

# Development of a simultaneous identification method for nine species of foodborne bacterial pathogens using real-time PCR assays and melting curve analysis

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Food safety is a global public health concern. The disease caused by foodborne pathogens often occurs throughout the world and produces considerable economic losses. Hence, rapid detection of these pathogens will enable immediate measures to be implemented for preventing widespread transmission. The present study was conducted to establish a novel multi-real-time polymerase chain reaction method to detect and quantify nine species of common bacterial pathogens simultaneously from food samples. The nine pairs of primers were designed according to the virulence-associated, high conservative, and specific genes of these pathogens. The products formed were identified based on a melting point temperature ( $T_m$ ) curve analysis. The food samples with seeded bacterial pathogens were examined. It took about three hours to perform detection. The results confirmed that the assay has good sensitivity and specificity, which can detect the limit to approximately  $7 \times 10^0$  copies per reaction respectively. This method would be a routine and practical protocol for identifying and quantifying bacterial pathogens from food or environmental samples and it should facilitate the detection and management of foodborne infectious pathogen outbreaks.

**Keywords:** foodborne bacterial pathogens; real-time PCR; melting curve analysis; detection and quantification.

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## Introduction

Food contaminated by infectious and toxigenic microorganisms has always been one of the most concerned public health issues [1]. Outbreaks of foodborne diseases are regularly reported in almost all countries in the world, from the most developed to the least developed countries [2]. Although possibly better prevention and control measures have contributed to this trend, there does appear to be an increasing health problem worldwide. During January 1, 2017 through December 31, 2017, a total of 841 outbreaks of foodborne disease were reported in the United States, resulting in 14,481 illnesses, 827

hospitalizations, and 20 deaths [3]. In China, hundreds of thousands of cases were caused by foodborne diseases every year, of which 40% of these cases involved pathogenic bacteria [4]. Furthermore, only a fraction of all foodborne infections has ever officially reported [5]. According to statistics, more than 200 different diseases have been known to be transmitted through food [6]. *Bacillus cereus*, *Vibrio parahaemolyticus*, *Escherichia coli* O157:H7, *Salmonella* spp, *Listeria monocytogenes*, *Shigella flexneri*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* are all the main pathogenic bacteria.

Rapid detection and accurate identification of pathogenic bacteria are the key to effectively control and prevent these diseases. Identification of the infective agent can reduce the possibility of misdiagnosis and change the treatment methods, thus reducing the morbidity and mortality [7]. Different microbial pathogens have different infectious doses (i.e., the number of infectious units required to cause an infection) [8, 9]. Determination of the types and numbers of different microbial pathogens in a sample is imperative for targeted treatment. In addition, the efficient enumeration of microbial pathogens in the pre-treatment and post-treatment of food samples can verify the effective evaluation of the treatment process [10, 11].

The traditional method of pathogen detection is to culture bacteria on agar culture plate, and then, detect the phenotype and serological characteristics of pathogens. However, these methods require several days for completion and are thus quite time consuming [12]. Some attempts have been made using biochemical tests, DNA homology, and protease variability. However, these techniques have some shortcomings, such as the need to isolate pathogens first, and the lack of sensitivity to detect low-level pathogens [13].

Molecular techniques such as polymerase chain reaction (PCR) can be employed to solve these problems and improve the sensitivity and specificity of pathogen detection. General PCR assays have been developed for the detection and identification of microbial pathogens. However, although the detection of bacterial pathogens based on PCR method is useful, its limitation is that only one or several bacteria can be detected at a time. When facing the complex distribution of various strains, species, and genera of pathogenic bacteria, a large number of general PCR assays would be needed. This may be a time-consuming process [14]. Oligonucleotide chips have been used for high throughput detection of common bacterial pathogens with high sensitivity and specificity [15-17]. However, these methods lack the ability to give

quantitative results. Although real-time PCR systems for quantitative analysis of bacterial pathogens have been developed, their limitations lie in that they either identify only one species or require a time-consuming process to detect multiple species [12]. The simultaneous detection of multiple pathogens by a single experimental cycle method would be relatively fast and effective. The above methods cannot meet the requirements of quantitative and simultaneous identification of different infectious agents at the same time.

