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# Simultaneous Detection of Food Pathogens, *Staphylococcus aureus, Salmonella* Enterica, *Bacillus cereus* and *Vibrio parahaemolyticus* by Multiplex Real-Time Polymerase Chain Reaction

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## ABSTRACT

The study established a multiplex real-time polymerase chain reaction (PCR) for simultaneous detection and quantitation of *Staphylococcus aureus, Salmonella* Enterica, *Bacillus cereus* and *Vibrio parahaemolyticus* in the same reaction at one time, thus reducing the amounts of reagents and cutting down on the labor and time. Four sets of genetic marker specific primers corresponding to SAOUHSC_02297, *inv*A, *hbl* and *tlh*A gene, and probes were labeled with FAM, HEX, TEXAS RED and Cy5 respectively. Multiplex real-time PCR was carried out with an iQ$^{TM}$ Multiplex Powermix Kit (BIO-RAD) Sequence Detection System. The result showed that the correlation coefficient between multiplex real-time PCR estimates and plate counts of 10-serial dilutions in specific bacteria was above 0.98, independent of $10^{1-4}$-fold numbers of three other pathogenic strains. With optimized conditions, the lowest detection concentrations of four pathogens were $10^{2.5}$ CFU/mL for *Sta. aureus, S.* Enterica and *V. parahaemolyticus* and $10^{3.5}$ CFU/mL for *B. cereus*. The duration of the entire experiment from DNA isolation and purification to PCR amplification was less than 12 h.

Key words: multiplex real-time polymerase chain reaction, *Staphylococcus aureus, Salmonella* Enterica, *Bacillus cereus, Vibrio parahaemolyticus*

## INTRODUCTION

Food-borne diseases remain a persistent challenge to public health. Food-borne disease outbreaks, which cause approximately 76 million cases of illnesses and 5,000 deaths every year[1], have more than doubled in the United States since 1987[2]. These outbreaks also cause serious economic loss for both consumer and industry. In Taiwan, during 1995 to 2001, 1,171 outbreaks of food-borne illness, including 109,884 cases, were reported in northern Taiwan, of which 735 (62.8%) were caused by bacterial infection. Bacterial pathogens, particularly *V. parahaemolyticus* (86.0%), *Sta. aureus* (7.6%), and *Salmonella* spp. (4.9%) were the main etiologic agents[3]. *Bacillus cereus* (41.2%) was the main etiologic agents in central Taiwan. These outbreaks were mainly caused by mishandling of food at home (41.2%) and in school (34.3%)[4]. These 4 responsible pathogens appeared different from those in Europe and the United States. It is important to establish a surveillance net of our own to prevent and control of food-borne disease outbreaks effectively.

Conventional standard detection methods for pathogenic bacteria are described in the *FDA Bacteriological Analytical Manual*[5]; such as culturing the organisms in selective agar and identifying isolates according to their biochemical or immunological characteristics. These protocols, however, are time-consuming and labor-intensive. Conventional PCR use genomic DNA as target to substantially amplify specific DNA fragments. However, conventional PCR assays with electrophoresis are primarily qualitative techniques and not appropriate for accurate quantification of a specific pathogenic bacteria[6]. Multiplex PCR is a technology that can amplify more than one target gene at the same time in the same reaction. It is able to monitor several pathogens simultaneously as compared to conventional PCRs. Multiplex PCR has been used to detect *Escherichia coli* O157 : H7[7], *Salmonella* and *Shigella* from

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apple cider[8] and ground beef[9]. However, quantitative detection of target genes is not feasible in multiplex PCR assays because amplified products only be visualized in agarose gels after the completion of PCR, and the strength of the target band cannot be measured correctly.

Real-time PCR for detection of pathogenic bacteria has properties of high specificity and sensitivity, and can be completed in one day. Real-time PCR utilizes the 5' - 3' nuclease activity of *Taq* DNA polymerase to digest an internal fluorogenic probe labeled with a fluorescent reporter dye and a fluorescent quencher dye, which is faster and more sensitive[10]. It has been successfully applied for detection of bacterial DNA in various environments, such as pathogenic bacteria in food[11-13], water[14] and feces[15].

