Multiplex PCR assay and lyophilization for detection of *Salmonella* spp., *Staphylococcus aureus* and *Bacillus cereus* in pork products

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Abstract Multiplex PCR (m-PCR) has the potential for more rapid detection of pathogens compared to simple PCR through the simultaneous amplification of multiple gene targets using several sets of specific primers. Here, we developed an m-PCR assay which combined dry reagent mixtures for ready-to-use simultaneous detection of *Salmonella* spp., *Bacillus cereus*, and *Staphylococcus aureus*. The assay did not show cross-reactivity with several common bacterial pathogens and the detection limit was 10^3 CFU/mL for mixed genomic DNA in pure culture. Lyophilized m-PCR reagents are stable for 2 months stored at 4 °C and for 1 month stored at 25 °C. Detection sensitivities of both dry and fresh mixes were able to simultaneously detect 10 CFU/mL of each pathogen in artificially inoculated samples after enrichment for 6 and 12 h. Results demonstrated that this method is both sensitive and specific and can be used for rapid detection and differentiation of foodborne diseases.

Keywords *Salmonella* · *Bacillus cereus* · *Staphylococcus aureus* · Multiplex PCR · Lyophilization

Introduction

Salmonella spp., *Staphylococcus aureus* and *Bacillus cereus* are the most common causes of foodborne pathogens in Thailand and other countries, and raw food are the main vehicles of transmission to humans such as pork products [1–3]. *Salmonella* spp. is a member of the family *Enterobacteriaceae* which includes many of the major foodborne pathogens. The disease mainly caused by *Salmonella* spp. through consumption of contaminated eggs and poultry products is salmonellosis which can manifest as bacterial diarrhea through to septicemia [4]. *S. aureus* often enters food via food handlers, animal skin, or dirty food preparation surfaces. Some strains of *S. aureus* have the ability to produce staphylococcal enterotoxins through growth in food which results in vomiting and diarrhea (without fever) 4–12 h after consumption [5]. *B. cereus* may cause gastrointestinal diseases that result in diarrhea and emesis. An emetic (vomiting) type is produced by cells growing in the food and acts rapidly (0.5–6 h). The diarrheal illness can act for 6–14 h after ingestion [6, 7]; therefore, the development of a rapid and ready-to-use method for simultaneous detection of these pathogens is of great importance to improve food safety and protect human health.

Various methods have been developed to detect foodborne pathogens including the culturing and characterization of micro-organisms [8]. However, this is both time-consuming and laborious. Recently, molecular based detection methods have become popular for early diagnosis. Multiplex PCR (m-PCR) assay has the potential for considerable time-saving, with reduced reagent costs and effort without compromising the results [9]. The liquid reagent used in the m-PCR assay must be refrigerated and a thawing step is required prior to experimentation.

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Elimination of the need for reagent preparation steps increases the reliability and reproducibility of the assay, and also enhances stability for handling in tropical conditions. Recently, lyophilization of reagent master mix PCR has been developed for rapid and ready-to-use detection of *Mycobacteria* spp., *Salmonella typhi* and *Vibrio cholera* [10–12].

Here, we developed an m-PCR assay for simultaneous detection of *Salmonella* spp., *S. aureus* and *B. cereus* using a dry mix containing all the reagents required for the m-PCR assay, with cryoprotectants to preserve the activity of the enzymes. Selection of the additives was based on previously reports [10]. The dry-reagent master mixtures were evaluated and compared with freshly prepared master mixture for the detection of food matrices artificially inoculated with three pathogens. In addition, we validated our developed proof-of-concept a m-PCR with dry reagent assay by testing with pork products.

Materials and methods

Bacterial strains

The bacterial strains used in this study (Table 1) included 33 target bacterial strains and 33 other bacterial strains. *Salmonella* spp. (ATCC 13311), *B. cereus* (BCC 6386) and *S. aureus* (TBRC 4930) were used as standard strains to develop the m-PCR assay. For bacterial growth, *Vibrio* spp. strains were cultured in tryptic soy broth (BD, USA) supplemented with 2.5% NaCl at 37 °C for 16 h, 150 rpm, and the other bacterial strains were cultured in nutrient broth (BD, USA) at 37 °C for 14 h, 200 rpm.

