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Establishment of PMA Real-Time PCR Method to Detect Viable Cells of *Listeria monocytogenes* and *Salmonella* spp. in Milk and Dairy Products

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Abstract

The infection with *Salmonella* spp., *Listeria monocytogenes* are the most contamination events in the milk and dairy products. There are many disadvantages of conventional culture-based methods, which are still recognized as the "gold standard" for identifying pathogenic bacteria. Thus, the aim of current study was to develop reliable and rapid method for the detection of bacteria causing foodborne. For the establishment of PMA Real-time PCR method for co-detection of *Salmonella* spp., *Listeria monocytogenes*, bacteria strains, including *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 19115; ATCC 19111), *Salmonella enterica* (ATCC 19115), *Staphylococcus aureus* (ATCC 25923), *Vibrio parahaemolyticus* (ATCC 17802), *Shigella flexneri* (ATCC 12022), *Bacillus aureus* (ATCC 11778) were enrolled into the establishment of current protocol. The concentration of PMA, primer concentration, probe concentration, and primer annealing temperature, specificity and sensitivity were evaluated. As the results, we successfully established the protocol of PMA Real-time PCR for co-detection of *Salmonella* spp. and *Listeria monocytogenes* on milk product. The concentration of PMA was determined as 50 µM. The sensitivity of established protocol were 10^1 CFU/ml for detection of *Salmonella* spp., and 10^2 CFU/ml for detection of *Listeria monocytogenes*. The current PMA Real-time PCR protocol was applied to detect the contamination of twenty local milk samples. No sample was co-contaminated with *Salmonella* spp. and *Listeria monocytogenes*. These results were similar to its performed by conventional culture-based methods. In summary, the current established PMA (50 µM) Real-time PCR could be applied for the co-detection of *Salmonella* spp. and *Listeria monocytogenes* on milk and dairy food.

Keywords: Dairy Products, *Listeria monocytogenes*, PMA Real-time PCR, *Salmonella* spp.

1. Introduction

Milk and dairy products play an important role in human daily nutrition and diversifying the diet. However, milk and dairy products are rich in nutrients, delivering high-quality proteins, micronutrients, vitamins, and energy-containing fats, etc. However, it could be easily contaminated by spoilage microorganisms and foodborne pathogens from various sources, food processing as well as consumption. *Salmonella* spp., *Listeria monocytogenes* are the most frequently potential pathogens associated with milk and dairy products[10]. Salmonella spp. is reported to be the second most common bacterial cause of foodborne[1]. According to report of FAO and WHO, the infection of *Listeria* is a relatively are but serious disease with high fatality rates of 20%-30%. Thus, it is essential

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to develop reliable and rapid method for the detection of bacteria causing foodborne.

The conventional culture-based methods are still recognized as the "gold standard" for identifying pathogenic bacteria. However, this technique has many disadvantages, such as time-consuming, labor-intensities, and false-negative results due to non-culturable pathogens[4,11]. Thus a rapid, highly sensitive, and inexpensive detection technique is required. Real-time PCR technique have developed for routine analysis of pathogenic bacteria with many outstanding characteristics, such as faster and sensitivity was greater than conventional culture-based methods[6]. Especially, the real-time method could be applied to co-detection of multiple bacterial pathogens by a single of reaction conditions, still, limitation still exists. The false-positive results could be occurred because the real-time PCR method could not distinguish whether bacteria is alive or dead after the sterilization step of food processing due to the persistence of DNA after cell death[2]. To circumvent this problem, Propidium-Mono-Azide (PMA) was applied prior to PCR analysis to circumvent this problem, allowing discriminate alive/dead bacteria[8]. The dye of PMA can enter bacteria via the damaged cell membranes and covalently bind to genomic DNA[7]. As the results, the interaction between PMA and genomic DNA lead the inability to be amplified, thus preventing the detection of dead cells. PMA/Real-time PCR or PMA/PCR has been applied to detection of *Salmonella* spp và *Listeria monocytogenes* in food[5,7,9] successfully applied PMA Real-time PCR in detection of viable *Salmonella Typhimurium* in lettuce at as low as 10^2 CFU/mL in pure culture and 10^3 CFU/g in lettuce[5]. The sensitivity reached to 10^1 CFU/g in lettuce within the 12-h treatment with PMA. In the study of Yang *et al.* (2012)[12] reported that PMA-mPCR (multiplex PCR) can simultaneously identify *S. Typhimurium*, *Paratyphi* B and *Typhi* in food with 1×10^6 CFU/g in spiked food products[12]. PMA real-time PCR was concluded as the suitable technique for the co-detection and co-quantification of viable pathogens, including *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes*, in vegetable samples[3]. Although PMA real-time PCR or PMA PCR has been applied in detection of food borne pathogens from various sources, it is still scarce in milk and dairy product. Therefore, current study was carried out to establish the simple, efficient and economical PMA real-time PCR using FAM dyes-labeled probe for simultaneous detection and quantification *Listeria monocytogenes* và *Salmonella* spp. in milk and dairy products.