Multiplex real-time PCR has been used for detection of different bacterial species, realizing the quantitation and identification of target strains at the same time. Such methods would be even more useful if they could simultaneously detect and quantify a mixture of pathogens in a sample. Molecular beacons (MB), due to their stable stem-and-loop structure, have been demonstrated to be significantly more specific than dyes such as SYBR Green I and other types of probes. Gubala and Proll[16] used MB for the detection of *V. cholera* by targeting four virulence and using multiplex real-time PCR assay. The specificity and sensitivity tested with pure culture and spiked environmental water samples were 10 CFU. Gillespie and Oliver[17] developed a multiplex real-time PCR method to simultaneously detect common mastitis pathogens *Sta. aureus, Streptococcus uberis,* and *Streptococcus agalactiae* directly from milk. The sensitivity of the procedure to correctly identify was 95.5%, and the specificity was 99.6%. Wang *et al*.[18] established a multiplex real-time PCR for the simultaneous quantitation of *E. coli* O157 : H7, *Salmonella*, and *Shigella*, and the final standard curves were calculated for each pathogen by plotting the threshold cycle value against the bacterial number *via* linear regression.

In this study, we detected pathogenic bacteria with new designing primers and probe specific for virulent gene of *Sta. aureus, S.* Enterica*, B. cereus* and *V. parahaemolyticus* with pure culture, and the specificity of this approach on the effect of mixture strains were also explored. Finally, pathogenic bacteria cells were detected by multiplex real-time PCR assay to determine the detection limit (sensitivity) of this method.

## MATERIALS AND METHODS

### I. *Bacterial Strains*

All the strains were obtained from the Bioresource Collection and Research Center (BCRC) of Food Industry Research and Development Institute (Hsinchu, Taiwan). The reference strains used as positive controls for the multiplex real-time PCR assay included *Sta. aureus* (BCRC 12990, 12656, 13957 and 13961), *S.* Enterica (BCRC 10744, 10747, 12459 and 12947), *B. cereus* (BCRC 10603, 11026, 15323 and 17039), *V. parahaemolyticus* (BCRC 10806, 12866, 12877 and 13028), and additional non-specific bacteria of *Sta. epidermidis* BCRC 10785, *E. coli* BCRC 10675, *B. thuringiensis* BCRC 14683 and *V. fluvialis* BCRC 12830 were used for negative control with four pathogen specific primers and fluorescent-labeled probe that targeted the specific gene.

### II. *Design of the Primers and Fluorescent-Labeled Probes*

The primers and fluorescent-labeled probes were synthesized by GenScript, Inc. (Piscataway, NJ. USA). TaqMan® probes were designed according to the target genes of the four pathogens using Beacon Designer 2.1 (Premier Biosoft International, PBI, Palo Alto, CA. USA) to prevent the cross homology of DNA sequence.

A genetic marker specific gene was used for primers and fluorescent-labeled probe design. The target for *Sta. aureus* primers and dual-labeled probe was selected from the SAOUHSC_02297 gene encoding the S1 RNA binding domain protein[19,20]. The *inv*A gene locus in invasion of the murine intestine was the target for primers and dual-labeled probe design for *S.* Enterica[21,22]. The target for *B. cereus* primers and dual-labeled probe was selected from the *hbl* gene encoding the binding component B, and two lytic components $L_1$ and $L_2$[23,24]. The target for *V. parahaemolyticus* primers and dual-labeled probe was selected from the *tlh*A gene encoding the thermolabile hemolysin[25].

Each probe was synthesized and labeled with a reporter dye and a quencher dye by PBI. For the *Sta. aureus*, the 5'end was labeled with FAM (6-carboxy-fluorescine), and 3'end was labeled with BHQ1 (Black Hole Quenchers 1). *S.* Enterica probe was labeled with HEX (hexachloro-6-carboxy-fluorescine) on the 5'end, and BHQ1 on the 3'end. The *B. cereus* probe was labeled with TEXAS RED on the 5'end and BHQ1 on the 3'end. The *V. parahaemolyticus* probe was labeled with Cy5 on the 5'end and BHQ1 on the 3'end, FAM, HEX, TEXAS RED and Cy5 are four different fluorescent dyes that can generate different signals. The DNA sequences, target genes, amplicon sizes, four pairs of primers and the probes used in this study are shown in Table 1.

