Original Article

Simultaneous Detection of *Bacillus cereus, Salmonella* spp. and *Vibrio parahaemolyticus* in Foodstuffs by Multiplex PCR

Phornphan Sornchuer*, Rattana Tiengtip **, Kittirat Saharat***

Abstract

Introduction: Food-borne disease is a serious public health concern worldwide. *Bacillus cereus, Salmonella* spp. and *Vibrio parahaemolyticus* are the most common examples of food-borne pathogens. The rapid detection of food-borne pathogens is becoming increasingly critical for ensuring the safety of consumers.

Methods: Current study, the multiplex polymerase chain reaction (PCR) assay was employed for simultaneous detection of *B. cereus, Salmonella* spp. and *V. parahaemolyticus* in various foodstuffs. A total of 90 samples were collected from various foodstuffs in Pathumthani Province of Thailand.

Results: The overall contamination rates of *B. cereus, Salmonella* spp. and *V. Parahaemolyticus* for food samples collected in this study were 87.8% (79/90), 4.4% (4/90) and 31.1% (28/90), respectively. Most of the food samples were contaminated with one species of pathogen except salads. Approximately 41.7% of salads were contaminated with at least 2 species of pathogen. Among these, three food-borne pathogens were simultaneously detected in one salad sample.

Conclusion: This study suggests that the multiplex PCR method is suitable for rapid identification of these three food-borne pathogens in foodstuffs in order to reduce the risk of food-borne disease outbreaks.

Keywords: Multiplex PCR, Food-borne pathogen, *Bacillus cereus*, *Salmonella*, *Vibrio parahaemolyticus*

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Introduction

Food poisoning is one of the important health problems that causes by several microorganisms especially bacteria. Among these, *Bacillus cereus, Salmonella* spp. and *Vibrio parahaemolyticus* are the common etiologic pathogens. These pathogens are also of major concern since they associate with certain food including ready-to-eat products.[1] *B. cereus* is a common food poisoning bacterium which can cause vomiting and diarrhea.[2, 3] It can produce heat resistant spores which allow the bacteria to survive at high temperature conditions. *B. cereus* contamination in various food products including dairy products has been reported.[4-6] The DNA gyrase subunit B (*gyrB*) gene is a phylogenetic discriminator for closely related species.[7] *Salmonella* is responsible for infection of the intestinal epithelium, known as salmonellosis. *Salmonella* spp. can cause food-borne illnesses which are the leading bacterial cause of acute gastrointestinal illness. The *invA* (invasion protein A), a gene of *Salmonella*, contains those sequences that presents in all pathogenic serovars and has been proved as a suitable PCR target with potential diagnostic applications.[8, 9] *V. parahaemolyticus* is a significant cause of food poisoning in seafood or related products. The toxin regulatory protein (*toxR*) gene appears to be well conserved among *Vibrio* species and has been used for the specific detection of *V. parahaemolyticus* in several studies.[1, 10]

The conventional methods for the identification of *B. cereus*, *Salmonella* spp. and *V. parahaemolyticus* are time-consuming and labor-intensive. Moreover, conventional phenotypic assays may fail to detect strains of bacteria that are present in the samples at low concentrations or that possess unusual phenotypic profiles.[11] Conventional PCR employ genomic DNA as target to amplify specific DNA fragment. This technique has been increasingly used for rapid detection of food-borne pathogens since it is simple, more sensitive and less time-consuming. Multiplex PCR assay is able to simultaneously amplify multiple gene targets by using several sets of primers in a single tube.[12] Multiplex PCR has been used to detect food-borne pathogens in various food products including ready-to-eat food[1], apple cider[13], and raw and ready-to-eat meat products.[14]

In the present study, we detected food-borne pathogenic bacteria in food samples collected from restaurant and flea market in Pathumthani Province, Thailand using multiplex PCR technique. Three pathogenic bacteria including *B. cereus*, *Salmonella* spp. and *V. parahaemolyticus* were simultaneously analyzed for their distribution in food samples.

Methods

Sample collection and treatment

A total of 90 food samples, including 36 salads, 37 stir-fried vegetables, 11 soups and curries, and 6 miscellaneous foods, were collected from restaurants and flea markets in Pathumthani Province, Thailand. Sampling was conducted between June and November 2018. Each 25-g sample was mixed with 225 ml of simultaneous enrichment broth (SEB) medium for simultaneous enrichment of *B. cereus, V. parahaemolyticus*, and *Salmonella* spp. as described previously[1]. After incubation for 18 h at 37°C, each 1 ml of culture broth was subjected to DNA isolation for the multiplex PCR assay. The schematic representation of the steps used in the multiplex PCR assay for the detection procedure of food-borne pathogens was demonstrated in Figure 1.