2. Materials and Methods

2.1 Bacterial Strains, Growth Condition

Bacterial strains: *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 19115; ATCC 19111), *Salmonella enterica* (ATCC 19115), *Staphylococcus aureus* (ATCC 25923), *Vibrio parahaemolyticus* (ATCC 17802), *Shigella flexneri* (ATCC 12022), *Bacillus aureus* (ATCC 11778), were purchased from Microbiologics Inc. (USA), grown on XLD Agar and BHI Agar, respectively, at 37°C for 24h.

2.2 DNA Isolation and Real-Time PCR Assay

DNA was isolated from pure cultures and food samples by using the commercial method Top PURE Genomic DNA/RNA extraction KIT (ABT, Vietnam) according to the guideline of manufacture. The primers and probes used in current were indicated in Table 1. For the detection and quantification *Listeria monocytogenes* và *Salmonella* spp., the protocol of real-time PCR assay was shown in Table 2.

Table 1. Probes and primers used in current study

Primers/ Probes	Sequences (5'-3')
Salmonella spp.	
Sal1598-F	AACGTGTTTCCGTGCGTAAT
Sal1859-R	TCCATCAAATTAGCGGAGGC
Sal1631-P	FAM-TGGAAGCGCTCGCATTGTGG-BHQ1
Listeria monocytogenes	
Lm835-F	AACTGGTTTCGTTAACGGTAAATACTTA
Lm998-R	TAGGCGCAGGTGTAGTTGCT
TxRd-P	FAM-CTACTACTCAA CAAGCTGCACCTGCTGC-BHQ1
Internal control	
dd-IAC-F	CTAACCTTCGTGATGAGCAATCG
dd-IAC-R	GATCAGCTACGTGAGGTCCTAC
dd-IAC-P	Hex-AGCTAGTCGATGCACTCCAGTCCT CCT-BHQ2

*Note: F: Forward primer, R: Reverse primer, P: Probe

Table 2. Thermal assay for real-time PCR

	Temperature (°C)	Time (seconds)
Step 1	95	300
Step 2 (x 40 cycles)	95	5
	60	30

2.3 Optimizing Primer Concentration, Probe Concentration, and Primer Annealing Temperature

To verify that optimizing the concentration of primers and probes, assay performance was tested at the primer concentrations of 200, 400, 600 nM, combined with each probe concentration of 50, 100, 150nM. The performance was carried on two concentrations, including 10^6 CFU/ml and 10^2 CFU/ml, of *Salmonella* spp. (ATCC 19115) and *Listeria monocytogenes* (ATCC 19115) (Replication: three times).

For optimizing the primer annealing temperature, the following temperatures, including 55°C, 58°C, 60°C, 62.5°C and 65°C, were tested on two concentrations of 10^6 CFU/ml and 10^2 CFU/ml, of *Salmonella* spp. (ATCC 19115) and *Listeria monocytogenes* (ATCC 19115) (Replication: three times).

2.4 Evaluation of Primers and Probes Specificity and Sensitivity

For the *in vitro* specificity validation, sets of primer and probe were tested by real-time PCR with target and non-target bacterial pathogens, including *Escherichia coli* (ATCC 25922), *Staphylococus aureus* (ATCC 25923), *Shigella flexneri* (ATCC 12022), *Bacillus aureus* (ATCC 11778), *Vibrio parahaemolyticus* (ATCC 17802), *Salmonella* spp. ATCC 19115; *Liseria monocytogenes* ATCC 19115.

For the *in vitro* sensitivity validation, mixture of 1 mL *Liseria monocytogenes* and 1 mL *Salmonella* spp. at following concentrations: 10^3, 10^2 and 10^1 CFU/mL were tested by real-time PCR.