### III. *Bacterial Strains and Culture Conditions*

*Sta. aureus*, *S.* Enterica and *B. cereus* were grown in nutrient broth (Nutrient broth; Difco, Sparks, MD, USA). *V. parahaemolyticus* were grown in TSB (Trypticase Soy Broth; Merck, Darmstadt, Germany) with adding 3% NaCl at 35°C for 12 - 20 h. The accurate cell counts of pathogenic bacteria were determined by specific agar culture methods. The cell cultures of *Sta. aureus* were transferred to BPA (Baird Parker agar; bioMeriux, Marcy I' Etoile, France) at 35°C for 24 h. *S.* Enterica were transferred to BSA (Bismuth sulfite agar, Merck, Darmstadt, Germany). *B. cereus* was transferred to MYP (Mannitol-Egg yolk-Polymyxin Agar; Difco, Sparks, MD, USA). The cell cultures of

**Table 1.** Oligonucleotide primers and probes used in multiplex real-time PCR

| Species | Target gene | | Sequence (5'-3') | Amplicon size (bp) |
|---|---|---|---|---|
| *Sta. aureus* | SAOUHSC_02297 | F[a] | CATATCGCTAATGGCTCTAACCC | |
| | | R[b] | ACAAATTACAACGTGTTGAAGACC | 112 |
| | | P[c] | FAM-ACGTTTCGCCTCAGTTGCCCTTGT-BHQ1 | |
| *S.* Enterica | *inv*A | F | ACGCCAAGTTTGTCCCCAATAG | |
| | | R | CGCCAAACGTCACGTAGAATTATC | 114 |
| | | P | HEX-CCGCTCCATGAAGTGCCCAAACGC-BHQ1 | |
| *B. cereus* | *hbl* | F | CCAGCCGCTGTTCCTAAACC | |
| | | R | GTGGATTGGGAGCAGCTATTTTAG | 145 |
| | | P | TEXAS RED-TGCACCAAGAGCCGAGAGTCCACC-BHQ1 | |
| *V. parahaemolyticus* | *tlh*A | F | ACATTAGATTTGGCGAACGAGAAC | |
| | | R | ATGCGTTAAAGATGTTGCCTGTATC | 171 |
| | | P | Cy5-CGTTCTTCGCCGCTGACAATCGCTT-BHQ1 | |

[a]F, forword [b]R, reverse [c]P, probe

*V. parahaemolyticus* were transferred to TCBS (Thiosulfate-citrate-bile salts-sucrose agar; Difco, Sparks, MD, USA). The 10-fold serial diluted solutions of target bacteria were mixed with equal volumes of three other pathogenic bacteria at 5.5 log CFU/ mL.

IV. *DNA Extraction and Purification of DNA from Bacterial Cultures*

After enrichment, $10^8$ CFU/mL of *Sta. aureus, S.* Enterica*, B. cereus, and V. parahaemolyticus* in log phase underwent seven 10-fold serial dilutions. Genomic DNA was isolated from each of bacterial strains: an aliquot of 1 mL bacterial suspension, centrifuged (at 8,500 ×g for 5 min.), discarded supernatants, and subjected to DNA isolation using magnetic bead technology[25] with the Chemagic DNA Bacteria Kit (Chemagen, Baseweiler, Germary) according to the manufacturer's instruction. Briefly, samples pellets (1 mL) of bacteria were incubated with 180 μL of lysis buffer (contained 20 mg/mL lysozyme) at 37°C for 60 min. Then, 25 μL of proteinase K (10 mg/mL final concentration) and 200 μL of lysis buffer were added to the mixture at 70°C for 60 min. After incubation, the mixture was centrifuged at 8,500 ×g for 5 min. The supernatant was mixed with 20 μL of magnetic beads and 600 μL of binding buffer at room temperature for 5 min. Then, the mixture was placed in a magnetic separator and supernatant discarded. The beat pellet was added 500 μL of washing buffer 3 and re-suspended at room temperature for 2 min. The mixture was placed in a magnetic separator and supernatant discarded. The beat pellet was added 500 μL of washing buffer 4 and same steps were applied to wash buffer 3. Then, the beat pellet was added 1000 μL of washing buffer 5 at room temperature for 1 min. The mixture was placed in a magnetic separator and supernatant discarded. The beat pellet was added 100 μL of elution buffer at 55°C for 10 min. Placed in magnetic separator for 2 min, the resulting mixture contained DNA extract.