DNA extraction

Following the manufacturer's instructions, genomic DNA templates were extracted from all culture strains using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan). The DNA pellet was dissolved in 50 µL of DNase-free water and DNA concentration and quality were measured by BioSpec-trometer kinetic (Eppendorf, Germany) at 260/280 and stored at − 20 °C until required for use.

PCR primers and design

A set of primers for m-PCR assay is shown in Table 2. Primers specific to the target gene were designed based on the *Salmonella* xcd gene, *B. cereus* hb1D gene and *S. aureus* vicK gene. To perform simultaneous detection, the primer sets for *B. cereus* (mp3L1R1F/R) and *S. aureus* (F-Sa5/R-Sa5) were obtained from Wehrle et al. [13] and Yu

et al. [14], respectively, while primer pairs for *Salmonella* (F-xcd/R-xcd) were designed according to a newer and more specific target of the xcd gene (GenBank accession no. CP016581.1) using Primer Premier 5.0 software (Premier Biosoft International), and tested for specificity by homology searches in the nucleotide database (NCBI, nucleotide BLAST). All primers used in this study were synthesized by Bio Basic Inc., Canada.

Multiplex PCR assay

The optimized m-PCR assay was performed according to the manual of the PCRBIO HS multiplex-PCR kit (PCR Biosystems Ltd, Royal college Street, London). The multiplex PCR reaction was carried out in a total volume of 50 µL containing 2X PCRBIO HS Taq Mix (HS Taq DNA polymerase, 6 mM MgCl₂ and 2 mM dNTPs), and four parameters were optimized individually including the final primer concentration for each target pathogen, annealing temperature, extension time and the cycling number. After optimization of the best amplification conditions were 0.14 µM of each primer and variable amounts of template DNA. The PCR condition consisted of an initial denaturation at 94 °C for 3 min, followed by 30 amplification cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) with the reaction terminated at 72 °C for 7 min. The product mixtures were analyzed by 2.5% agarose gel electrophoresis and visualized using a UV transilluminator.

Specificity testing of the m-PCR assay

The 66 bacterial strains (Table 1) including 33 target and 33 others were used to test the specificity of the m-PCR assay. The DNA templates isolated from the bacterial cultures described earlier were subjected to the optimum m-PCR assay obtained from 2.5% gel electrophoresis analysis.

Determination of detection limit of the m-PCR assay in pure culture

Simultaneous detection of the m-PCR assay was evaluated using the reference strains of *Salmonella* spp. (ATCC 13311), *S. aureus* (TBRC 4930), and *B. cereus* (BCC 6386) detected in pure culture and determined as previously described [15] with some modifications. Briefly, the bacteria were grown on tryptic soy agar (TSA) agar overnight, then small inocula were grown in tryptic soy broth (TSB; Difco) and incubated at 37 °C with shaking at 225 rev/min to obtain mid-log phase cells (OD_{600nm} = 0.5). Then, serial tenfold dilutions of the cultures were prepared in phosphate buffer saline solution (PBS). For DNA preparation, 100 µL of each bacterial dilution from pure culture were extracted