In this study, we developed a sensitive, specific, quantitative, and high throughput method for rapid and simultaneous detection of nine common pathogens using real-time PCR assays and melting curve analysis. This paper described the specific primers design protocol, primers sequences, methodologies for DNA extraction, amplification, and the preliminary application in the detection of pathogenic bacteria from food samples.

## Materials and Methods

### Bacterial strains, culture media, and DNA extraction

The bacterial strains used for real-time PCR analysis in this study were standard strains and isolated strains as shown in Table 1. All strains were obtained from the National Centers for Medical Culture Collection (CMCC) (Beijing, China). The target bacterial cultures were serially diluted to appropriate inoculum levels and confirmed by plate count in triplicate. Artificially contaminated food samples were also detected by conventional culture methods.

In order to extract bacterial DNA, a single colony was picked up from LB agar plate, and then, inoculated in 3 mL LB broth (10 g peptone, 5 g yeast extract, and 5 g NaCl in 1 L water) in a flask. The bacteria were cultured at 37°C for 18 h with shaking. To harvest the bacterial cells, 1 mL bacterial cultures (about  $10^8$  copies/mL) were centrifuged at 5,000 g for 10 mins. The bacterial

**Table 1.** Main bacteria used in this study.

Bacteria	Reference strain(s)*	Isolated strain(s)
<i>Bacillus cereus</i>	4	2
<i>Campylobacter jejuni</i>	6	3
<i>Escherichia coli</i> O157:H7	12	6
<i>Klebsiella pneumoniae</i>	3	1
<i>Listeria monocytogenes</i>	5	4
<i>Pseudomonas aeruginosa</i>	10	4
<i>Salmonella</i> ssp	44	28
<i>Shigella flexneri</i>	16	3
<i>Vibrio parahaemolyticus</i>	10	7

\*Purchased from the National Center for Medical Culture Collection (CMCC) (Beijing, China).

**Table 2.** Specific primers used for real-time PCR analysis in this study.

Species	Primers	Sequence 5'-3'	Target gene	Locus	Product size (bp)
<i>Bacillus cereus</i>	Bc-gyrB-1	GCTTCACCATCTTGTGG	gyrB	NC_004722	123
	Bc-gyrB-2	CGCCATTATCCGTTACA			
<i>Vibrio parahaemolyticus</i>	Vp-tdh-1	AATGGTTGACATCCTACATGACTG	tdh	AY044113	112
	Vp-tdh-2	ACTTGACCTGATTTTACGAACACA			
<i>EHEC</i> O157:H7	0157-eae-1	TTACCAGCGATACCAAGAGC	uidA	AF305917	132
	0157-eae-2	CAACATGACCGATGACAAGG			
<i>Salmonella</i> ssp	Sal-fim-1	TACCAACCGCAAGGCATAA	fimY	NC_003198	120
	Sal-fim-2	GACACCGCGTTTAAAGAAATG			
<i>Listeria monocytogenes</i>	Lm-hly-1	GGGAAATCTGTCTCAGGTGATGT	hly	NC_002973	106
	Lm-hly-2	CGATGATTTGAACCTCATCTTTTGC			
<i>Shigella flexneri</i>	Sf-ipa-1	CCGGGATAAAGTCAGAACTC	ipaH_1	NC_008258	131
	Sf-ipa-2	CTCCCGACACGCCATAGAAA			
<i>Campylobacter jejuni</i>	Cj-htp-1	CTGAATTTGATACCTTAAGTGCAGC	hypothetical protein	NC_009839	86
	Cj-htp-2	AGGCACGCCTAACCTATAGCT			
<i>Pseudomonas aeruginosa</i>	Pa-gyr-1	CAAGCCCTACAAGAAATCCG	gyrA	NC_009656	159
	Pa-gyr-2	TCCACCGAACCGAAGTTG			
<i>Klebsiella pneumoniae</i>	Kp-pho-1	TGCCAGACCGATAACTTTA	phoE	NC_009648	142
	Kp-pho-2	CTGTTTCTTCGCTTCACGG			

pellets were subjected to DNA extraction using lysis by boiling [17, 18]. The bacterial DNA samples (in sterile redistilled water) were stored at 4°C for future use. 1 µL of bacterial DNA was used in the PCR manipulation described below.

