 Detection and Identification of Six Foodborne Bacteria by Two-tube Multiplex Real Time PCR and Melting Curve Analysis

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Abstract

Objective This study is aimed to develop a two-tube melting curve-based multiplex real time PCR assay (MCMRT-PCR) for the simultaneous detection of six common foodborne pathogenic bacteria (diarrhoeagenic Escherichia coli, Salmonella, and Shigella in tube 1, Staphylococcus aureus, Vibrio parahaemolyticus, and Listeria monocytogenes in tube 2).

Methods A two-tube MCMRT-PCR assay was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Amplification by PCR was optimized to obtain high efficiency. The sensitivity and specificity of assays were investigated.

Results The detection limit of optimized MCMRT-PCR assay was 3.9×10^2 CFU/mL for S. aureus, 4.4×10^2 CFU/mL for L. monocytogenes, 3.0×10^2 CFU/mL for Salmonella, 2.5×10^2 CFU/mL for Shigella, 2.1×10^2 CFU/mL for V. parahaemolyticus, and 1.2×10^2 CFU/mL for E. coli. The feasibility of MCMRT-PCR was further evaluated using artificially contaminated milk, the sensitivity was at the level of 10^5 CFU/mL.

Conclusion A two-tube MCMRT-PCR assay using six primer sets was developed for detection of multiple pathogens. Our findings demonstrates that the proposed two-tube assay is reliable, useful and rapid for simultaneous detection of six foodborne pathogenic bacteria with an intended application in provincial Centers for Diseases Control and Prevention (CDC).

Key words: Detection; Real-time PCR; Melting curve; Bacteria

INTRODUCTION

Foodborne disease caused by pathogenic bacteria presents a serious threaten to public health in both developing countries and industrialized nations.[1-2] Staphylococcus aureus (S. aureus), Vibrio parahaemolyticus (V. parahaemolyticus), Listeria monocytogenes (L. monocytogenes), diarrhoeagenic Escherichia coli (E. coli), Salmonella, and Shigella are six of the common foodborne pathogenic bacteria.[1,3-4]. E. coli has emerged as a serious gastrointestinal pathogen which can cause diarrhoeal diseases in many countries.[5-6]. It has been proved that the eaeA gene encodes an outer membrane adhesin (intimin), which mediates intimate attachment to host cell and is essential for establishment of attaching and effacing lesion. It is also involved in pathogenicity. In

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this study, eaeA is used as target gene in identification of *E. coli. Salmonella*, one of the most serious pathogens, also can cause serious salmonellosis, both in animals and humans[7]. It is reported that nearly 2000 *Salmonella* serovars contain the invA gene, which is a virulence gene encoding an invasion protein. Generally, the invA gene has been proven to be Salmonella-specific, which is proved to exist only in *Salmonella*[8-9]. *Shigella* is an important source of bacterial diarrhoea[10-12]. The invasion plasmid antigen H (ipaH) gene is carried by four *Shigella* species. It has been considered that the ipaH gene is the main target for detection of *Shigella* in many researches[13-14].

*L. monocytogenes*, a gram-positive rod, could cause severe infections including encephalitis, meningitis, and septicemia[15-16]. The hlyA encoding a 58-kDa listerio lysin O, which has been classically regarded as one of the three significant virulence markers, is unique to the species *L. monocytogenes*[17-19].

*V. Parahaemolyticus* is a major pathogen of food-borne gastroenteritis in many Asian countries, including Taiwan, China, and Japan[20-22]. The tlh gene (thermolabile hemolysin gene) is a species-specific marker of *V. parahaemolyticus*[23]. *S. aureus* is one of the most prevalent causes of gastroenteritis worldwide. Human is common carrier of *S. aureus* in the nose, throat, and skin infections[3]. *S. aureus* produces an extracellular thermostable nuclease [thermonuclease (TNase)]. Enzymatic test for TNase production and its encoded gene (nuc gene) are used in many laboratories in identification of *S. aureus* isolate[24-25].

Molecular detection methods based on DNA analysis, such as PCR[26-28], DNA hybridization[29-31], and biochip[32] have been applied in detection of food pathogens. Probe-based real time PCR is a method that can simultaneously identify more than one pathogen. The high specificity and accuracy makes it a commonly adopted resolution in food testing[33-34]. However, these methods are either time consuming or expensive, which makes them not suitable for less equipped laboratories in developing countries. There is therefore a growing demand for simple and cost-effective molecular tests.