V. *Preparation of Multiplex Real-Time PCR Quantification from Pure Strain and Mixture Strains*

In order to ensure the reliability of the bacterial DNA quantitative analysis, the culture of *S.* Enterica*, B. cereus*, *V. parahaemolyticus* and *Sta. aureus* were 10-fold serially diluted.

Each of the 4 pure strains of bacteria (target bacteria) of 10-fold serial dilutions with the equivalent of $10^{0.5}$ to $10^{6.5}$ CFU/mL was detected in four sets of primers and probes in real-time PCR assays.

For mixture strains of multiplex real-time PCR assays, one bacterial strain (target bacteria) of 10-fold serial dilutions with the equivalent of $10^{0.5}$ to $10^{6.5}$ CFU/mL was mixed with another three pathogenic bacteria (served as non-target bacteria) with the equivalent of $10^{5.5}$ CFU/mL was detected in four sets of primers and probes in real-time PCR assays.

The multiplex real-time PCR assays were used according to the manufacturer's instruction of iQ™ Multiplex Powermix Kit (BIO-RAD). Each reaction mixture in a final volume of 25 μL, contained 12.5 μL of iQ™ Multiplex Powermix Kit (dNTPs including dUTP, 12 mM $MgCl_2$, iTaq DNA polymerase, and stabilizers), 250 nM of each primer and dual-labeled probe, bacterial DNA, and sterile water.

VI. *Multiplex Real-Time PCR Optimization and Conditions*

The iCycler iQ multiplex real-time PCR detection system (BIO-RAD) was used for the multiplex real-time PCR assay. Each well contains the four extracted DNA of pathogenic bacteria. The process was 95°C for 3 min followed by 40 cycles of 95°C for 10 sec and 55°C for 45 sec. The fluorescence for each probe was measured during the

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45 sec-hold at 55°C, and then directly analyzed by using iQ$^{TM}$5 Optical System software vers. 2.0 (BIO-RAD). For each sample, a cycle threshold ($C_t$) was calculated based on the baseline cycles and threshold value which is 10 times the mean standard deviation of fluorescence in all wells over baseline cycles. The standard curves were calculated by plotting the Ct value against log CFU/mL by linear regression to analyze the results for each pathogen.

VII. *Data Analysis*

The results of PCR assay were converted to the average amount of target bacterial genome in 1 mL of cultural suspension. Logarithms of cell count in the 10-fold diluted solutions and estimates of real-time PCR were plotted on the horizontal and vertical axes, respectively. The accuracy of real-time PCR was analyzed by computing the correlation coefficient between the two sets of values using the signed rank and Wilcoxon signed test. SAS 6.12 software was used to do all statistical analyses.

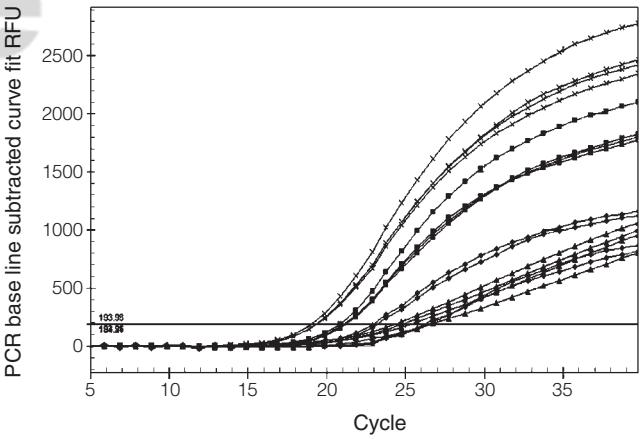


**Figure 1.** The signal map of FAM, HEX, TEXAS RED and Cy5 to simultaneously detect *Sta. aureus* BCRC 12990(▲), 12656(▲), 13957(▲), and 13961(▲); *S.* Enterica BCRC 10744(X), 10747(X), 12459(X) and 12947(X); *B. cereus* BCRC 10603(◆), 11026(◆), 15323(◆) and 17039(◆) and *V. parahaemolyticus* BCRC 10806(■), 12866(■), 12877(■) and 13028(■) at 4 reactions by multiplex real-time PCR at one time.

