Korean J. Food Sci. An. Vol. 34, No. 5, pp. 717~723 (2014)
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DOI http://dx.doi.org/10.5851/kosfa.2014.34.5.717
ISSN 1225-8563 eISSN 2234-246X

ARTICLE

# Simultaneous Detection of *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Bacillus cereus*, *Salmonella* spp., and *Staphylococcus aureus* in Low-fatted Milk by Multiplex PCR

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**Abstract**

A rapid and specific PCR assay for the simultaneous detection of *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Bacillus cereus*, *Salmonella* spp., and *Staphylococcus aureus* in foods was developed to reduce the detection time and to increase sensitivity. Multiplex PCR developed in this study produced only *actA*, *fliC*, *hbl*, *invA, ileS* amplicons, but did not produce any non-specific amplicon. The primer sets successfully amplified the target genes in the multiplex PCR without any non-specific or additional bands on the other strains. The multiplex PCR assays also amplified some target genes from five pathogens, and multiplex amplification was obtained from as little as 1 pg of DNA. According to the results from the sensitivity evaluation, the multiplex PCR developed in this study detected 10 cells/mL of the pathogens inoculated in milk samples, respectively. The results suggested that multiplex PCR was an effective assay demonstrating high specificity for the simultaneous detection of five target pathogens in food system.

**Keywords:** simultaneous detection, multiplex PCR, pathogens, low-fatted milk

## Introduction

In these days, the microbial safety of food is getting significant concern in consumers and food industry. Food microbial contamination is not often discovered by individual hygiene and hygiene reinforcement in food production steps. However, there is still a risk of microbial contamination of food throughout the processing before consumption. The rapid and accurate identification of bacterial pathogens in food is important and have been studied by many researchers, both for quality assurance and trace of bacterial pathogens within the food supply (Bhagwat, 2003; Settanni and Corsetti, 2007; Yang *et al*., 2013a). Current methods for the detection of food-borne pathogens generally involve the following: (a) colony isolation on selective media, (b) the use of biochemical tests, and (c) serotyping using antibodies against specific bacterial antigens. However, these traditional culture-based methods are both cumbersome and time consuming (Kim *et al*., 2010). Recently, a polymerase chain reaction (PCR) method has been developed and applied frequently for detection of pathogenic microorganisms due to its rapidity and more feasibility for industrial application. There has been a lot of discussion about using multiplex PCR assay for the detection of pathogenic microorganisms in previous studies (Abd-Elmagid *et al*., 2013; Mukhopadhyay and Mukhopadhyay, 2007; Omiccioli *et al*., 2009).

*Bacillus cereus* and *Staphylococcus aureus* are gram-positive, spore-forming bacteria. Due to their spore-forming ability, they can pose problems in the food industry. In addition to this, *Staphylococcus aureus* food poisoning resembles *Bacillus cereus* food-borne intoxication in its symptoms and incubation period (Abd-Elmagid *et al*., 2013; Cremonesi *et al*., 2005; Kumar *et al*., 2009; Stenfors Amesen *et al*., 2007). *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. are dangerous food-borne pathogens and important pathogens spreading through various foods, afflicting human health worldwide (Amagliani *et al*., 2004; Yuan *et al*., 2009).

Milk has been known to have high nutritional value and be contaminated easily with microorganisms. In particu-

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lar, dairy products contaminated with pathogens can be fatal to babies, immunocompromised patients, and pregnant women. Therefore, new approaches in safety of dairy products are needed to find fast and efficient detection technique with high detection sensitivity in the area of milk and milk products.