Multiplex real time PCR method coupled with melting curve analysis and SYBR green I, the most commonly adopted dye, was used in the detection of multiple genes[35], however, few study of simultaneous detection of foodborne pathogens using this method was reported. Additionally, SYBR green I was reported to inhibit PCR in a concentration-dependent manner[36-37], affect melting temperature (Tm) of PCR products[38], and prefers certain amplicons to others[39], while DNA-intercalating dye SYTO 9 does not inhibit PCR in a broader range of concentration, and is unbiased among different amplicons[40-41].

This study is aimed to develop a two-tube melting curve-based multiplex real time PCR assay (MCMRT-PCR) method with SYTO to simultaneously detect *S. aureus*, *V. parahaemolyticus*, *L. monocytogenes*, *E. coli*, *Salmonella*, and *Shigella*. The potential application of this method for pathogen detection is also discussed.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Media**

Cultured *E. coli, V. parahaemolyticus, S. aureus*, *L. monocytogenes*, *Salmonella*, and *Shigella* were used as target bacteria in this study. These bacterial strains were obtained from American Type Culture Collection (ATCC), Chinese Medical Current Content (CMCC), and China Center of Industrial Culture Collection (CICC). *E. coli* was collected and preserved by Chinese Center for Disease Control and Prevention (Table 1).

**Enrichment Procedure**

All bacterial strains were grown for 12 h in LB broth at 37 °C in a Shaking Water Bath (approximately 10⁸ CFU/mL), collected into 2 mL microcentrifuge tubes, and resuspended in 1.5 mL PBS, followed by 10-fold serial dilutions using PBS. These dilutions were used in further sensitivity and quantification tests. For each dilution, 100 μL suspension was plated and incubated at 37 °C for 16 h, followed by counting of colonies.

**Table 1. Reference Bacteria Strains Used in This Study**

<table>
<thead>
<tr>
<th>Taget Bacteria</th>
<th>Strains/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>diarrhoeagenic</td>
<td><em>Escherichia coli</em> O157:H7/</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Xuizhou21/EDL933</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td><em>Salmonella</em> Enteritidis/CMMC50041</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>*Shigella flexneri/CMMC51537</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>CMCC54004</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>CICC21617</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC29213</td>
</tr>
</tbody>
</table>
**Genomic DNA Extraction**

For each dilution mentioned above, 1 mL cell suspension was processed as follows to extract genomic DNA. All bacterial samples were centrifuged at 13,000×g for 15 min. The cell pellets were resuspended in 50 µL of 50 mmol/L NaOH and placed in a 95 °C heating block for 10 min. After the mixtures were cooled at room temperature, 10 µL of 1 mol/L Tris (pH 8.0) was added, followed by centrifuge at 13,000×g for 5 min. The supernatants were used as PCR templates and stored at -20 °C.

**Primers**

Specific primers for *E. coli*, *Salmonella*, *L. monocytogenes* and *V. parahaemolyticus* were previously reported.[19,23,42-43] Reference sequences of *nuc* (region: 894264-894950, numbering based on NC_002758) and *ipaH* (region: 74821-75075, numbering based on NC_017319) were used to modify and design specific primers for *S. aureus* and *Shigella* using Primer Premier 5.0, respectively. The specificities of all the primers were verified by comparison with the GenBank database through BLASTn search. All of the primers were synthesized and HPLC-purified by Sangon Biotech (Shanghai, China) Co., Ltd.. The primer information is listed in Table 2.

**Multiplex PCR and Melting Curve Analysis**

A Two-tube MCMRT-PCR assay was developed for the detection of six foodborne pathogens. In each tube, 10 µL reaction system was constructed with 5 µL of 2×Multiplex PCR Master Mix (Qiagen), 1.6 µmol/L SYTO9 green fluorescence dye (Invitrogen, USA), 0.5 µL each of DNA template, and the following amount of primer pair, 150 nmol/L *invA*, 250 nmol/L *ipaH*, and 250 nmol/L *eaeA* in tube 1, 300 nmol/L each of *nuc* and *hlyA* and 100 nmol/L *tlh* in tube 2. All the reactions were performed on 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The cycling conditions were one cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 s, 57 °C for 30 s, 72 °C for 30 s. Dissociation analysis was performed by incubating the reaction at 95 °C for 15 s, annealing at 60 °C for 15 s, followed by increasing the temperature to 95 °C over 20 min. Four replicates were performed for each real-time PCR run and each run was repeated at least twice.