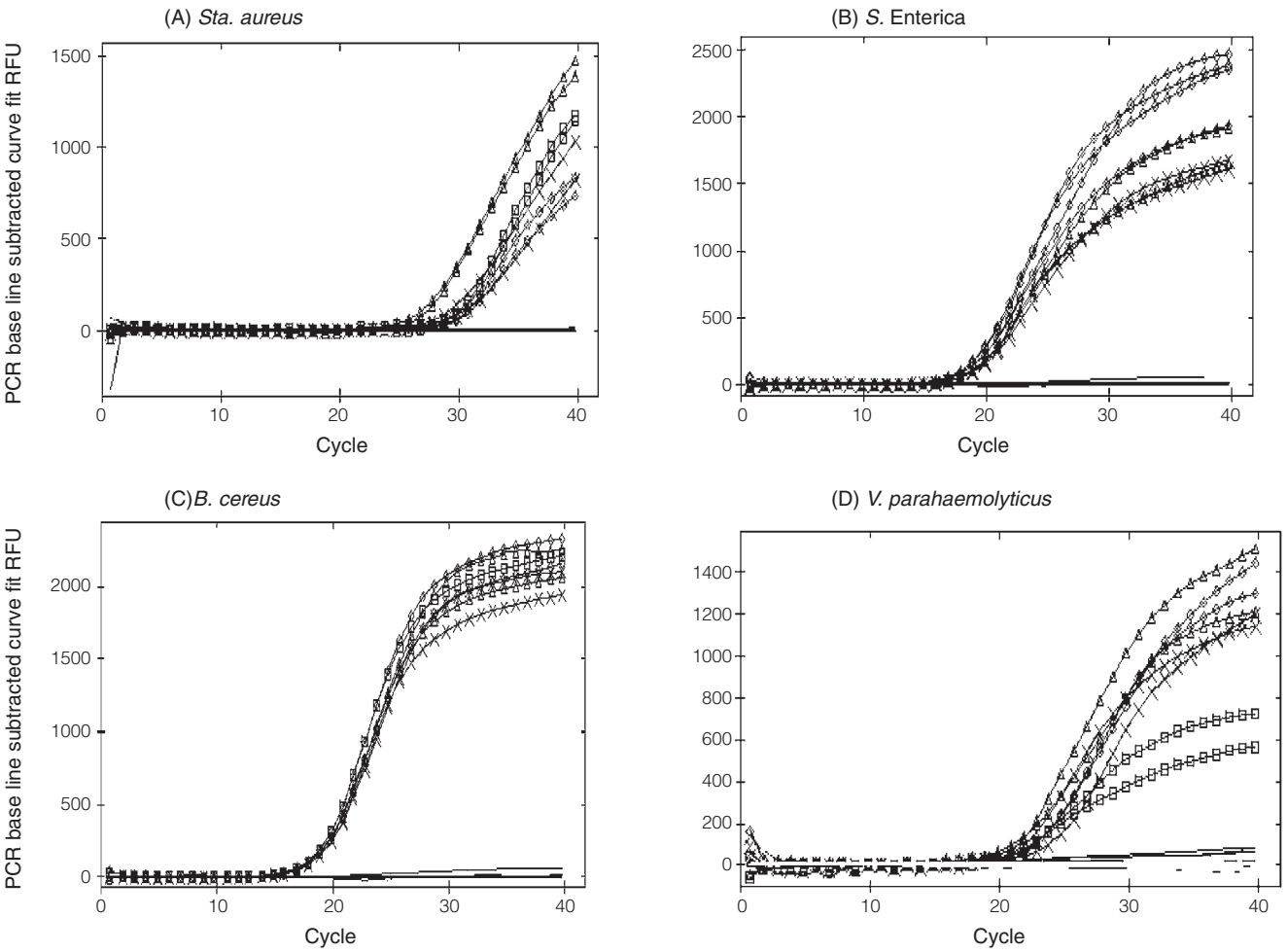


**Figure 2.** Representative PCR amplification profile obtained from the fourplex real-time PCR. Analysis of specific genes amplified from each $10^{7\text{-}8}$ CFU/mL, (A) *Sta. aureus* BCRC 12990 (▲), 12656 (X), 13957 (◆) and 13961 (■), (B) *S.* Enterica BCRC 10744 (▲), 10747 (X), 12459 (◆)and 12947 (■), (C) *B. cereus* BCRC 10603 (▲), 11026 (X), 15323 (◆) and 17039 (■), (D) *V. parahaemolyticus* BCRC 10806 (▲), 12866 (X), 12877 (◆) and 13028 (■). No signal for negative control. Esch analysis was repeated multiple times to ensure the reproducibility of results.

## RESULTS

I. *Specificity of Multiplex Real-Time PCR for 4 Pathogenic Bacteria in Mixture Strains*

To check the specificity of primers and probes designed by the PBI software for multiplex real-time PCR analysis, DNA from pure cultures of 16 strains was examined. Figure 1 is the signal map of FAM, HEX, TEXAS RED and Cy5 to simultaneously detect *Sta. aureus, S.* Enterica*, B. cereus* and *V. parahaemolyticus* at the 4 reactions by multiplex real-time PCR at one time. Based on the 16 strains tested, the results showed that the primers and probes were