The direct detection of pathogenic bacteria in food samples is hampered by the presence of PCR-inhibitory substances frequently associated with enrichment media, DNA isolation reagents, and the food matrix itself (Oikarinen *et al*., 2009). In the present study, a species-specific primer set that can selectively amplify regions of geno-

**Table 1. List of pathogen and non-pathogen bacterial strains used for specificity tests**

| Type | No. | Strain | Source |
|---|---|---|---|
| Pathogen | 1 | *Bacillus cereus* | Lab collection |
| | 2 | *Bacillus cereus* | KCCM 11341 |
| | 3 | *Bacillus cereus* | KCCM 40935 |
| | 4 | *Escherichia coli* O157:H7 | Lab collection |
| | 5 | *Escherichia coli* O157:H7 | ATCC 43894 |
| | 6 | *Escherichia coli* O157:H7 | ATCC 43895 |
| | 7 | *Escherichia coli* O157:H7 FRIK125 | From ISU[a] |
| | 8 | *Escherichia coli* O157:H7 93-062 | From ISU[a] |
| | 9 | *Staphylococcus aureus* | KCCM 32395 |
| | 10 | *Staphylococcus aureus* | ATCC 25923 |
| | 11 | *Staphylococcus aureus* | KCCM 40510 |
| | 12 | *Salmonella* Enteritidis | KCCM 12021 |
| | 13 | *Salmonella* Typhimurium | ATCC 14802 |
| | 14 | *Salmonella* Gallinarum | ATCC 9184 |
| | 15 | *Salmonella* Enteritidis | ATCC 13076 |
| | 16 | *Salmonella* Typhimurium | Lab collection |
| | 17 | *Salmonella* st. paul | From ISU[a] |
| | 18 | *Salmonella* Gaminara 8324 | From ISU[a] |
| | 19 | *Salmonella* Oranienbury 9329 | From ISU[a] |
| | 20 | *Listeria monocytogenes* | ATCC 15313 |
| | 21 | *Listeria monocytogenes* ScottA | From ISU[a] |
| | 22 | *Listeria monocytogenes* H7969 | From ISU[a] |
| | 23 | *Listeria monocytogenes* H7962 | From ISU[a] |
| | 24 | *Listeria monocytogenes* H7596 | From ISU[a] |
| | 25 | *Listeria monocytogenes* H7762 | From ISU[a] |
| Non-pathogen | 26 | *Bacillus subtilis* | IFO 12113 |
| | 27 | *Bacillus subtilis* BR40 | Lab collection |
| | 28 | *Bacillus thuringiensis* | Lab collection |
| | 29 | *Bacillus* spp. MY2 | Lab collection |
| | 30 | *Bacillus amyloliquefaciens* KU801 | Lab collection |
| | 31 | *Escherichia coli* | KCCM 32396 |
| | 32 | *Escherichia coli* | ATCC 25922 |
| | 33 | *Escherichia coli* (-) control | Lab collection |
| | 34 | *Escherichia coli* | ATCC 10536 |
| | 35 | *Escherichia coli* DH5a | Lab collection |
| | 36 | *Staphylococcus epidermidis* | ATCC 12228 |
| | 37 | *Staphylococcus chromogenes* 19 | Lab collection |
| | 38 | *Staphylococcus xylosus* 29 | Lab collection |
| | 39 | *Staphylococcus hylococcus* 54 | Lab collection |
| | 40 | *Staphylococcus simulans* 78 | Lab collection |
| | 41 | *Listeria grayi* | KCTC 3443 |
| | 42 | *Listeria ivanovii* subsp. *ivanovii* | KCTC 3444 |
| | 43 | *Listeria grayi* | KCTC 3581 |
| | 44 | *Listeria welshimeri* | KCTC 3587 |
| | 45 | *Listeria seeligeri* | KCTC 3591 |

[a]From Aubrey F. Mendonca, Department of Food Science and Human Nutrition, Iowa State University, Ames, IA

mic DNA in simplex and multiplex PCR reactions was designed. The PCR assay was validated by species specific amplification of known amount of DNA with same set of primers.

The major focus of this study was to investigate the specificity of a multiplex PCR designed for the simultaneous detection of five major foodborne pathogens to be found in milk (*Listeria monocytogenes*, *Escherichia coli* O157:H7, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella* spp.) and its sensitivity by measuring the detection limit of bacterial cell cultures as a single pathogen in milk.