**Specificity of the Multiplex PCR Assays**

To evaluate the specificity of the primer pairs for their respective target genes, PCR assays were carried out to test all the strains reported in Table 1 by using the primers in Table 2. Deionized water was included in each PCR assay as a negative control.

**Determination of Detection Sensitivity and Linear Range of Multiplex Real-time PCR**

To determine the sensitivity and quantification capability of this test, DNA was extracted from cell pellets quantified by colony counting followed by 10-fold dilution from 10^8 to 10^2 CFU/mL and used as templates. The detection sensitivity was determined for each template individually and equally premixed templates in both of the two tubes in a multiple format. For each template, a standard curve was plotted and $R^2$ was calculated to describe the linear range and quantification capability of the test.

<table>
<thead>
<tr>
<th>Taget Bacteria</th>
<th>Target Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Tm of PCR Product (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>diarrhoeagenic Escherichia coli</td>
<td>eaeA</td>
<td>AGGTCGCTGTCGTCTCCTGTA CCCTGCTGCTGCTTGGTTG</td>
<td>80.5</td>
<td>[42]</td>
</tr>
<tr>
<td>Salmonella</td>
<td>invA</td>
<td>GTGAAATTATCCGCACACCTCGGACCACCA TCATGCACCGTGCAAAGGACC</td>
<td>86.2</td>
<td>[43]</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>hlyA</td>
<td>ATGGCCGAATTTGGTAC CGCCACACCTGGAGATAT</td>
<td>81.0</td>
<td>[19]</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>tlh</td>
<td>ACTCAACACAAAGAGAGATCGACCAA GATGAGGCGTTGATGTCCAA</td>
<td>84.8</td>
<td>[23]</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>nuc</td>
<td>GCCATTGGATGGTATGATACGGTG ACTTGCTTCAGGACCAT</td>
<td>77.5</td>
<td>Modified</td>
</tr>
<tr>
<td>Shigella</td>
<td>ipaH</td>
<td>TGACAAACATTACCCGGACTT CGACAATGGATTCCCTGA</td>
<td>78.0</td>
<td>Modified</td>
</tr>
</tbody>
</table>
**Determination of Detection Sensitivity of Multiplex Real-time PCR in Artificially Contaminated Food Sample**

Cell pellets quantified by colony counting were 10-fold diluted from $10^8$ to $10^2$ CFU/mL and spiked into milk. DNA extraction from spiked milk followed the protocol of genomic DNA extraction of the pure culture. Sensitivity and linear correlation tests were also performed with spiked milk.

**RESULTS**

**Specificity of the Primer Sets and Tm of PCR Products**

Six pairs of real time PCR primers (Table 2) were used to detect the following bacteria respectively: *E. coli*, *Salmonella*, *L. monocytogenes*, *V. parahaemolyticus*, *S. aureus* and *Shigella* targeting genes *eaeA*, *invA*, *hlyA*, *th*, *nuc*, and *ipaH*, respectively.

Genomic DNA of reference strains listed in Table 1 was used to test the specificity of primers and to determine Tm value of each amplicon. Tm values of PCR products were listed in Table 2. All six pairs of primers successfully amplified the corresponding genes as expected, with dissociation plot of single peak.

According to Tm values determined above, the six pairs of primers were divided into two groups to construct a two-tube triplex real time PCR assay, with primers of *E. coli*, *Salmonella*, and *Shigella* in tube 1 and primers for *S. aureus*, *L. monocytogenes* and *V. parahaemolyticus* in tube 2, which can be used to detect and differentiate all six bacteria simultaneously. The negative controls produced no dissociation profile.

**Sensitivity for Selected Primer Sets**

Sensitivity of multiplex real time PCR was evaluated with reference strains (as listed in Table 1), which had been quantified using colony counting. Both single pathogen and equally combined mixture of the six quantified pathogens were used in detection limit test, and standard curves were plotted for each single pathogen test. Standard curves generated by plotting the Ct values against log of the CFU/mL were shown in Figure 1. Detection limits of single and multiplex pathogens and correlation coefficient ($R^2$ values) are listed in Table 3, indicating the detection limits of multiple template tests were of no difference with those of single template tests. Figure 2 demonstrated the amplification plots and melting curves of multiple pathogen assays.

**Detection of Artificially Contaminated Sample**

To evaluate the validity of the multiplex real time PCR method in detection of pathogens in food products, milk samples artificially contaminated with six food pathogens were tested. As expected, Tm value and dissociation curve detected from spiked food sample were of the same from pure culture (data not shown).