specific for the target four pathogens. Figure 2 is the specificity from the multiplex real-time PCR analysis. Only 4 *Sta. aureus* strains (BCRC 12990, 12656, 13957 and 13961) tested positive with SAOUHSC_02297 gene and FAM-labeled probes in the multiplex real-time PCR assay (Figure 2A). Only *inv*A gene and HEX-labeled probe specific for 4 *S.* Enterica strains (BCRC 10744, 10747, 12459 and 12947) were positive (Figure 2B). Only 4 *B. cereus* strains (BCRC 10603, 11026, 15323 and 17039) tested positive with *hbl* gene and TEXAS RED labeled probes (Figure 2C). Only 4 *V. parahaemolyticus* strains (BCRC 10806, 12866, 12877 and 13028) tested positive with *tlh*A gene and Cy5 labeled (Figure 2D). Amplification of DNA from other bacterial strains (*Sta. epidermidis* BCRC 10785, *E. coli* BCRC 10675, *B. thuringiensis* BCRC 14683 and *V. fluvialis* BCRC 12830) resulted in no fluorescence.

II. *Construction of Standard Curves from Pure Strain and Mixture Strains Using Multiplex Real-Time PCR*

Figure 3 presents standard curve from pure strain and mixture strains for quantitative detection of 4 pathogenic bacteria by multiplex real-time PCR analysis. Triplicates of