2.5 Inactivation of Bacterial Cells

10^7 cells/mL *Salmonella* spp. or *Liseria monocytogenes* was heated at 100°C, 5 minutes or 90°C, 5 minutes to validate the optimized temperature for inactivation of bacterial cells. The resulting heat-treated was cooled to room temperature, then culturing on XLD Agar (for *Salmonella* spp. cultivation), BHI Agar (for *Liseria monocytogenes* cultivation) and incubated in the dark at room temperature for 24-48 hours.

2.6 Determination of the Optimized Concentration of PMA

PMA was examined at two following concentrations: 25 µM and 50 µM. Assay were carried on three groups of bacteria, including live bacteria, dead bacteria (heat-killed cells), and mixtures of live and dead bacteria by real-time PCR (treated/untreated with PMA). To investigate the optimal concentration of PMA, the value ΔCt was calculated according to ΔCt = Ct(PMA) – Ct(0). The optimal concentration of PMA was chosen by which the largest value of ΔCt had.

2.7 PMA Real-Time PCR Assay on Bacteria Strains and Artificially Bacterial Pathogens-Contaminated Food Samples

In order to determine whether PMA real-time PCR assay could specifically detect only alive bacterial cells but not dead bacterial cell (heat-killed cells), *Salmonella* spp. (ATCC 14028), or *Listeria monocytogenes* (ATCC 19111) at the concentration between 10^1 CFU/mL to 10^7 CFU/mL was treated with 50 µM PMA or untreated with PMA. Then, PMA-treated and PMA-untreated samples were processed identically for DNA extraction and real-time PCR.

The pasteurized milk samples, which were confirmed without contaminated by conventional culture-based method on XLD Agar and BHI Agar, were collected from local market. For artificial contamination, the pasteurized milk samples were artificially contaminated with *Salmonella* spp. (ATCC 14028), or *Listeria monocytogenes* (ATCC 19111) at the concentration of 10^1 CFU/ml and 10^7 CFU/ml. Extracted genomic DNA from PMA treat and non-treated was analyzed using PMA real-time PCR. The value ΔCt was calculated according to ΔCt = Ct(PMA) – Ct(0).

2.8 PMA Real-Time PCR Assay on Local Milk Samples

Total of 20 milk samples were tested in current study were analyzed for the presence and quantitative detection of *Listeria monocytogenes* và *Salmonella* spp. were collected in local market. The DNA extraction was performed

according to the guideline of TopPURE® Genomic DNA extraction kit. The experiments were conducted by two independent assays: 1. conventional culture-based methods; and 2. PMA real-time PCR assay (samples were treated and untreated with PMA 50 µM).

3. Results and Discussion

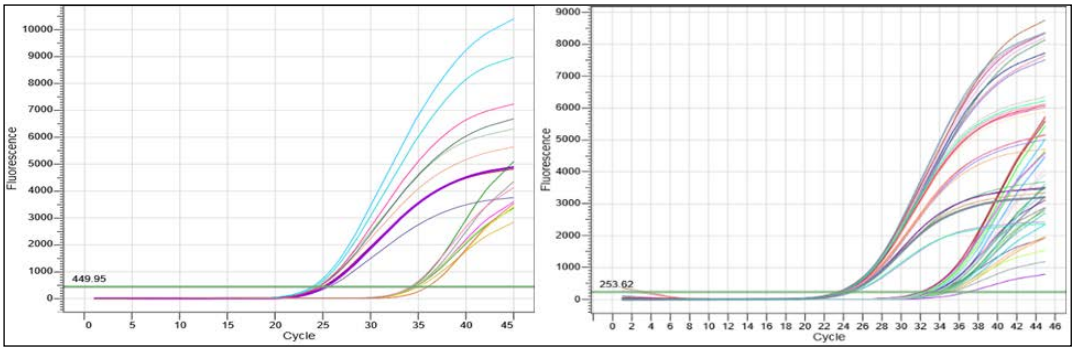
3.1 Evaluation of Primer, Probe Concentration, and Primer Annealing Temperature

There are no difference between the assay performances, which were tested at primer concentration of 200, 400, 600 nM, combined with each probe concentration of 50, 100, 150 nM. According to the primer concentration of 400 nM combined with the probe concentration of 100 nM, the Ct value of bacterial pathogens at density of 10^6 CFU/ml and 10^2 CFU/ml were 24.89 ± 0.06 and 34.92 ± 0.08 (for *Salmonella* spp.), and 24.01 ± 0.05 and 34.98 ± 1.16 (for *Listeria monocytogenes*), respectively (Figure 1). The optimal concentration of primers and probes were 400 nM and 100 nM, respectively.