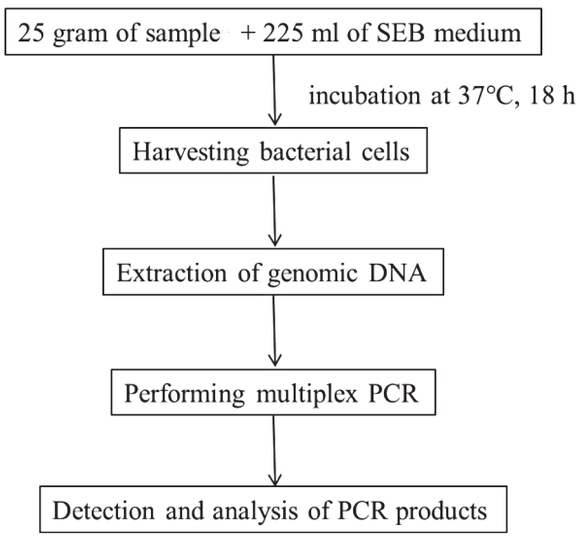

Figure 1 The scheme of multiplex PCR assay for simultaneous detection of *B. cereus, Salmonella* spp., and *V. parahaemolyticus* in food

DNA isolation

DNA extraction was performed by boiling method as described previously with several modifications.[15] After 18h incubation at 37°C, 1.0 ml of the culture was collected and cells were harvested by centrifugation at 12,000 × g for 3 min. The pellet was re-suspended by vortex in sterile deionized water and then boiled in 100°C for 5 min. After centrifugation at 12,000 × g for 2 min, the supernatants were collected. Aliquots of the sample were kept at -20°C until used for PCR reactions.

Multiplex PCR assays

The target genes selected for their characteristics were the *gyrB* (DNA gyrase subunit B) gene in *B. cereus*, the *invA* (invasion protein A) gene in *Salmonella* spp. and the *toxR* (toxin regulatory protein) gene in *V. parahaemolyticus*, all of which have been reported as the most specific and reliable genetic targets for these pathogens. The 16S rRNA gene was also targeted as an internal control of the presence of amplifiable bacterial DNA. The oligonucleotide primers used in this study are shown in Table 1. The multiplex PCR was performed in 25 µl reaction mixture containing 12.5 µl 2X KAPA2G Fast Multiplex Mix (Kapa Biosystems, MA, USA), 10 µM of each primer, and 4 µl of template DNA. Amplification was performed using the MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The cycling conditions were as follows: denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. The PCR products were then separated by electrophoresis on a 1.5% agarose gel alongside a 50 bp DNA ladder (Goldbio, St Louis, MO, USA), and visualized using Ecodye™ Nucleic Acid Staining Solution (Biofact, Daejeon, South Korea). Agarose gel electrophoresis was performed in 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM ethylenediaminetetraacetic acid, pH 8.4). The amplified DNA fragments were visualized under an ultraviolet transilluminator (AlphaImager HP, Santa Clara, CA, USA) and photographed using the gel documentation system.

Table 1 Sequences of oligonucleotide primers used in this study

Species	Sequence (5′→3′)	Target gene	Amplicon size (bp)	Reference
B. cereus	F= GAATTCCATTACGAAGGTGG R= CGACGTGTCAATTCACGCGC	*gyrB*	540	[1]
Salmonella spp.	F= ACAGTGCTCGTTTACGACCTGAAT R= AGACGACTGGTACTGATCGATAAT	*invA*	284	[16]
V. parahaemolyticus	F= TCATTTGTACTGTTGAACGCCTA R= AATAGAAGGCAACCAGTTGTTGAT	*toxR*	375	[10]
Bacterial DNA	F= AGAGTTTGATCATGGCTCAGG R= GGACTACCAGGGTATCTAATT	16S rRNA gene	720	[15]

