the presence of SYBR Green for simultaneous detection of *Salmonella enteritica* and *Campylobacter jejuni* in chicken meat and eggs. Denis, Bielińska, Wieczorek, and Osek (2016) recently reported the development of two-tube mRT-PCR consisting of one tube for STEC detection and another tube for *Salmonella* spp. and *L. monocytogenes* detection in milk, under the same thermal cycling conditions. He et al. (2016) developed a one-tube mRT-PCR process for the simultaneous detection of five foodborne pathogenic bacteria (*S. aureus*, *L. monocytogenes*, *Salmonella enterica*, *Vibrio parahaemolyticus* and *Shigella* spp.) in foods. However, based on our knowledge, there has been no research on the development of EvaGreen-based single-tube mRT-PCR for the specifically simultaneous detection of the three target bacterial pathogens (STEC, *L. monocytogenes* and *Salmonella* spp.) in foods. Therefore, in the present study, a friendly new platform was developed using EvaGreen-based single-tube mRT-PCR assay combined with culturing in simultaneous enrichment broth for the simultaneous detection of the three target bacterial pathogens in foods. The application and efficiency of the developed method for the detection of the three main target pathogens were evaluated using artificially and naturally contaminated foods.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In total, 57 bacterial strains used in the present study consisting of 31 target and 26 non-target bacteria (Table 1). All the bacterial strains were stored in cryo-beads (Cryoinstant®, Scharlau, Barcelona, Spain) as frozen stock culture at −20 °C until use. Reactivation of the bacterial culture involved one cryo-bead from the frozen stock culture being aseptically transferred into tryptic soy broth (TSB) (Merck, Darmstadt, Germany) and incubated without shaking at 37 °C for 18 h. However, *V. parahaemolyticus* was cultured in TSB supplemented with 3% NaCl (Merck, Darmstadt, Germany) and *L. monocytogenes* was grown in TSB supplement with 0.6% yeast extract (Merck, Darmstadt, Germany).

2.2. DNA extraction methods

2.2.1. DNeasy Blood and Tissue kit

A sample of 1 mL of freshly grown bacterial culture was centrifuged at 8000 × *g* for 10 min. The cell pellets obtained were subjected to DNA extraction using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. After DNA extraction, the DNA quantity (A260) and quality (ratio of A260/A280) were determined using a Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The pure genomic DNA obtained from the kit was used as a template for the evaluation of primer specificity and sensitivity for each target pathogenic bacterium under singleplex or mRT-PCR conditions. In addition, such genomic DNA was used as a positive control when the developed mRT-PCR was investigated with artificially or naturally contaminated food samples. The pure DNA was maintained in frozen storage at −20 °C prior to use.

2.2.2. Boiling method

DNA was extracted using simple boiling to Trevanich, Tiyapongpattana, and Miyamoto (2010) with a few modifications. The boiling method was used for DNA extraction from the pure cultures of bacteria or food samples after enrichment in simultaneous enrichment broth (SEB). Briefly, 1 mL of sample was centrifuged at 12,000 × *g* for 3 min. The resulting pellets were washed twice and resuspended in 100 µL distilled water. The suspensions were boiled in a water bath for 10 min and then centrifuged at 12,000 × *g* for 3 min. The obtained supernatant was used as a DNA template for further evaluation of singleplex or mRT-PCR efficiency. The DNA quantity and quality were determined as described above.

2.3. Target genes and primers

All target genes and primers used in this study are presented in Table 2. The newly designed primers specified to *stx*1 and *stx*2 genes of STEC were developed in our laboratory. Only Stx1-F and Stx2-F named Stx1,2-F were designed as a degenerate primer. The DNA sequences of the *stx*1 and *stx*2 genes from the STEC were retrieved from Gen-Bank (www.ncbi.nlm.nih.gov/Genbank/) and aligned using the CLUSTAL W