## Materials and Methods

### Bacterial strains and culture conditions

A total of 45 pathogenic and non-pathogenic bacterial strains were used in this study as listed in Table 1. The cultures were maintained on Tryptic Soy Agar (TSA; Difco Laboratories, USA). The plates were incubated at 37°C for 18-24 h in order to allow the microbial growth. A single colony was selected and grown overnight at 37°C in Tryptic Soy Broth (TSB; Difco Laboratories, USA).

### Genomic DNA extraction

Three different methods for genomic DNA extraction from pure culture were used. Bacteria grown overnight in TSB were centrifuged at 8,900 *g* for 15 min. The pelleted cells were then used for DNA extraction. The cells were re-suspended in 100 μL of Tris-EDTA (TE) buffer and boiled for 10 min. After cooling on ice for 15 min, the collected cells were centrifuged at 16,600 *g* for 1 min. Finally, the supernatant was used for the PCR (Germini *et al*., 2009).

Pure genomic DNA was extracted with an *AccuPrep*® genomic DNA extraction kit (Bioneer Co., Korea) in accordance with the manufacturer's direction, along with lysozyme (Sigma Chemical Co., USA).

The isolation of bacterial genomic DNA was performed with some modifications of the method described (Neumann *et al*., 1992; Pospiech and Neumann, 1995). The pellet was re-suspended in 5 mL of SET buffer (50 mM NaCl, 25 mM EDTA, 20 mM Tris-Cl, pH 7.5) containing 10 mg/mL of lysozyme. After an incubation for 1 h at 37°C, 2.5 mL of 0.5 M EDTA was added, and incubated for 5 min at room temperature. Then, 4 mL of 10% (m/v) SDS and proteinase K was added to a final concentration of 1 mg/mL, and incubated at 65°C for 30 min. Equal volume of phenol:chloroform:isoamylalcohole (25:24:1, PCI) were added, and gently mixed. The mixture was centrifuged at 8,900 *g* for 15 min, the upper aqueous phase was transferred to a new tube, and RNase A was added to a final concentration of 20 μg/mL, and incubated at 37°C for 1 h. The solution was extracted with an equal volume of PCI. Then, 5 M NaCl added to final concentration of 0.1 M and two volumes of absolute ethanol were added and gently mixed. The DNA was spooled out, washed with 70% ethanol, dried, and dissolved in a suitable volume of TE buffer (pH 8.0).

The concentration of DNA was estimated by $A_{260}$, and the quality and purity of DNA was evaluated by $A_{260}/A_{280}$ and electrophoretic analysis.

### Primer design

All the primers used for this study were chosen for biomarker and were designed indigenously by using the Gen Bank database (Table 2). The design and theoretical analysis of primers with respect to self-complementarity, inter-primer annealing, and optimum annealing temperatures were accomplished by means of the FastPCR software program (http://primerdigital.com/fastpcr.html). Primers were tested for PCR amplification at five different annealing temperatures.

**Table 2. Primers designed for the multiplex PCR**

| Microorganism | Acc. number | Primer sequence (5'-3') | Target gene | Amplicons (bp) |
|---|---|---|---|---|
| *L. monocytogenes* | AE017262 | TAGTTCGCTGAATAGTGGCGA<br>*T*TGCTTTTTCGTCTTCTGCAC | *actA* | 611 |
| *E. coli* O157:H7 | AE005174 | ACATCTTTACTCACTGTAGCCTG<br>*A*ACTACCGATGCTGCATTCGA | *fliC* | 519 |
| *B. cereus* | AE016877 | TCATTGATTTGCCGTTGCGTAT<br>*G*TCACATCCATTGTAACTGGAGGA | *hbl* | 437 |
| *Salmonella* spp. | AM933172 | TTCTCTTGGCGCCCACAATGCGAG<br>*T*CCATCAGCAAGGTAGCAGTC | *invA* | 338 |
| *S. aureus* | NC_003923.1 | CATACAGCACCAGGTCACGGGGAA<br>*G*TTCTCCAGTCGTGTGGATAGC | *ileS* | 227 |

**Specificity and detection limit assays**

The specificity of the PCR assay was tested by using, as a template, the extracted DNA by a boiling method of bacteria listed in Table 1. Non-pathogen bacteria made a group (*Bacillus* spp. group, *Escherichia* spp. group, *Staphylococcus* spp. group, and *Listeria* spp. group) and carried out multiplex PCR in multiple primers. Pathogen bacteria were assayed using multiplex PCR in single and multiple primers.