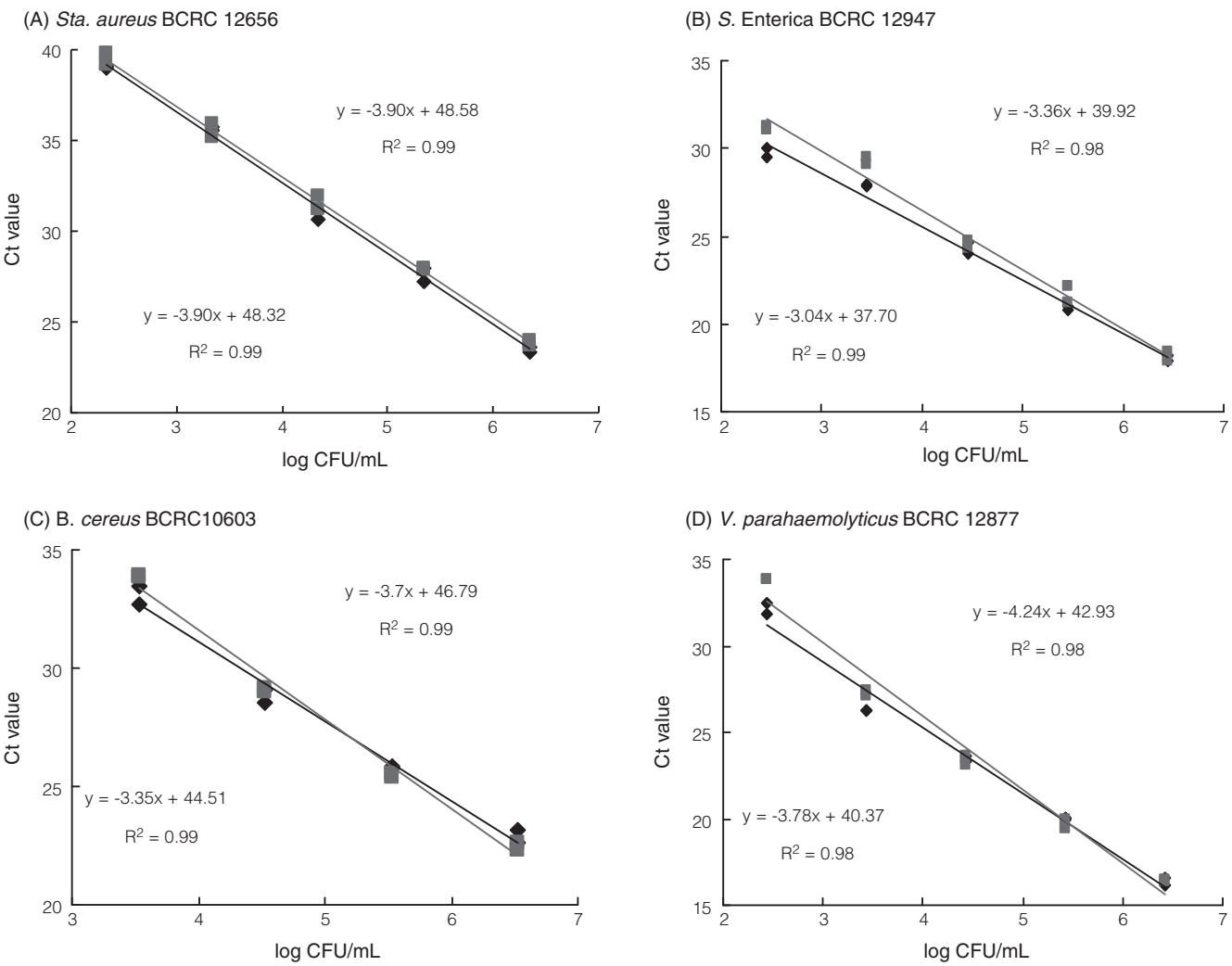


**Figure 3.** Standard curve from pure strain and mixture strains for quantitative detection of 4 pathogenic bacteria by multiplex real-time PCR analysis. (A)*Sta. aureus* BCRC 12656, (B) *S.* Enterica BCRC 12947, (C) *B. cereus* BCRC 10603, (D) *V. parahaemolyticus*12877 for multiplex real-time PCR analysis. (◆) pure culture; (■) culture mixed with 3 other pathogenic bacteria. All experiments were repeated three times; the average results are displayed.

each analysis were produced to ensure the reproducibility of results. When multiplex real-time PCR was applied to pure strain and to the four mixtures strains of pathogens, the standard curves were similar, the detection efficiencies were $10^{2.5}$ to $10^{6.5}$ CFU/mL for *Sta. aureus, S.* Enterica, and *V. parahaemolyticus* with pure culture of $R^2$ that were 0.99, 0.98 and 0.98, respectively and with mixture strains of $R^2$ that were 0.99, 0.99 and 0.98, respectively, and $10^{3.5}$ to $10^{6.5}$ CFU/mL for *B. cereus* with pure strains and mixture strains of $R^2$ were 0.99 and 0.99. The detection limit of this multiplex assay, the cells were $10^{2.5}$ CFU/mL for *Sta. aureus, S.* Enterica and *V. parahaemolyticus* and $10^{3.5}$ CFU/mL for *B. cereus.*

## DISCUSSION

The multiplex real-time PCR assay can be used as a rapid quantitative approach with high specificity for detecting several pathogens simultaneously by amplifying more than one target gene in a single reaction. Therefore, some studies utilizing simultaneous detection of foodborne pathogens by multiplex real-time PCR have been published, including some focused on *Bacteroides* spp.and faecal enterococci in waters[27], *Salmonella* and *Cronobacter* in powdered infant formula[28], *Escherichia coli* O157 : H7, *Salmonella* spp. and *Sta. aureus* in fresh, minimally processed vegetables[29], *E. coli* O157:H7, *Salmonella*, and *Shigella* in ground beef[18], total and pathogenic *V. parahaemolyticus* bacteria in oysters[30], *Campylobacter* and *Salmonella* in chicken rinse fluid[31], *Salmonella* and *L. monocytogenes* in raw sausage meat[32], *Salmonella* serovars and *L. monocytogenes*[33] and *Salmonella* strains and *E. coli* O157 : H7[34] in single enrichment broth culture.