Table 1 Genomic DNA of bacterial isolates used for m-PCR assay in this study

Bacterial strains	Source	Multiplex PCR detection		
		<i>Salmonella</i>	<i>S. aureus</i>	<i>B. cereus</i>
<i>Salmonella enterica</i> strains (n = 27)				
<i>S. Typhimurium</i>	ATCC 13311	+	—	—
<i>S. Typhimurium</i>	RMUTTS-153	+	—	—
<i>S. Wadar</i>	RMUTTS-4	+	—	—
<i>S. Saintpaul</i>	RMUTTS-33	+	—	—
<i>S. Welterreden</i>	RMUTTS-71	+	—	—
<i>S. Risen</i>	RMUTTS-16	+	—	—
<i>S. Ohio</i>	RMUTTS-139	+	—	—
<i>S. Stanley</i>	RMUTTS-17	+	—	—
<i>S. Antum</i>	RMUTTS-35	+	—	—
<i>S. Brunei</i>	RMUTTS-141	+	—	—
<i>S. Corvallis</i>	RMUTTS-2	+	—	—
<i>S. ParatyphiB</i>	RMUTTS-155	+	—	—
<i>S. Mbandaka</i>	DMST 17377	+	—	—
<i>S. Mbandaka</i>	RMUTTS-47	+	—	—
<i>S. Hadar</i>	DMST 10634	+	—	—
<i>S. Hadar</i>	RMUTTS-11	+	—	—
<i>S. Hritlingfoss</i>	RMUTTS-75	+	—	—
<i>S. Agona</i>	RMUTTS-60	+	—	—
<i>S. Orion</i>	RMUTTS-144	+	—	—
<i>S. Livingstone</i>	RMUTTS-67	+	—	—
<i>S. Bardo</i>	RMUTTS-56	+	—	—
<i>S. Choleraesuis</i>	DMST 5580	+	—	—
<i>S. Dublin</i>	DMST 30404	+	—	—
<i>S. Enteritidis</i>	ATCC 13076	+	—	—
<i>S. Infantis</i>	DMST 26426	+	—	—
<i>S. Senftenberg</i>	DMST 17013	+	—	—
<i>S. Virchow</i>	DMST 17013	+	—	—
<i>Bacillus cereus</i> (n = 2)				
<i>B. cereus</i>	RMUTT-BC2	—	—	+
<i>B. cereus</i>	BCC 6386	—	—	+
<i>Staphylococcus aureus</i> (n = 4)				
<i>S. aureus</i>	TBRC 4930	—	+	—
<i>S. aureus</i>	RA 024122	—	+	—
<i>S. aureus</i>	RA 024123	—	+	—
<i>S. aureus</i>	RA 024121	—	+	—
Other strains (n = 33)				
<i>V. parahaemolyticus</i>	ATCC 17802	—	—	—
<i>V. mimicus</i>	DMST 22089	—	—	—
<i>V. vulnificus</i>	DMST 5258	—	—	—
<i>V. fluvialis</i>	DMST 24049	—	—	—
<i>V. fluvialis</i>	DMST 22085	—	—	—
<i>E. coli</i> 0157:H7	ATCC 35150	—	—	—
<i>E. coli</i>	RMUTTE-120116-4	—	—	—
<i>E. coli</i>	RMUTTE-120118-5	—	—	—
<i>E. coli</i>	RMUTTE-120113-4	—	—	—
<i>E. coli</i>	RMUTTE-120113-45	—	—	—
<i>E. coli</i>	RMUTTE-120201-4	—	—	—

Table 1 continued

Bacterial strains	Source	Multiplex PCR detection		
		<i>Salmonella</i>	<i>S. aureus</i>	<i>B. cereus</i>
<i>Enterobacter aerogenes</i>	DMST 1333	—	—	—
<i>Enterococcus faecium</i>	RMUTTEn-110909-8	—	—	—
<i>Enterococcus faecium</i>	RMUTTEn-110909-4	—	—	—
<i>Enterococcus faecium</i>	RMUTTEn-110825-4	—	—	—
<i>Enterococcus faecium</i>	RMUTTEn-110909-2	—	—	—
<i>Enterococcus faecalis</i>	RMUTTEn-110512-4.2	—	—	—
<i>Enterococcus faecalis</i>	RMUTTEn-110124-6	—	—	—
<i>Enterococcus faecalis</i>	RMUTTEn-110205-8	—	—	—
<i>Enterococcus faecalis</i>	RMUTTEn-120112-1	—	—	—
<i>Enterococcus faecalis</i>	RMUTTEn-120201-7	—	—	—
<i>Enterococcus faecalis</i>	RMUTTEn-110205-4	—	—	—
<i>Listeria monocytogenes</i>	ATCC 700402	—	—	—
<i>Listeria monocytogenes</i>	RMUTT-Lis	—	—	—
<i>Listeria ivanovii</i>	ATCC 700402	—	—	—
<i>Listeria innocua</i>	DMST 9011	—	—	—
<i>Listeria welshimeri</i>	DMST 20559	—	—	—
<i>Yersinia enterocolitica</i>	RMUTT-Yer	—	—	—
<i>Cronobacter sakazaki</i>	RMUTT-Cro	—	—	—
<i>Lactococcus lactis</i>	RMUTT-Lac	—	—	—
<i>Pseudomonas</i> sp.	RMUTT-Pse120111-5	—	—	—
<i>Pseudomonas</i> sp.	RMUTT-Pse100913-4	—	—	—
<i>Pseudomonas</i> sp.	RMUTT-Pse110427-1	—	—	—