To determine the detection limit of the PCR, using extracted DNA and cultured cell. Serial dilutions (1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL, and 1 fg/μL) of each extracted DNA tested using the multiplex PCR. The DNA extraction used a phenol/chloroform method in five pathogen bacteria (*Bacillus cereus* KCCM 11341, *Escherichia coli* O157:H7 Lab collection, *Staphylococcus aureus* ATCC 25923, *Salmonella* Enteritidis KCCM 12021, and *Listeria monocytogenes* ATCC 15313). Cultured cell was used to colony PCR with single and multiple primers. The concentration of cultured cells was estimated by $A_{625}$, reached between 0.080.1, it made the density $10^8$ cells/mL. Serial dilutions ($10^7$-$10^1$ cells/mL) of each cultured cell tested using the colony PCR.

**PCR conditions**

Reactions with DNA as template were carried out in 50 μL volumes containing 10 pmol of each primer, 0.2 mM of each dNTPs, 5 μL of 10 × PCR buffer, 30 μg of bovine serum albumin (BSA), and *Taq* DNA polymerase. And reactions with cultured cell as template were carried out in 25 μL volumes containing 10 cells/μL to $10^3$ cells/μL of each cultured cells, 10 pmol of each primers, and 12.5 μL of 2 × GoTaq® Green Master Mix (Promega, USA).

Thermocycling conditions included an initial denaturation 5 min at 94°C, then a denaturation step at 94°C for 45 sec, annealing at 55°C for 45 sec, and a 45 sec extension at 72°C for total of 40 cycles. The final extension was done at 72°C for 10 min. The amplified PCR products were resolved via electrophoresis in a 1% agarose gel.

**Inoculated food**

The commercial low-fatted milk (Namyang Co., Korea) which purchased in a local market, was used as experimental subject (food). The samples to be tested were prepared as follows: 1 mL of milk was inoculated $10^4$, $10^3$, and $10^2$ cells/mL. Then, 9 mL of TSB was added into the inoculated milk samples to get the final cell numbers of $10^3$-$10^1$ per mL. The samples were incubated at 37°C for 18 h and were used for PCR assay. Before PCR reaction, cultured cells were washed with peptone water.

## Results and Discussion

The simplex and multiplex PCRs were carried out on DNA extracted by boiling method to verify the specificity of the primers. In the preliminary study (data not shown), primers were not cross-reacted with those of other pathogens and, were designed to target specific genes in the presence of pathogenic bacteria. Target genes were selectively amplified by designed-primers using the NCBI BLAST test results. As a result, it appeared that a product amplified by primers was not confirmed in non-pathogenic bacteria and that these are effective to differentiate the strains. These results with a good specificity were similar to those in other researches that carried out for the simultaneous detection of pathogens in foods using multiplex PCR (Chiang, 2012; Omiccioli, 2009; Yang *et al*., 2013b).

In this study, the specific primers for *L. monocytogenes* amplified an expected 611 bp fragment from DNA of *L. monocytogenes* and no amplification was found with *E. coli* O157:H7, *B. cereus, Salmonella* spp., and *S. aureus*. Similarly, each primer produced specific fragments of 519 bp for *Escherichia coli* O157:H7, 437 bp for *B. cereus*,