There are no difference between the assay performances, which were tested at annealing temperature of 55°C, 58°C, 60°C, 62.5°C and 65°C. At the annealing temperature of 60°C, the Ct value of bacterial pathogens at density of 10^6 CFU/ml and 10^2 CFU/ml were 24 and 34 (for *Salmonella* spp.), and 24 and 33 (for *Listeria monocytogenes*), respectively (Figure 2). The optimal annealing temperature was 60°C.

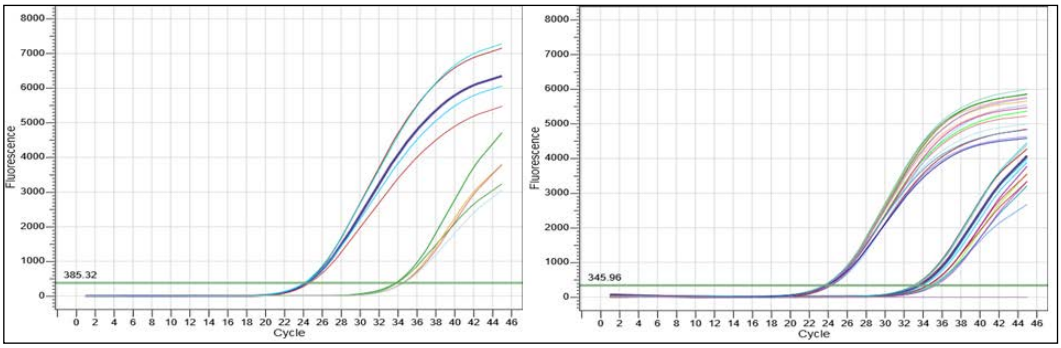
3.2 Evaluation of Specificity

No target fluorescence signal and the Ct of 25 (IAC) were observed in the control group. Target signal was not observed in the samples of ATCC. Only positive controls (*Salmonella* spp. ATCC 19115; *Liseria monocytogenes* ATCC 19115) were positive. Thus, it could be concluded the primers and probes were specific to *Salmonella* spp



(A) (B)

Figure 1. The amplification blot generated by qPCR at gradient concentration of primers and probesat bacterial pathogens density of 10^6 CFU/ml and 10^2 CFU/ml. (A) *Salmonella* spp. (ATCC 14028); (B) *Listeria monocytogenes* (ATCC 19111).



(A) (B)

Figure 2. The amplification blot generated by qPCR at gradient annealing temperature at bacterial pathogens density of 10^6 CFU/ml and 10^2 CFU/ml. (A) *Salmonella* spp. (ATCC 14028); (B) *Listeria monocytogenes* (ATCC 19111).

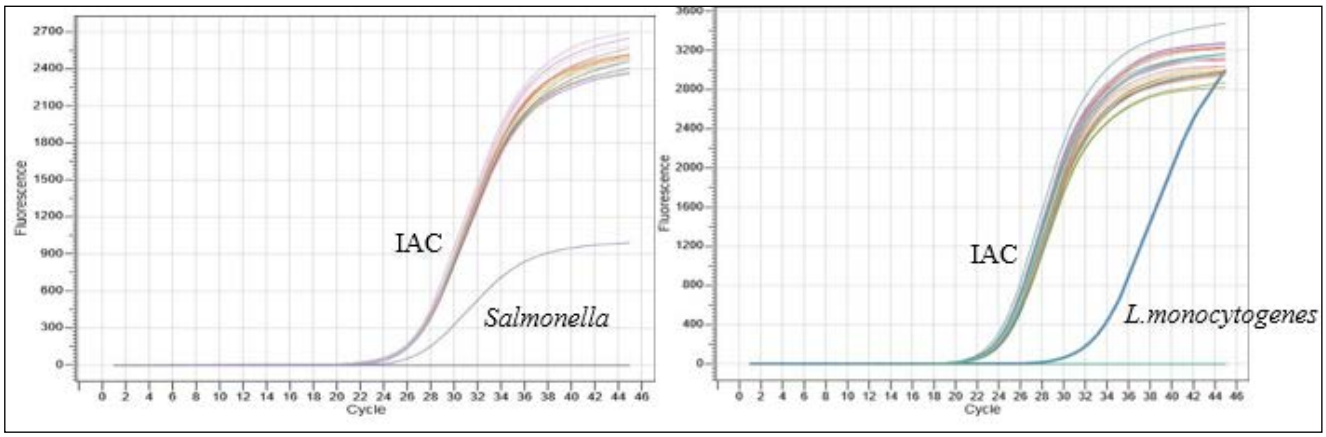
and *Liseria monocytogenes*, meant that did not pair with *E. coli, S. aureus, V. parahaemolyticu, Shigella flexneri* and *Bacilus* (Figure 3). Additionally, no cross-positive was observed in the strain of *Salmonella* spp. ATCC 19115; *Liseria monocytogenes* ATCC 19115.