ATCC American Type Culture Collection (Manassas, Va), DMST Department of Medical Sciences, Thailand, RA Clinical microbiology unit, Ramathibodi Hospital, Bangkok, Thailand, BCC BIOTEC Culture Collection, Pathum Thani, Thailand, TBRC Thailand Bioresource Research Center, Pathum Thani, Thailand, RMUTT Department of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathum Thani, Thailand

Table 2 Primers used in this study

Primer name	Sequence (5'-3')	Target Gene	Product size (bp)	References
F-xcd	CCACCCGGTCGCTGAAAAAC	xcd	117	This study
R-xcd	TGATACTGTGTCTGCGTCCCC			
mp3L1R1F	AGTTATTGCAGCTATTGGAGG	hb1D	148	Wehrle et al. [13]
mp3L1R1R	GTCCATATGCTTAGATGCTGTGA			
F-Sa5	AAAGGTGTAGGTTGAAAAGTAGAAG	vicK	176	Yu et al. [14]
R-Sa5	GTTACAGGCATTTTGTCTTTAGGTG			

using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit, as described above. Sensitivity tests were conducted in triplicate. The last dilution with all three samples that tested positive was considered as the detection limit.

In parallel with the latter, and to count the bacteria, 100 µL aliquots of appropriate dilutions were spread on tryptic soy agar (TSA) in duplicate and incubated overnight at 37 °C. The bacterial colonies were counted at the

dilution yielding 30–300 colony-forming units (CFUs), and then the CFU mL⁻¹ of bacterial suspension was calculated.

Dry m-PCR mixture preparation and stability determination of the lyophilized reagent

The determination of the optimum concentration of cryoprotectants was conducted as previously reported with

minor modifications [10]. In brief, the dry mixes contained 0.14 μM of each primer, 2X PCRBIO HS Taq Mix (HS Taq DNA Polymerase, 6 mM MgCl_2 and 2 mM dNTPs), 1–10% w/v of trehalose (Merck, USA) and distilled water in a total volume of 50 μL . The reagents were lyophilized for 4 h in Modulyo[®] Freeze Dryers (Thermo Electron Corporation, USA). The m-PCR reagent without cryoprotectants was also lyophilized as a negative control. After lyophilization, the dry reagent underwent m-PCR assay using fresh preparation mixtures with and without cryoprotectants. For reconstitution, 44 μL of distilled water was added into the tube to resuspend the lyophilized reagents in individual vials, with variable amounts of template DNA for multiplex PCR detection in a total volume of 50 μL . The result was analyzed by 2.5% agarose gel electrophoresis, and the optimum concentration of cryoprotectants was selected for further study.

The storage temperature of dry reagent has a great impact on its amplification effect and practical application. To determine their stability, the lyophilized reagents were stored at 4, 25 and 37 °C after preservation for 7, 14, 30 and 60 days. The reagents were tested for multiplex PCR detection and the products were analyzed by 2.5% agarose gel electrophoresis.

Evaluation of the assay using artificially contaminated food matrices

We evaluated the m-PCR assay for the detection of a target *Salmonella* spp., *B. cereus* and *S. aureus* using artificially contaminated food matrices. Briefly, Vietnamese pork sausage samples were purchased at a local market in Pathum Thani, Thailand. To confirm that the Vietnamese pork sausage samples were negative for *Salmonella* spp., *B. cereus* and *S. aureus*, a 25 g sausage sample was homogenized with 225 mL of sterile buffer peptone water (BPW), followed by isolation and identification of *Salmonella* spp., *B. cereus* and *S. aureus* by ISO6579:2002(E), BAM (Chapter 14) and BAM (Chapter 12), respectively.