### Preparation of the artificially contaminated food samples

In the experiment of artificially contaminated food samples, only those food samples that were confirmed to be pathogens negative by both culture and PCR methods were used. Food

samples were inoculated with a strain or mixed species of bacteria. The DNA of various bacteria in food samples were extracted using lysis by boiling [17, 18] and were used as the templates for PCR amplification described below.

### Primer design

A total of nine primer pairs were used in this study as shown in Table 2. All primers were designed according to the target gene sequence published in the GenBank. The target genes were the virulence-related, highly conserved, and

specific genes of these pathogens. The design of primers was performed using Primer Premier 5 (Premier Biosoft, San Francisco, CA, USA) according to the recommendations of the software. In order to run PCR for all target strains separately in the same PCR operation, primers were designed with a relatively adjacent annealing temperature. Primers were tested for specificity using a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primers were synthesized by GenScript Co. (Nanjing, Jiangsu Province, China). The specificity of the primers had also been analyzed and evaluated by cross-reactions among the pathogens listed in Table 1. Specificity of primer was further affirmed by carrying out melting peak analysis of real time PCR assays and agarose gel electrophoresis on the amplicon.

#### PCR system

PCR was performed under the following conditions. The final reaction system was determined by the preliminary experiment with the PCR solution containing 2.5  $\mu$ L of 10 $\times$  PCR Buffer, 150  $\mu$ M of dNTP, 0.3  $\mu$ M of each primer, 500 ng of DNA template, and 1 Unit of Taq DNA Polymerase with the total volume made to 25  $\mu$ L using sterile distilled water. The reaction was conducted at 94°C for 3 mins, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by 72°C for 5 mins. Electrophoresis of amplification products was done on 1.2% agarose gels (containing 0.5  $\mu$ g/mL ethidium bromide) in which 5  $\mu$ L reaction products were loaded with 1  $\mu$ L loading buffer. Gels were run for 30 mins at 100 V, visualized on a UV transilluminator and photographed by ImageQuant 350 Imager (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

#### Multi-real-time PCR amplification

Multi-real-time PCR was performed on the nine foodborne bacterial pathogens samples synchronously. The PCR was carried out using the ABI PRISM<sup>®</sup> 7500 real-time quantitative PCR (Applied Biosystems, Carlsbad, CA, USA). The final reaction mixture contained 1  $\mu$ L of each 5  $\mu$ M primer, 12.5  $\mu$ L 2 $\times$  SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (Takara, Dalian, Liaoning Province, China), 0.5  $\mu$ L

of 50 $\times$  ROX Reference Dye II, 1  $\mu$ L of template with the total volume made to 25  $\mu$ L by sterile distilled water [19]. The procedure of PCR was as follows: 10 s at 95°C to activate the Hot Start Taq DNA polymerase, followed by 45 cycles of 5 s at 95°C, 34 s at 55°C. Fluorescence was monitored during the 55°C annealing phase. A melting curve analysis was done immediately after above cycles by rapid cooling to 60°C and then the temperature was raised to 94°C by continuous fluorescence measurement.

#### Sensitivity of assays

The sensitivity of real-time PCR assays for nine foodborne bacterial pathogens was evaluated. For determination, a single colony was picked up from fresh culture and suspended in sterile distilled water. Ten-fold serial dilutions were made and 50  $\mu$ L of each dilution was spread on LB agar in triplicate. The plates were incubated at 37°C for 48 h. The colonies on the plates were counted. A series of seven 10-fold dilutions water samples were prepared starting from 7 $\times$ 10<sup>9</sup> copies/mL. Detection was carried out under the same conditions as above to evaluate the end-point sensitivity of real-time PCR analysis.