3.3 Evaluation of Sensitivity

The result of evaluating sensitivity was performed in the sample of *Salmonella* spp. and artificial contaminated with *Salmonella* spp., were shown in Table 3 and Figure 4. As the result, the Ct of 37.11±0.45 và 34.76±0.77 were observed in in the concentration of 10^1 CFU/mlof the sample of *Salmonella* spp. and artificial contaminated with *Salmonella* spp., respectively. The positive result of artificial contaminated with *Salmonella* spp. of concentration of 10^1 CFU/ml was confirmed by conventional culture-based method on XLD Agar, as the result, it was similar to the PMA Real-time PCR method (Figure 4). Thus, the sensitivity of *Salmonella* spp. detection was 10^1 CFU/ml.

The result of evaluating sensitivity was performed in the sample of *Liseria monocytogenes* ATCC 19115 and artificial contaminated with *Salmonella* spp., were shown in Table 4 and Figure 5. As the result, the Ct of 37.17±0.41 và 37.71±0.66 were observed in the concentration of 10^1 CFU/ml of the sample of *Liseria monocytogenes* and artificial contaminated with *Liseria monocytogenes*, respectively. The positive result of artificial contaminated with *Salmonella* spp. of concentration of 10^1 CFU/ml was confirmed by conventional culture-based method on BHI Agar, as the result, it was similar to the PMA Real-time PCR method (Figure 5). Thus, the sensitivity of *Salmonella* spp. detection was 10^1 CFU/ml.

In the case of co-detection of artificial contaminated with *Salmonella* spp. and *Liseria monocytogenes*on the samples, the FAM green signal, which indicated to *Liseria monocytogenes*, and Texas red signal, which indicated to *Salmonella* spp., were observed (Figure 6). In detail, the Ct value were 32.13; 35.16 and 38.78 in the concentration of 10^3, 10^2 and 10^1 CFU/ml of *Salmonella* spp., respectively; the Ct value were 31.87; 35.97 and 37.15 in the concentration of 10^3, 10^2 and 10^1 CFU/ml of *Liseria monocytogenes*, respectively. Thus, the sensitivity of the codetection of *Salmonella* spp. and *Liseria monocytogenes* was 10^1 CFU/ml. This result was similar to Ding *et al.* (2017).

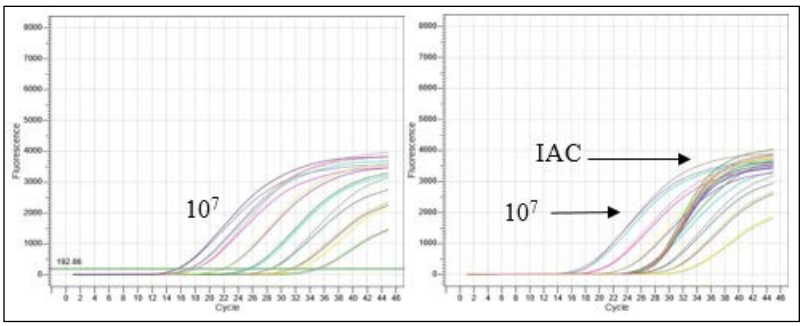


(A) (B)

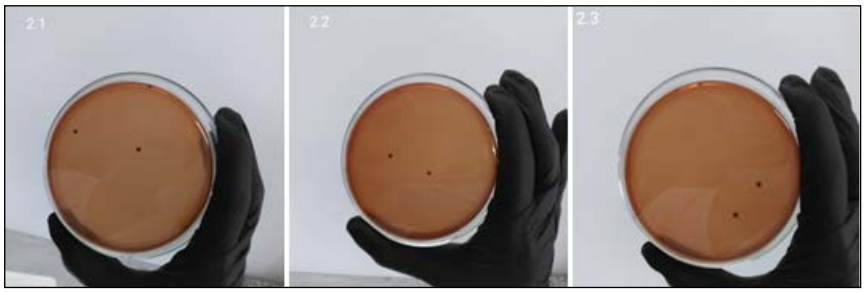
Figure 3. The amplification blot generated by qPCR at evaluation of the primers and probes' specificity. (A) *Salmonella* spp. (ATCC 14028); (B) *Listeria monocytogenes* (ATCC 19111).