The sensitivity of the m-PCR assay for simultaneous detection in spiked Vietnamese pork sausage samples was determined by using known amounts those bacteria as described above. Firstly, 90 mL of BPW was added to 10 g of Vietnamese pork sausage and homogenized thoroughly for 1 min using a stomacher (BagMixer 400P Lab Blenders, Interscience USA). Serial tenfold dilutions of the mid-log phase of each bacteria were prepared as described earlier. One milliliter of each dilution of these bacteria was spiked into 90 mL of each Vietnamese pork sausage homogenate. Then, each homogenate was incubated at 37 °C with shaking at 200 rev/min for 0, 6 and 12 h. After that, 1 mL of co-enrichment medium of each dilution was extracted by FavorPrep[™] Tissue Genomic DNA

Extraction Mini Kit, as described above. Two microliters of each supernatant were then used as a template DNA for m-PCR assay. Sensitivity tests were conducted in duplicate, also tested with dry reagent, and the last dilution with all two samples that tested positive was considered as the detection limit.

Validation of the developed multiplex PCR freeze drying assay for simultaneously detecting three foodborne pathogens in pork products

To validate multiplex detection using the m-PCR freeze dry assay, raw meat pork obtained from supermarkets ($n = 15$) and traditional market ($n = 15$) were used. The samples were tested by the conventional culture method as described above for each target pathogens. The multiplex PCR with dry reagent assay for the detection of *Salmonella* spp., *S. aureus* and *B. cereus* was evaluated with individual samples. A 10 g of each sample was transferred into a 90 mL BPW and blended for 1 min using a stomacher (BagMixer 400P Lab Blenders, Interscience USA). The homogenized samples were cultured at 37 °C for 6 h without shaking, and 1 mL supernatant was collected for DNA extraction and subjected to m-PCR with dry reagent assay. Afterwards, the number of positive results of each pathogens were compared for culture base method and freeze drying m-PCR system.

Results and discussion

Specificity of *Salmonella* spp., *S. aureus* and *B. cereus*

The expected amplification size of the newly developed multiplex PCR product was 117 bp for *Salmonella* spp., 148 bp for *B. cereus* and 176 bp for *S. aureus* reference strains (Fig. 1). When this PCR protocol was applied to examine all the test strains of these three species, a specific amplicon was also shown in each case (Table 1). A total of 27 *Salmonella* spp., 4 *S. aureus* and 2 *B. cereus* strains, were correctly identified, while no amplicon of specific size was observed for non-targets of the other bacterial species ($n = 33$) (Table 1). Non-specific amplicons of different size did not appear in any case. Previously, a specificity of 100% was reported in a singleplex PCR assay developed with primers mp3L1R1F/R for amplification of *B. cereus* [13], and primers F-Sa5/R-Sa5 for amplification of *S. aureus* [14]. Newer primers for *Salmonella* spp. with multiplex PCR were designed according to the new *S. enterica*-specific marker 3335471(SC9) that assured 100% inclusivity for 30 salmonella genomes and 100% exclusivity for 16 non-Salmonella enterica genomes by

Fig. 1 Result of agarose gel electrophoresis after single PCR and multiplex PCR. Lane M: 2 log DNA marker, Lane 1: 176 bp for *S. aureus*, Lane 2: 148 bp for *B. cereus*, Lane 3: 117 bp for *Salmonella* spp., Lane 4: multiplex PCR and Lane N: negative control

verification of the PCR assay [16]. Thus, the developed m-PCR assay was highly specific.

Sensitivity of the multiplex PCR

After culturing the three standard strains overnight on their corresponding culture media plates at 37 °C, the detection limit for pure cell cultures of *Salmonella* spp., *S. aureus* and *B. cereus* was 10^3 CFU/mL (Fig. 2). The detection limit of multiplex PCR in other studies varied widely, ranging from 10^3 to 10^5 CFU/mL. Yu et al. [14] determined the detection limit of m-PCR assay as 10^3 CFU/mL for *S. aureus*, *Salmonella* spp. and *L. monocytogenes*, while Silva et al. [17] recorded m-PCR detection sensitivity as approximately 10^3 CFU/mL for *Salmonella* Enteritidis and *Salmonella* Agona in phosphate buffered peptone water.