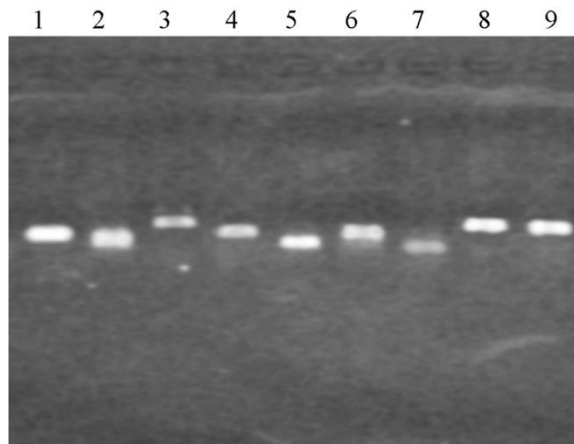
#### Detection of double-blind artificial contaminated samples by multi-real-time PCR

Ten double-blind artificial contaminated samples were processed and detected by the developed multi-real-time PCR. The isolates were confirmed by traditional methods and the VITEK test system (BioMerieux, Shanghai, China). DNA extraction and PCR amplification were performed as described above. Statistical analysis of real-time PCR results was performed by using SPSS 12.0 for Windows (IBM, Armonk, New York, USA).

## Results and Discussion

#### Specificity of primers

The primers of real-time PCR assay were searched in BLAST N database to find out the similarity of sequences. There was no known DNA sequence of non-target strains homologous to the primers in BLAST N databases. To further



**Figure 1.** Agarose gel electrophoresis profile of PCR products. The PCR template of each lane contained all bacterial species listed in Table 1. Primer pairs utilized from lane 1 to 9 are as follows. Lane 1: Bc-gyrB-1/2; Lane 2: Vp-tdh-1/2; Lane 3: O157-eae-1/2; Lane 4: Sal-fim-1/2; Lane 5: Lm-hly-1/2; Lane 6: Sf-ipa-1/2; Lane 7: Cj-htp-1/2; Lane 8: Pa-gyr-1/2; Lane 9: Kp-pho-1/2.

evaluate the specificity of the primers, DNA was extracted from a mixture of samples containing all bacterial species listed in Table 1 as a template for detection. Specificity of primers and absence of unspecific products or primer dimers were analyzed by agarose gel electrophoresis (Figure 1). On the electrophoretogram, each of the tested target strain was positive and produced the expected amplification bands. No other bands were visible. The specificity of primers was also detected by the SYBR Green I-based real-time PCR. The results were shown in Figure 2. There was a unique peak of  $T_m$  value on the melting curve of the amplified products of each target strain. These results suggested that the primers were specific to each target strain.