bp, base pairs; F, forward primer; R, reverse primer

Results

Multiplex PCR protocol detection of food samples

The multiplex PCR assay for simultaneous detection of *B. cereus, Salmonella* spp., and *V. parahaemolyticus* was performed in 90 food samples collected from Pathumthani Province, Thailand. The multiplex PCR products were 540 bp for *B. cereus*, 284 bp for *Salmonella* spp., 375 bp for *V. parahaemolyticus* and an internal control of the presence of 720 bp for 16S rRNA of bacterial DNA, as indicated in Table 1. Each primer set was determined individually by using the DNA template purified from the reference strain to ensure its specificity. All pathogen-specific primer pairs generated a single PCR product with an expected product size, indicating species specificity of the used primers (Figure 2). Multiplex PCR was performed on the mix of DNA extracts from the three reference strains as shown in lane 5 of Figure 2. The results indicated that these three primer pairs in the multiplex PCR assay could distinguish the three pathogens from each other, demonstrating the specificity of this method. Then, all primer pairs and the multiplex PCR conditions were used in subsequent experiment.

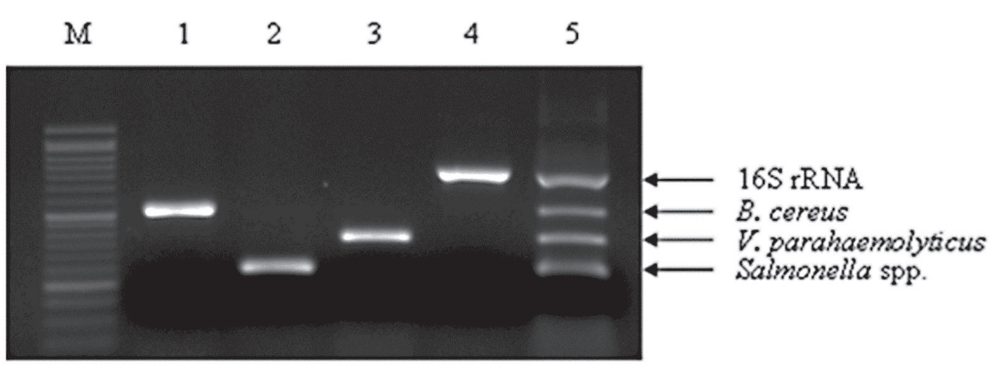

Figure 2 Agarose gel electrophoresis showing monoplex and multiplex PCR-amplified products. M, 50-bp DNA marker; lane 1: *B. cereus*; lane 2: *Salmonella* spp.; lane 3: *V. parahaemolyticus*; lane 4: 16S rRNA of bacterial DNA; lane 5: The multiplex PCR with four targets

Prevalence of *B. cereus, Salmonella* spp., and *V. parahaemolyticus* in food samples

The overall contamination rates of *B. cereus, Salmonella* spp. and *V. Parahaemolyticus* for various samples collected in this study were 87.8% (79/90), 4.4% (4/90) and 31.1% (28/90), respectively (Table 2). One or more of the bacterial pathogens were detected in 79 of 90 (87.8%) samples, with one pathogen detected in 48 (53.3%) samples, two pathogens detected in 30 (33.3%) samples, and three pathogens detected in one (1.1%) sample. Most of the food categories were contaminated with one species of pathogen except salads (Figure 3). Moreover, three food-borne pathogens were simultaneously detected in one salad sample. All food samples in the category of stir-fried vegetables were contaminated with at least one pathogen and all of them were positive for *B. cereus*. Approximately 22.2%, 18.2%, and 16.7% of salads, soups and curries, and miscellaneous foods, were negative for *B. cereus*, *V. parahaemolyticus*, and *Salmonella* spp. using multiplex PCR detection after 18h enrichment.

Table 2 Multiplex PCR results of three pathogens from food samples

Sample type	Number of samples	Number of positive (%)		
		B. cereus	*V. parahaemolyticus*	*Salmonella* spp.
Salads	36	28	15	1
Stir-fried vegetables	37	37	12	3
Soups and curries	11	9	1	0
Miscellaneous	6	5	0	0
Total	90	79 (87.8)	28 (31.1)	4 (4.4)