Fig. 2 The sensitivity of multiplex PCR assay for the simultaneous detection of *Salmonella* spp., *B. cereus* and *S. aureus* using 10-fold serial dilution of DNA extracted from a culture of 3 different bacteria gradiented from 10^8 –1 CFU/mL. Lane M: 2 log DNA marker and N: negative control

Moreover, a study by Taylor et al. [18] for multiplex detection assay identified *S. typhimurium* as 10^4 CFU/mL, *L. monocytogenes* as 10^3 CFU/mL, *C. jejuni* as 10^5 CFU/mL and *E. coli* O157:H7 as 10^4 CFU/mL. Consequently, our results were similar to previous reports.

Evaluation of the lyophilized reagents

Tests for the optimum cryoprotectant concentration, determined using 10 ng to 10 pg of each bacterial DNA (*Salmonella* spp., *S. aureus* and *B. cereus*) as templates, revealed that 5% w/v of trehalose gave a slightly better result (100 pg) by gel electrophoresis than 1 or 10% w/v (10 ng and 1 ng) [Fig. 3(B), (C), (D)]. However, the dry reagents without cryoprotectants showed no amplification [Fig. 3(A)]. Therefore, 5% w/v was chosen for the subsequent dry reagent of m-PCR assays. A previous study determined that the sensitivity test of a dry reagent triplex PCR assay was 100 pg of the genomic DNA in pure culture [12]. Thus, our development method also showed similar sensitivity.

To evaluate the stability of dry reagents for m-PCR assay, three different temperatures of 4, 25 and 37 °C and times of 7, 14, 30 and 60 days were tested with different combinations. The m-PCR assay produced the three expected bands for *Salmonella* spp., *S. aureus* and *B. cereus* after 30 days at 4 and 25 °C. Furthermore, the dry reagents produced similar results after 60 days at 4 °C (Table 3). However, amplification results of the m-PCR were not satisfied after preservation at 7 days at 37 °C and 60 days at 25 °C, respectively (Table 3). This approach has also been performed for *Mycobacteria* spp., *Salmonella typhi* and *Vibrio cholera* [10–12], and can, therefore, be applied to overcome local problems relating to transport, storage, cost and freeze-thaw issues.

Fig. 3 Optimization of trehalose using in the dry mixes for detection of multiplex PCR (m-PCR) using 10-fold serial dilution of DNA extracted from a culture of 3 different bacteria gradiented as template. A gel electrophoresis analysis of m-PCR performed with the addition of trehalose at concentrations of 0% w/v (A), 1% w/v (B), 5% w/v (C), 10% w/v (D). Lane M: 2 log DNA marker and N: negative control

Table 3 The sensitivity of dry reagents stored at 4, 25 and 37 °C

Storage Temp	Storage time (days)				
	0	7	14	30	60
4 °C	+	+	+	+	+
25 °C	+	+	+	+	—
37 °C	+	—	—	—	—

Detection of three food-borne pathogens in spikes samples

To determine the detection limit of the m-PCR assay for *Salmonella* spp., *S. aureus* and *B. cereus*, bacterial genomic DNA of spiked Vietnamese pork sausage samples were used in the m-PCR reaction. Based on the initial inoculum of three bacterial samples (10^9 CFU/mL), multiplex PCR products were analyzed using gel electrophoresis. Results showed that the detection limit of m-PCR for *Salmonella* spp., *S. aureus* and *B. cereus* was 10^5 CFU/mL [Fig. 4(A)] with no incubation of the spiked samples. For incubation times of 6 and 12 h the sensitivity was 10 CFU/mL [Fig. 4(B), (C)] (Table 4). These results were similar to detection using the dry reagents preserved for 30 days at 25 °C (Table 4).