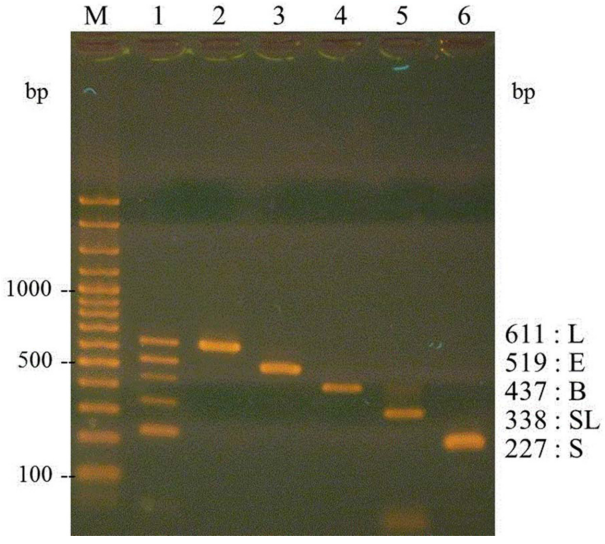


**Fig. 1. Specificity of multiplex PCR by multiple primer.** The size of the PCR products obtained for *Listeria monocytogenes* (611 bp), *Escherichia coli* O157:H7 (519 bp), *Bacillus cereus* (437 bp), *Salmonella* Enteritidis (338 bp), and *Staphylococcus aureus* (227 bp), respectively. Lane M, 100 bp DNA ladder marker; lane 1, multiple DNA; lane 2, *Listeria monocytogenes*; lane 3, *Escherichia coli* O157:H7; lane 4, *Bacillus cereus*; lane 5, *Salmonella* Enteritidis; lane 6, *Staphylococcus aureus*.

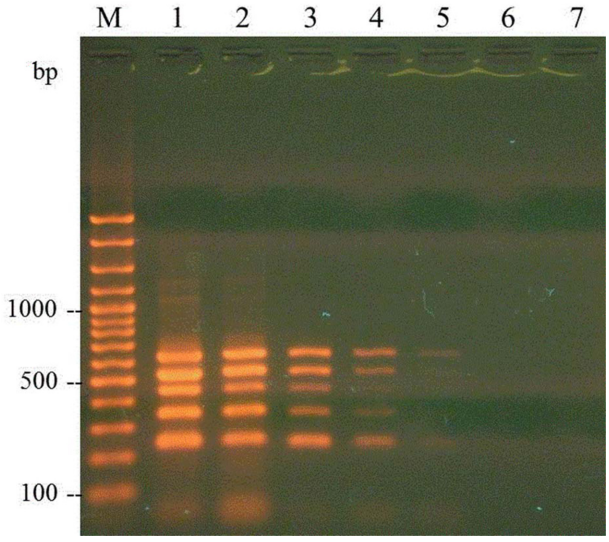


**Fig. 2. The detection limits of serially diluted DNA by multiplex PCR assay.** Each DNA was included by the density of line 1-7. Lane M, 100 bp DNA ladder marker; lane 1, 1 ng/μL; lane 2, 100 pg/μL; lane 3, 10 pg/μL; lane 4, 1 pg/μL; lane 5, 100 fg/μL; lane 6, 10 fg/μL; lane 7, 1 fg/μL.

338 bp for *Salmonella* spp., and 227 bp for *S. aureus* (Fig. 1). From the results, each primer was applied to identify the five pathogenic strains tested in this study (*B. cereus* KCCM 11341, *E. coli* O157:H7 Lab collection, *S. aureus* ATCC 25923, *S.* Enteritidis KCCM 12021, and *L. monocytogenes* ATCC 15313).

DNA extracted by the phenol/chloroform method and the genomic DNA extraction kit method showed the enough density to confirm detection limit. In addition, it was found that a purity of DNA in phenol/chloroform method was similar as those of genomic DNA extraction kit method. During the PCR reaction, it have been known that, by adding BSA, *Taq* DNA polymerase can react more reliably and the reaction can be more sensitive and stable to detect pathogenic bacteria (Hyun *et al*., 2005; Oikarinen *et al*., 2009; Strien *et al*., 2013). According to the results from the multiplex PCR carried out on serial diluted DNA containing 30 μg of BSA, it was found that the minimum concentration of template DNA required for the multiplex PCR reaction was approximately 1 pg/μL, and that amplification within a lower density than those in a previous study was possible (Yang *et al*., 2013b). A product amplified was not confirmed with DNA less than 100 fg in detail (Fig. 2).