#### Results of the multi-real-time PCR assay

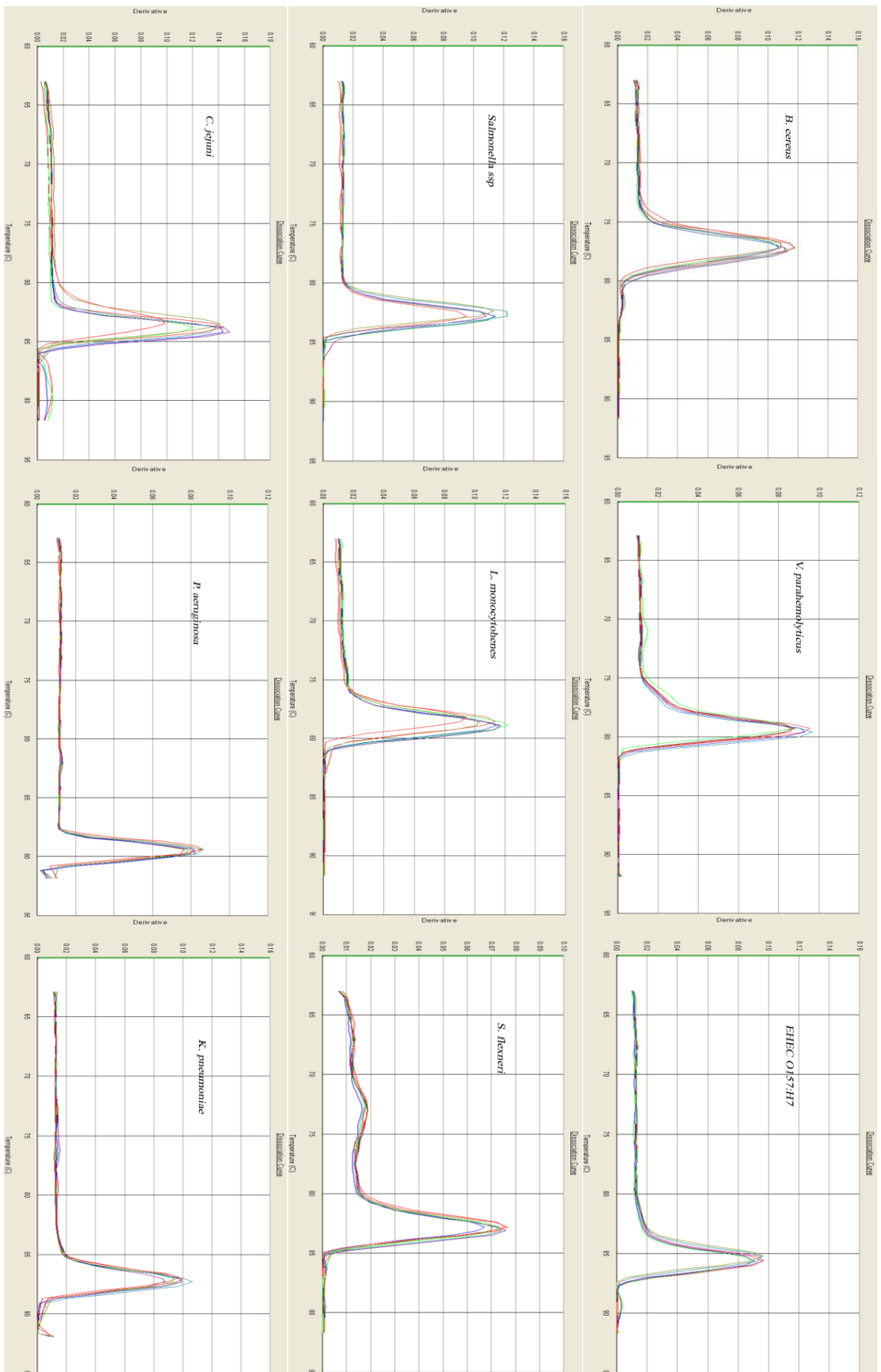
The amplification plots of SYBR Green I-based real-time PCR assay for simultaneous detection of nine species of foodborne bacterial pathogens were shown in Figure 3. The angle and linearity of each curve is related to the efficiency of the PCR reaction with straight and nearly vertical slopes representing more nearly optimal PCR conditions [19, 20]. The same distance between curves from curve 1 to curve 6 (10-fold serial dilution water samples of each pathogen) suggested that the linear range for quantification of this real-time PCR assay was from  $7 \times 10^6$  to

$7 \times 10^1$  copies per reaction. The real-time PCR system was also evaluated for sensitivity using a series of 10-fold diluted. There were typical amplification plots appeared from curve 1 to curve 7 corresponding to  $7 \times 10^6$  to  $7 \times 10^0$  copies per reaction. The curve 8 corresponding to the negative control could not be amplified in the initial 45 cycles in SYBR Green I-based real-time PCR assay. The results indicate that the detection limit of this method can reach approximately  $7 \times 10^0$  copies per reaction. The method is suitable as a qualitative and quantitative assay to simultaneously detection of nine species of foodborne bacterial pathogens.