Fig. 4 The limit detection of multiplex PCR assay (m-PCR) for the simultaneous detection of *Salmonella* spp., *B. cereus* and *S. aureus* with spiked vietnamese pork sausage samples at the initial inoculum different concentration of three bacterial. The result were tested with m-PCR assay for no-incubation (A), 6 h (B) and 12 h (C) after spiked samples. Lane M: 2 log DNA marker, Lane P: positive control, Lane No spike: non-spiked bacterial and Lane N: negative control

Table 4 Detection limit of DNA extracted from spike samples testing by the fresh m-PCR mixtures and Dry reagents mixtures

Time for incubation (h)	Preparations assay	
	Fresh m-PCR mixtures (CFU/mL)	Dry reagents mixtures (30 days at 25 °C) (CFU/mL)
0	10^5	10^5
6	10	10
12	10	10

For spiked Vietnamese pork sausage samples, the sensitivity of m-PCR assay after 6–12 h preincubation was comparable to that of the PCR assay for *S. aureus* in spiked Dairy Products as 10 CFU/mL [19], *Salmonella* spp. in spiked chicken meat and whole milk as 1–9 CFU/g [20] and m-PCR assay for *B. cereus* in three different spiked food matrices as 10 CFU/g [13]. Therefore, the m-PCR

Fig. 5 The results of the multiplex PCR with dry mixes for detection of *Salmonella* spp., *S. aureus* and *B. cereus* in raw pork meat samples from two types of market. Lane 1–15: obtained from traditional market, Lane 16–30: obtained from supermarket, Lane M: 2 log DNA marker, Lane P: positive control and Lane N: negative control

assay presented here was recommended as similarly sensitive with other PCR assays.

Comparison of simultaneously three foodborne pathogens detection using m-PCR freeze drying and culture base methods in pork products

A total of 30 raw meat pork from two types of market were tested by m-PCR with dry reagent assay and conventional culture base methods for detection of *Salmonella* spp., *S. aureus* and *B. cereus*. The m-PCR dry reagents system were used for this experiment by storing at room temperature for 30 days. The samples tested by the m-PCR with dry reagent assay, 13 of the 30 samples

(9 traditional market and 4 supermarket) (43.3%) were positive for *Salmonella* spp., 2 samples (only traditional market) (6.6%) were positive for *S. aureus*, and 1 sample (only traditional market) (3.3%) were positive for *B. cereus* (Fig. 5), in accordance with the results detected by culture methods (Table 5). The results demonstrated that the developed assay, was able to correctly identify the presence of the three food-borne pathogens including *Salmonella* spp., *S. aureus* and *B. cereus*, which are among the prevalent food-borne pathogens in several countries [1–3]. In contrast, the m-PCR with dry reagent assay in this study was more rapid, time-saving and cheaper than conventional culture base methods. Moreover, these assays also have the enhancement for ease of use and eliminates user error than conventional multiplex PCR assay.

Although the reagents are slightly more expensive, m-PCR dry reagents system offers considerable advantages over conventional m-PCR assay for application in resource-poor countries. The use of them, which are stable at room temperature for 1 month and 4 °C for at least 2 month, facilitates shipment and storage. Further, the reduced number of work steps decreases the performance time and minimizes the risk of contamination.

In conclusion, the multiplex PCR assay and dry mixes developed here represented the first assay for ready-to-use simultaneous detection of *Salmonella* spp., *S. aureus* and *B. cereus*. Lyophilization and artificially contaminated food testing results suggest that this assay will be a useful technique for *Salmonella* spp., *S. aureus* and *B. cereus* detection during reagent handling and storage. Furthermore, our method offers a valuable diagnostic tool for the rapid and sensitive detection of these three pathogenic bacteria in perishable foods such as Vietnamese pork sausage and pork products.

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Table 5 Comparison the result of standard culture method and freeze drying m-PCR assay for detection of *Salmonella* spp., *S. aureus* and *B. cereus* in raw pork meat samples

Market type	No. of samples	Number and (%) positive results					
		Culture methods			Freeze Dry m-PCR		
		<i>Salmonella</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>Salmonella</i>	<i>S. aureus</i>	<i>B. cereus</i>
Traditional market	15	9 (60.0)	2 (13.3)	1 (6.66)	9 (60.0)	2 (13.3)	1 (6.66)
Supermarket	15	4 (26.6)	0 (0.0)	0 (0.0)	4 (26.6)	0 (0.0)	0 (0.0)
Total	30	13 (43.3)	2 (6.6)	1 (3.33)	13 (43.3)	2 (6.6)	1 (3.3)

Agriculture and Food Research Organization (NAFR), Tsukuba, Ibaraki, Japan that provided the bacterial isolates RMUTT used in this study.

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