The sensitivity of the multiplex PCR for simultaneous detection of the tested bacteria was performed using the detection limit as presented in Fig. 3 and 4. The detection limits of pathogenic bacteria in a single primer (Fig. 3) were compared to those of pathogenic bacteria by multiple primers (Fig. 4). In case of the cultured cells using colony PCR without going through the process of DNA extraction, amplification occurs when using only the target of each primer. When using multiple primers, it was less efficient than before, confirmation was possible in all cases except for *B. cereus* and *S. aureus*. The results suggest that this method can be efficient in terms of using multiple primers without significant difference.

Milk is consumed in a variety of patterns, so pay attention to food safety. It has a characteristic to be contaminated in a low density of pathogens. Thus, the situation made by inoculating various concentrations of pathogen bacteria in the milk. Cultured milk samples without extracting genomic DNA was tested by colony PCR using master mix (Packeiser *et al*., 2013). The results showed that after 18 h incubation, the multiplex PCR assay was able to correctly identify the presence of the five pathogens at all the different contamination levels and down to the lowest concentration of $10^2$ cells/mL (Fig. 5). The results for the detection limit within this work were com-

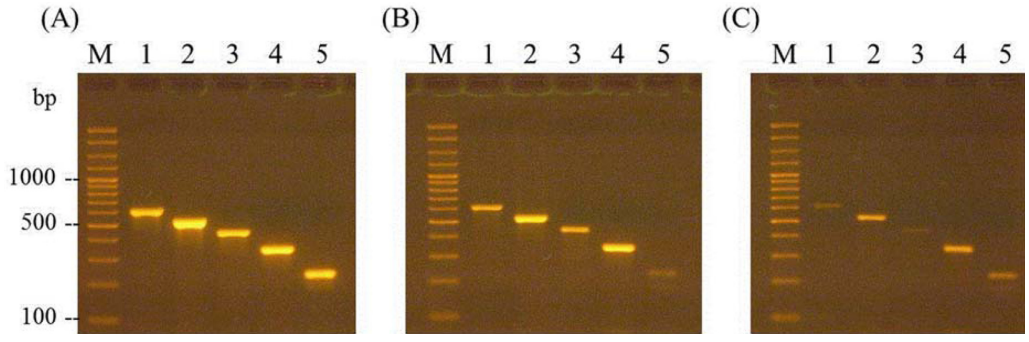


**Fig. 3. The detection limits of pathogenic bacteria by single primer.** (A) $10^3$ cells/mL; (B) $10^2$ cells/mL; (C) $10^1$ cells/mL. Lane M, 100 bp DNA ladder marker; lane 1, *Listeria monocytogenes*; lane 2, *Escherichia coli* O157:H7; lane 3, *Bacillus cereus*; lane 4, *Salmonella* Enteritidis; lane 5, *Staphylococcus aureus*.