#### Detection of the double-blind artificial contaminated samples

Ten double-blind artificial contaminated samples were treated and detected according to the above methods. All ten groups of strains were distinguished by this method. The comprehensive identification results by classical methods are regarded as the final standards. All results were consistent between the real-time PCR assay and the conventional methods. The consistency was 100% (data not shown).

DNA microarrays and multiplex PCR can also be used in the detection of several pathogens simultaneously [5, 15, 21]. However, there are



**Figure 2.** Melting curve (fluorescence versus temperature) of amplification products obtained from sample. The melting temperatures of *B. cereus*, *V. parahaemolyticus*, *EHEC O157:H7*, *Salmonella ssp*, *L. monocytogenes*, *S. flexneri*, *C. jejuni*, *P. aeruginosa*, and *K. pneumoniae* occur at 77.1°C, 79.5°C, 85.4°C, 82.6°C, 78.6°C, 82.8°C, 83.7°C, 89.5°C, and 87.2°C, respectively. No nonspecific peaks are present in each reaction.

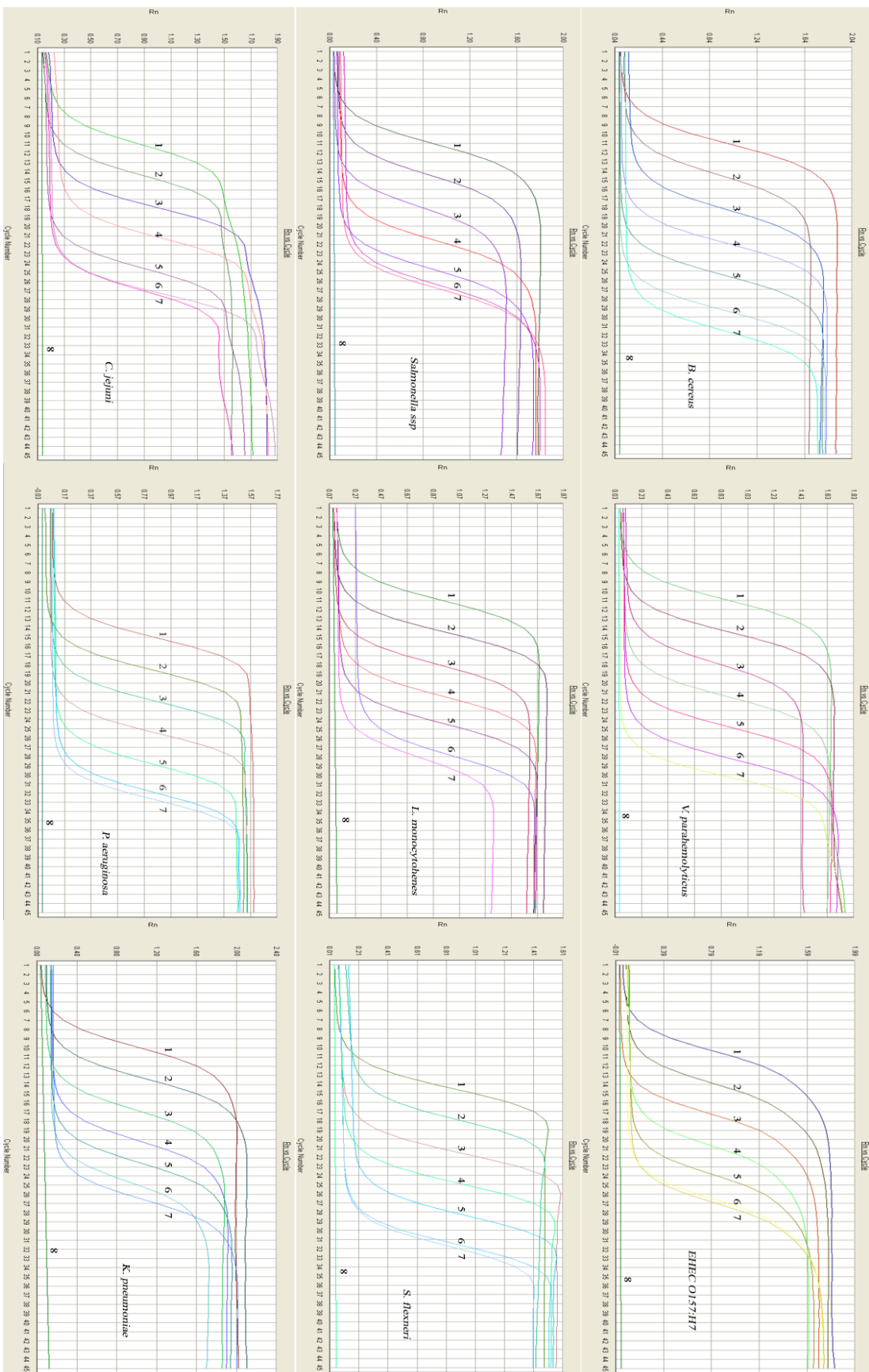


Figure 3. The amplification profile (fluorescence versus cycle number) of 10-fold serial dilution of foodborne pathogens in real-time PCR, respectively. Assay utilized concentrations of bacteria ranging from  $10^7$  to  $10^1$  copies per reaction. Assay 1:  $7 \times 10^6$ , Assay 2:  $7 \times 10^5$ , Assay 3:  $7 \times 10^4$ , Assay 4:  $7 \times 10^3$ , Assay 5:  $7 \times 10^2$ , Assay 6:  $7 \times 10^1$ , Assay 7:  $7 \times 10^0$ , Assay 8: negative control (no bacteria).

some shortcomings in those techniques. The effectiveness of microarray technology depends largely on the oligonucleotide probes residing on the gene chip. It requires careful selection of target genes and carefully designed oligonucleotide probes, especially in terms of species, sequence, and number, which are the cardinal factors in designing effective gene chips [22]. In multiplex PCR with a mixture of many primers, it requires post-PCR steps and no cross reaction among primers. This will greatly increase the complexity of analysis. In addition, those methods are incapable to quantify the pathogens.