Of all the examined target genes in individual real time PCR, the detection limits of artificially contaminated samples were of no difference from those of the pure cultures, the correlation coefficients of spiked samples for each pathogen are slightly lower than those with pure culture (Table 4). However, sensitivity decreased in the testing of mixed pathogens, suggesting that the two-tube MCMRT-PCR assay was able to simultaneously detect six food pathogens in the artificially contaminated milk when six pathogens are present at the level of $10^5$ CFU/mL (Table 4).

**DISCUSSION**

Rapid and accurate methods for simultaneous identification of foodborne pathogens are becoming increasingly important. In previous study, real time PCR methods have been used for the detection and quantification of target organisms with TaqMan probes or molecular beacons to generate a fluorescent signal. With this method, *E. coli* [47], *Salmonella*, *Shigella* [46], *V. parahaemolyticus* [23], *S. aureus* [47], and *L. monocytogenes* [29,48] were successfully detected from food products. To detect more than one target gene at the same time, the method would be at a high cost. High-resolution melting (HRM) analysis is another very effective method for genotyping and mutation scanning [49-50], however the high cost of HRM equipment limits its wide use. A potentially cheaper alternative to HRM is to follow real-time PCR by melting curve analysis (MCA) which can be performed on the conventional real-time PCR equipment already available in most laboratories in local CDCs of China. MCA provides a much faster and cheaper alternative to gel electrophoresis and sequencing, and greatly decreases chances of contamination [42]. Generally, MCA only adds up to 30 min compared to Taqman probe-based assays,
**Figure 1.** Standard curves generated by plotting the Ct values against log of the CFU/mL of six single pure cultures (A-F) and spiked milk (a-f) by real time PCR and formulas for linear regression of the data. 

A/(a), diarrhoeagenic *Escherichia coli* $Y=2.37x+34.01$; B/(b), *Salmonella* $Y=-3.82x+37.78$; C/(c), *Shigella* $Y=-3.66x+40.40$; D/(d), *Listeria monocytogenes* $Y=-3.23x+37.40$; E/(e), *Vibrio parahaemolyticus* $Y=-2.89x+33.09$; F/(f), *Staphylococcus aureus* $Y=-3.03x+38.83$.

**Table 3.** Sensitivity and Linear Correlation of Two-tube Multiplex Real-time PCR Test with Pure Culture

<table>
<thead>
<tr>
<th>Target Pathogen</th>
<th>Detection Limit (CFU/mL)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>diarrhoeagenic Escherichia coli</em></td>
<td>$1.2 \times 10^2$</td>
<td>0.997</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>$3.0 \times 10^2$</td>
<td>0.998</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>$2.5 \times 10^2$</td>
<td>0.984</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>$4.4 \times 10^2$</td>
<td>0.990</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>$2.1 \times 10^2$</td>
<td>0.995</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>$3.9 \times 10^2$</td>
<td></td>
</tr>
</tbody>
</table>
Detection and identification of six foodborne bacteria by MCMRT-PCR assay

but greatly improves adaptability and reduces cost, since it omits the need of probe designing and requires only the conventional fluorescence channel, which is equipped in all of the real-time PCR machines.

In the present study, a MCMRT-PCR assay was developed to simultaneously detect *E. coli*, *Salmonella*, *Shigella*, *L. monocytogenes*, *V. parahaemolyticus*, and *S. aureus*. The *eaeA* (intimin-encoding gene), *invA* (invasion protein A gene) and *ipaH* (invasion plasmid antigen H gene) were targeted to allow simultaneous detection of *E. coli*, *Salmonella* and *Shigella* in tube 1. *hlyA* (listeriolysin O gene), *tlh* (thermolabile hemolysin gene) and *nuc* (heat stable nuclease gene) genes were targeted for simultaneous detection of *L. monocytogenes*, *V. Parahaemolyticus*, and *S. aureus* in tube 2.

**Figure 2.** Multiplex real-time PCR amplification for simultaneous detection of six foodborne pathogens in quadruplicate. (A) amplification plots in tube 1, of 10-fold serial mixed dilution of diarrhoeagenic *Escherichia coli*, *Salmonella*, and *Shigella*. a-f, $10^2$ to $10^5$ CFU/mL; g, negative control. (B) dissociation plots obtained from (A). a, *ipaH* of *Shigella*; b, *eaeA* of *Escherichia coli*; c, *invA* of *Salmonella*; d, negative control. (C) amplification plots in tube 2, of 10-fold serial mixed dilution of *Staphylococcus aureus*, *Listeria monocytogenes* and *Vibrio parahaemolyticus*. a-f, $10^2$ to $10^5$ CFU/mL; g, negative control. (D) dissociation plots obtained from (C). a, *nuc* of *Staphylococcus aureus*; b, *hlyA* of *Listeria monocytogenes*; c, *tlh* of *Vibrio parahaemolyticus*; d, negative control.