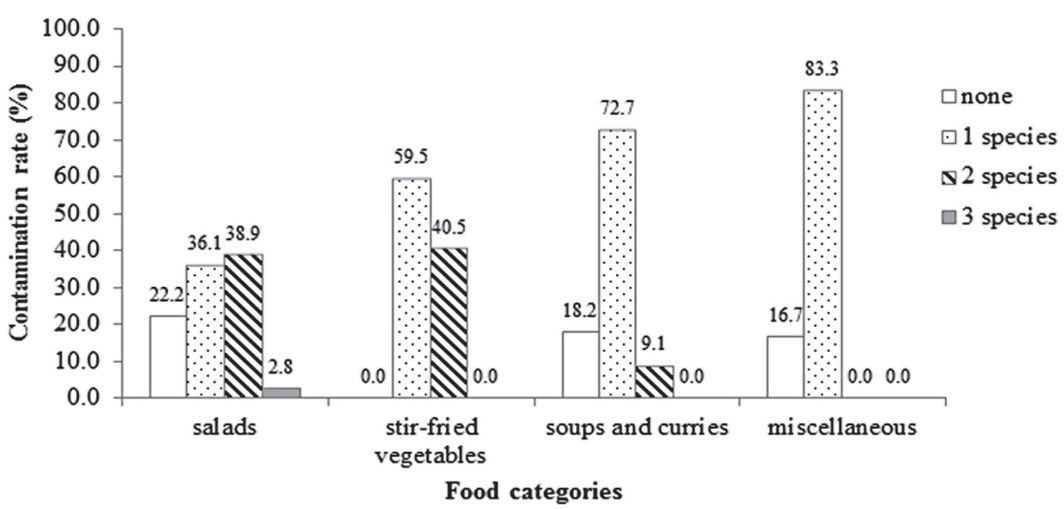

Figure 3 Prevalence of food-borne pathogens in salad, stir-fried vegetables, soups and curries, and miscellaneous food samples. 1, 2 and 3 species represents the number of food-borne pathogen species detected in food samples while none means no pathogen was detected.

Discussion

The multiplex PCR assay has largely been used to detect different species of food-borne pathogens. This method provides the detection of species that are present at low levels that can remain undetected by plating.[17, 18] Several multiplex PCR assays have been developed for simultaneous detection of food-borne pathogens.[1, 15, 19] Three bacterial pathogens composed of *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*, were identified by multiplex PCR.[15] The simultaneous detection of all three target strains could be achieved down to 10^2 CFU/ml. Moreover, this method could correctly identify the presence of the three food-borne pathogens in food samples those enriched in SEB medium for 12 h at the inoculated levels of 10^1 CFU/ml. A multiplex PCR for simultaneous detection of five food-borne pathogens including *Staphylococcus aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Salmonella* Enteritidis and *Shigella flexneri*, was developed.[19] The multiplex PCR developed was capable of detecting all five strains simultaneously in the spiked pork at the level of 10^1 CFU/g after 24h enrichment. Moreover, a multiple PCR assay was developed for simultaneous detection of six food-borne pathogens which were *E. coli* O157:H7, *B. cereus, V. parahaemolyticus*, *Salmonella* spp., *L. monocytogenes*, and *S. aureus* in artificially inoculated ready-to-eat food samples.[1] The assay was able to specifically simultaneously detect as low as 1 CFU/ml of each strain after enrichment for 12 h. Taken together, multiplex PCR assay was suitable for reliable and rapid detection of food-borne pathogens in contaminated samples. However, the limitation of PCR method is its inability to differentiate the DNA from viable and non-viable cells. Therefore, the DNA amplified directly from a food sample may not be a good indicator of the presence of viable pathogenic bacteria.

B. cereus can spread to many types of food products including meat, vegetables, grains and dairy products. As *B. cereus* spores are an integral component of soil microflora, they can easily contaminate cereal grain products. Moreover, this food-borne pathogen is able to attach to both meat surfaces and meat processing equipment.[20] In addition, *B. cereus* is capable to grow after cooking and heat treatment can induce spore germination. The prevalence of *B. cereus* in various food products such as herbs and spices, breakfast cereals, pasta, rice, pasteurized milk, and cheeses was reported to be 33.8% in Poland.[21] In this study, the contamination rate of *B. cereus* in food samples was approximately 87.8% which was higher than previous report in Poland. However, the incidence of *B. cereus* isolated from ready-to-eat food samples including béchamel sauce, bolognaise sauce and lasagna in Belgium was 70-81%.[22] Food sources of *Salmonella* included mainly eggs, meat, unpasteurized milk, cheese, contaminated raw fruits and vegetables, spices and nuts.[23] The prevalence of *Salmonella* spp. in food samples was 4.4% in this study which was lower than that in Southern Tunisia. Using the combined enrichment/real-time PCR method, *Salmonella* spp. was detected in 26.8%, 22.7% and 21.3% of cakes, dairy products, and cooked meals, respectively.[24]