Multiplex real-time PCR methods involved using Beacon Designer of four pairs of new specific primers and four probes corresponding SAOUHSC_02297, *inv*A, *hbl* and *tlh*A gene to generate a fluorescent signal for the detection, as well as quantitation of target pathogenic bacteria. Its ability to monitor several pathogens is simultaneous in order to reduce the amounts of reagents and cutting down the labor and time of analysis. When multiplex real-time PCR was applied to pure strain and to the four mixture strains, the detection efficiencies were $10^{2.5}$ to $10^{6.5}$ CFU/mL for *Sta. aureus, S.* Enterica and *V. parahaemolyticus*, and $10^{3.5}$ to $10^{6.5}$ CFU/mL for *B. cereus* in this report. The result was comparable with Wang *et al.*[17]. They established a multiplex real-time PCR for the simultaneous quantitation of *E. coli* O157:H7, *Salmonella*, and *Shigella* with optimized conditions, the quantitative detection range was $10^2$ to $10^9$ CFU/mL for *E. coli* O157:H7, $10^3$ to $10^9$ CFU/mL for *Salmonella*, and $10^1$ to $10^8$ CFU/mL for *Shigella*.

The detection limit of this multiplex assay, the cells were $10^{2.5}$ CFU/mL for *Sta. aureus, S.* Enterica and *V. parahaemolyticus* and $10^{3.5}$ CFU/mL for *B. cereus.* Therefore, compared to previous studies, our result was consistent with those of studies by Wolffs, *et al.*[31], Elizaquıvel and Aznar[29] assessed a multiplex real-time PCR assay, which was capable of detecting at $10^3$ CFU/ml for *Sal.* and *Cronobacter*, $3.0 \times 10^3$ CFU/ml for *Campylobacter* and *Salmonella*[31], $10^3$ CFU/mL for *E. coli* O157 : H7, *Salmonella* spp. and *Sta. aureus*[29]. The result was not consistent with a study by Wang *et al.*[18] and Gillespie and Oliver[17], which detecting limits were $10^5$ CFU/g for *E. coli* O157:H7, $10^3$ CFU/g for *Salmonella*, and $10^4$ CFU/g for *Shigella*[18], $10^3$ CFU/mL for *Sta. aureus*, and was $10^2$ CFU/mL for *Strep. agalactiae* and *Strep. uberis*[17] by multiplex real-time PCR methods without any preceding procedure of enrichment.

The pure strain and mixture strains were with excellent linear correlation coefficient $R^2$ (0.98 - 0.99). The standard curves of mixture strains showed that the correlation coefficient between multiplex real-time PCR estimates and conventional plate counts of 0.98, independently of $10^{1\text{-}4}$-fold numbers of another 3 pathogenic bacterial counts. Therefore, the multiplex real-time PCR method performed well with mixture strains and pure strain, and the sensitivity and the efficiency were not decreased when this assay was applied to mixture strains (Figure 3).

Although the multiplex real-time PCR method performed well with mixtures of pure cultures (*Sta. aureus, S.* Enterica and *V. parahaemolyticus*), the sensitivity was not sensitive enough to detect *B. cereus*. The possible reason for the decrease in sensitivity might be resulted in (I) the variation in DNA recovery of Gram positive bacteria, different efficiency of DNA extraction from mixture strains[35], (II) the presence of primer dimer, (III) interference of DNAs from a huge number of contaminating bacteria[36].

The multiplex real-time PCR assay is a simple, rapid and efficient technique, it could potentially facilitate presumptively and simultaneously detect 4 pathogenic bacteria. The duration of the entire experiment from DNA isolation and purification to PCR amplification was less than 12 h. This protocol may be combined with a DNA extraction step to further improve sensitivity without any preceding procedure of enrichment.

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