Real-time PCR assay has similar sensitivity to classical PCR method, but it takes less time and labor intensity. Another advantage of this method is that it does not require post-PCR steps, thus avoiding the possibility of cross-contamination due to PCR products [23]. Real-time PCR technology has significantly improved and simplified the quantification of nucleic acids. It has become a valuable tool for scientists in many different disciplines, especially in the field of molecular detection [24]. However, only one or few kinds of bacteria can be detected at a time in the present applied research of real-time PCR technology.

In this study, a real-time PCR method was developed to realize simultaneously detection of multiple pathogens. Primers designed with a relatively adjacent annealing temperature made it possible. Real-time PCR for various targets could be run synchronously in the same PCR run.

This coinstantaneous SYBR Green I-based real-time PCR protocol described herein provides a rapid, sensitive, specific, and high throughput means for the identification and quantification of foodborne pathogens. This result is of special significance for surveillance and management of foodborne infectious pathogen outbreaks. Such protocols can be further developed to include other important pathogens (e.g. hepatitis A virus, rotavirus, *Vibrio cholerae*, etc.) and extended to examination of other environmental samples.

## Conclusions

Through smart design, we have developed a method that has the potential to identify and quantify nine common foodborne bacterial pathogenic species simultaneously including *B. cereus*, *V. parahemolyticus*, *E. coli* O157:H7, *Salmonella* spp, *L. monocytogenes*, *S. flexneri*, *C. jejuni*, *P. aeruginosa*, and *K. pneumoniae*. This method achieved good specificity and sensitivity. The limit of detection reached  $7 \times 10^0$  copies per reaction. The ability to rapidly monitor various microbial pathogens would be extremely useful not only for routine clinical diagnosis, but also for assessments of food safety.

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