**Table 4.** Sensitivity and Correlation of Two-tube Multiplex Real-time PCR Test with Artificially Spiked Milk

<table>
<thead>
<tr>
<th>Target Pathogen</th>
<th>Detection Limit (CFU/mL)</th>
<th>Single pathogen</th>
<th>Mixed pathogen</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>diarrhoeagenic <em>Escherichia coli</em></td>
<td>1.2x10^2</td>
<td>1.2x10^2</td>
<td>0.987</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>3.0x10^2</td>
<td>3.0x10^2</td>
<td>0.995</td>
<td></td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>2.5x10^2</td>
<td>2.5x10^2</td>
<td>0.981</td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>4.4x10^2</td>
<td>4.4x10^2</td>
<td>0.973</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>2.1x10^2</td>
<td>2.1x10^2</td>
<td>0.979</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>3.9x10^2</td>
<td>3.9x10^5</td>
<td>0.981</td>
<td></td>
</tr>
</tbody>
</table>
Specificity of the primers is a key part of the process, eaeA, invA, ipaH, hlyA, tli, and nuc genes were adopted for detection of E. coli, Salmonella, Shigella, L. monocytogenes, V. Parahaemolyticus, and S. aureus, respectively. To achieve the highest amplification efficiency, concentrations of all primer sets were adjusted. Amplicon Tm value is another factor that has to be taken into account. In this system, the Tm value of all amplicons ranged from 77.5-86.2 °C, making it possible to separate the six target genes in two tubes. In addition, a simple and rapid alkaline lysis method was used to extract bacterial DNA from the food samples. When MCMRT-PCR was applied to the pure cultures of the six pathogens, the detection limit was 3.9×10^2 CFU/mL for S. aureus, 4.4×10^2 CFU/mL for L. monocytogenes, 3.0×10^2 CFU/mL for Salmonella, 2.5×10^2 CFU/mL for Shigella, 2.1×10^2 CFU/mL for V. parahaemolyticus, 1.2×10^2 CFU/mL for E. coli, with correlation coefficient (R^2) all above 0.99 with a dynamic range from 10^2 to 10^8. The application of this method in artificially contaminated samples was also tested. Although the real-time method performed well with mixtures of pure cultures, as well as in single pathogen challenged milk, both sensitivity and efficiency decreased when applied to multiple pathogens spiked milk. The presence of various food constituents, such as fats, glycosgen, organic and phenolic compounds, may have influenced the amplification of DNA in the PCR.

A major limitation of this study for the simultaneous detection of six important foodborne pathogenic bacteria is that the specificities of the targeted gene and the primer for each pathogen was not thoroughly investigated using species closely related to target organisms due to limited availability of bacterial strains in our laboratory. However, all of the six targeted genes in this study were well-documented previously[8-9,23,51], and accordingly four pairs of specific primers for V. parahaemolyticus, L. monocytogenes, E. coli, Salmonella were cited in this study, the published primers for S. aureus and Shigella[13,24] were slightly modified in this study to meet the requirement of MCA. Nevertheless, given the main purpose of this study is to develop an improved multiple real time PCR method (probe-free), the grouping of six reported primer pairs into two tubes under the proposed optimized working condition does work functionally as evidenced by the sensitivity and quantification results obtained from this study using pure cultures.

Another limitation of this study is that the DNA extraction method based on alkaline lysis and isopropanol precipitation remains a hindrance in high throughput application, particularly due to its poor performance in the DNA enrichment of Gram-positive strains (S. aureus, L. monocytogenes). As our current preliminary study has showed the MasterPure Complete DNA purification kit (Epicenter Technologies, Madison, WI) worked well for the DNA extraction form both Gram-positive and negative strains (data not shown), this kit will be tested to refine the DNA extraction method.

The MCMRT-PCR assay is a simple, rapid and efficient technique. Further evaluation of this proposed assay with a large number of representative strains and contaminated milk is needed before this method can potentially be optimized for use with other food products. The optimized MCMRT-PCR assay will improve the accessibility to track food-contaminated outbreaks for minimally equipped laboratories in local CDCs, reducing the risks of infection from the food and helping researchers formulate effective surveillance systems that meet public health goals and safety objectives implemented by risk-informed policy.

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