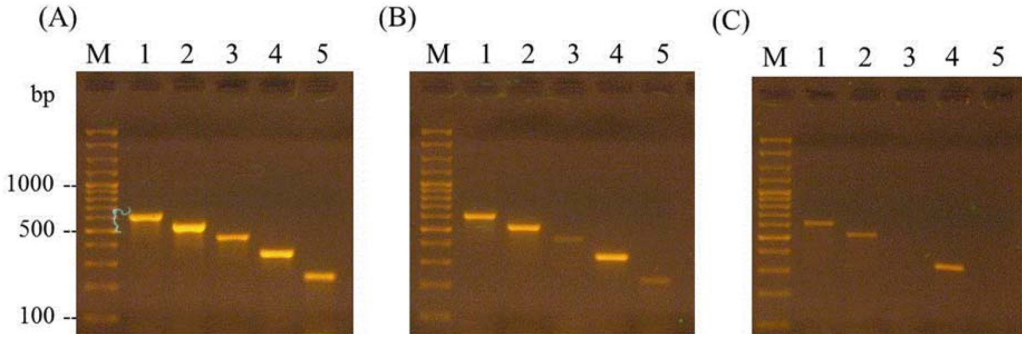


**Fig. 4. The detection limits of pathogenic bacteria by multiple primer.** (A) $10^3$ cells/mL; (B) $10^2$ cells/mL; (C) $10^1$ cells/mL. Lane M, 100 bp DNA ladder marker; lane 1, *Listeria monocytogenes*; lane 2, *Escherichia coli* O157:H7; lane 3, *Bacillus cereus*; lane 4, *Salmonella* Enteritidis; lane 5, *Staphylococcus aureus*.

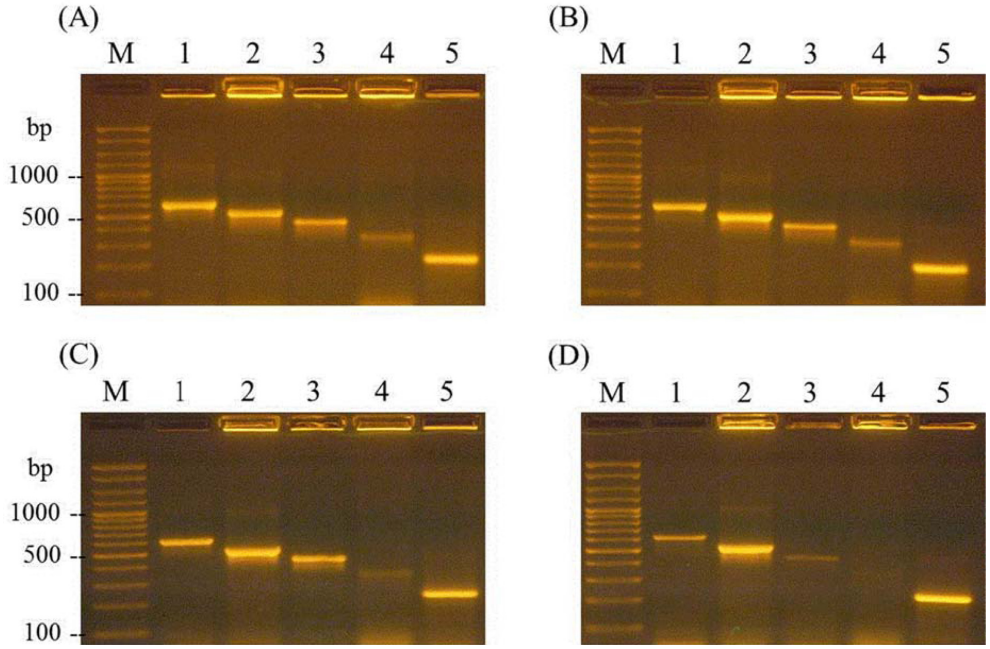


**Fig. 5. The detection limits of pathogenic bacteria in milk.** (A) $10^4$ cells/mL; (B) $10^3$ cells/mL; (C) $10^2$ cells/mL; (D) $10^1$ cells/mL. Lane M, 100 bp DNA ladder marker; lane 1, *Listeria monocytogenes*; lane 2, *Escherichia coli* O157:H7; lane 3, *Bacillus cereus*; lane 4, *Salmonella* Enteritidis; lane 5, *Staphylococcus aureus*.

parable with those of other similar researches by Chiang *et al.* (2012) and Yang *et al*. (2013b). And the result of $10^1$ cells/mL is vague, but can confirm a result amplified all. Therefore, each pathogenic bacterial contamination applied with, all pathogenic bacteria can be detected in the experiment.

In conclusion, primers used in this study were enough to amplify five kinds of pathogenic bacteria at the same time. The experiments on food had acceptable result for multiplex PCR. Therefore, multiplex PCR development in this study may be applied to foods such as dairy products and meat products in a wide range of possibilities.

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(Received 2014.2.14/Revised 2014.6.12/Accepted 2014.7.28)