V. parahaemolyticus is an important human pathogen which can cause food poisoning when consumed in raw or partially-cooked seafood or related products. The incidence of *V. parahaemolyticus* in seafood was found to be approximately 47.5% with the highest prevalence rate in oysters (63.4%) followed by clams (52.9%), fish (51.0%), shrimps (48.3%), and mussels, scallop and periwinkle (28.0%).[25] In this study, the prevalence of *V. parahaemolyticus* in food samples was approximately 31.1% (28/90). The contamination of *V. parahaemolyticus* in salads, stir-fried vegetables, and soups and curries,

was approximately 16.7, 13.3, and 1.1%, respectively. Generally, *V. parahaemolyticus* is contaminated in seafood and related products such as shellfish, fish, shrimp, crab, and lobsters. However, foodstuffs can be contaminated with the bacteria if it has not been properly cooked, handling or storage.[26]

The multiplex PCR assay described in this study can simultaneously detect three food-borne pathogens including *B. cereus*, *Salmonella* spp., and *V. parahaemolyticus*, in food samples. Our results demonstrated that three common food-borne pathogens are prevalent in foodstuffs in Pathumthani Province, Thailand. The use of rapid detection for routinely screening of bacterial pathogen in various food products may reduce the potential risk of food-borne disease outbreaks.

Acknowledgments

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Conflict of interest.

All authors report no conflicts of interest relevant to this article.

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บทคัดย่อ

การตรวจสอบเชื้อ *Bacillus cereus, Salmonella* spp. และ *Vibrio parahaemolyticus* ในอาหารโดยวิธีมัลติเพล็กซ์พีซีอาร์

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บทนำ: โรคที่เกิดจากอาหารเป็นสื่อเป็นปัญหาทางด้านสาธารณสุขที่สำคัญอย่างหนึ่งที่เกิดขึ้นทั่วโลก เชื้อ *Bacillus cereus, Salmonella* spp. และ *Vibrio parahaemolyticus* เป็นเชื้อแบคทีเรียที่พบได้บ่อยว่าเป็นสาเหตุของโรคนี้ การตรวจหาเชื้อที่เป็นสาเหตุของโรคที่เกิดจากอาหารเป็นสื่อจึงมีความสำคัญเพื่อช่วยยืนยันความปลอดภัยต่อผู้บริโภค

วิธีการศึกษา: การศึกษานี้ได้ใช้เทคนิคมัลติเพล็กซ์พีซีอาร์เพื่อตรวจสอบเชื้อ *B. cereus*, *Salmonella* spp. และ *V. parahaemolyticus* ในตัวอย่างอาหาร จำนวน 90 ตัวอย่างที่เก็บในจังหวัดปทุมธานี ประเทศไทย

ผลการศึกษา: อัตราการปนเปื้อนของเชื้อ *B. cereus, Salmonella* spp. และ *V. parahaemolyticus* ในตัวอย่างอาหารที่นำมาศึกษาในครั้งนี้เท่ากับ 87.8% (79/90), 4.4% (4/90) และ 31.1% (28/90) ตามลำดับ ตัวอย่างอาหารส่วนมากมีการปนเปื้อนเชื้อก่อโรคเพียงชนิดเดียวยกเว้นอาหารประเภทสลัด โดยที่อาหารประเภทสลัดที่มีการปนเปื้อนเชื้อก่อโรคอย่างน้อย 2 ชนิดมีเท่ากับ 41.7% และพบว่ามีอาหารประเภทสลัดที่ตรวจพบเชื้อก่อโรคทั้งสามชนิดจำนวน 1 ตัวอย่าง

สรุปผลงานวิจัย: การศึกษาครั้งนี้ชี้แนะว่าเทคนิคมัลติเพล็กซ์พีซีอาร์เหมาะสำหรับการทดสอบเชื้อแบคทีเรียก่อโรคในอาหารทั้ง 3 สายพันธุ์อย่างรวดเร็ว ทั้งนี้เพื่อช่วยลดโอกาสเสี่ยงการแพร่ระบาดของโรคที่เกิดจากอาหารเป็นสื่อ

คำสำคัญ: มัลติเพล็กซ์พีซีอาร์, จุลินทรีย์ที่ทำให้เกิดโรคจากอาหารเป็นสื่อ, บาซิลลัส ซีเรียส, ซัลโมเนลลา, วิบริโอ พาราฮีโมไลติคัส