Table 3. Results of PMA Real-time PCR on the samples of Salmonella spp. ATCC14028 and artificial contaminated samples

***Salmonella* spp. (CFU/ml)**						
10^7	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**
18.88±0.9	19.08±0.47	22.91±0.47	26.71±0.44	29.52±0.48	33.5±0.54	37.11±0.45
19.01±0.3	21.13±0.6	24.29±0.6	26.89±0.55	29.3±0.63	31.24±0.59	34.76±0.77

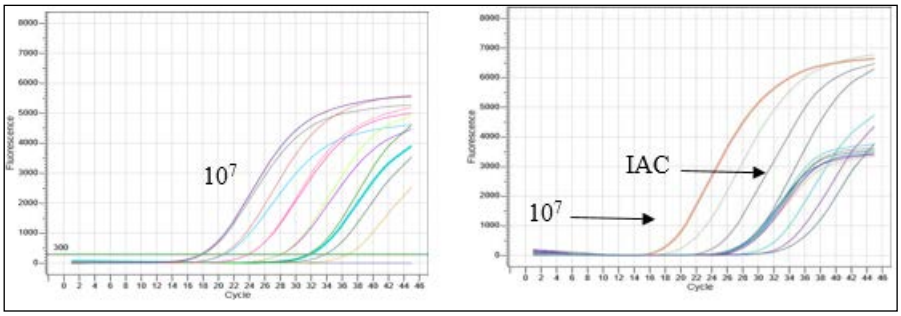


(A) (B)

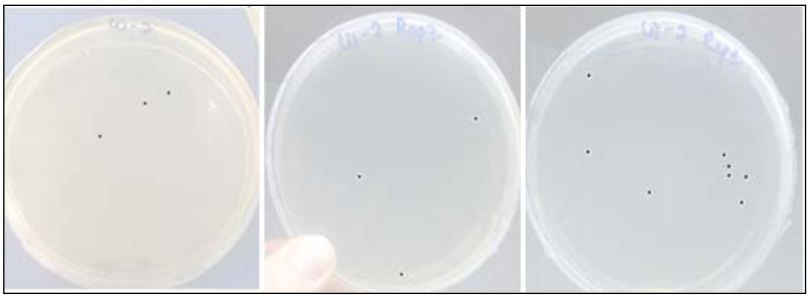


(C)

Figure 4. The amplification blot generated by Real-time qPCR at (A) *Salmonella* spp. ATCC14028; (B) artificial contaminated with *Salmonella* spp.; and (C) The conventional culture-based method on XLD Agar result of 10^1 CFU/ml of artificial contaminated with *Salmonella* spp.



(A) (B)



(C)

Figure 5. The amplification blot generated by Real-time qPCR at (A) *Liseria monocytogenes* ATCC 19115; (B) artificial contaminated with *Liseria monocytogenes*; and (C) The conventional culture-based method on BHI Agar result of 10^1 CFU/ml of artificial contaminated with *Liseria monocytogenes*.

4. Establishment of PMA Real-Time PCR to Co-Detect *Salmonella* spp. and *Liseria monocytogenes* on Milk

4.1 Evaluation of the Heat Inactivation of Bacterial Cells

The heat-treated *Salmonella* spp. or *Liseria monocytogenes* was incubated in the dark at room temperature for 24-48 hours. As the results, 90°C-treated *Liseria monocytogenes* formed colonies in culturing BHI Agar. 90°C-treated or 100°C-treated *Salmonella* spp. for 5 minutes, and 100°C-treared *Liseria monocytogenes* did not grow in the culturing Agar (Figure 7). Thus, the temperature 100°C was used for inactivation of both *Salmonella* spp. and *Liseria monocytogenes*.

4.2 Evaluation of the Concentration of PMA

Two concentrations of PMA 25 µM and 50 µM were examined at three groups of bacteria, including live

Table 4. Results of PMA real-time PCR on the samples of *Salmonella* spp. ATCC14028 and artificial contaminated samples

***L. monocytogenes* (CFU/ml)**						
10^7	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**
18.39±0.28	21.2±0.62	24.25±0.77	28,51±0.76	32.3±0.45	34,2±0.49	37.17±0.41
19.71±0.61	22.54±0.15	26.62±0.59	29,45±0.37	32.56±0.53	34,03±0.58	37.71±0.66

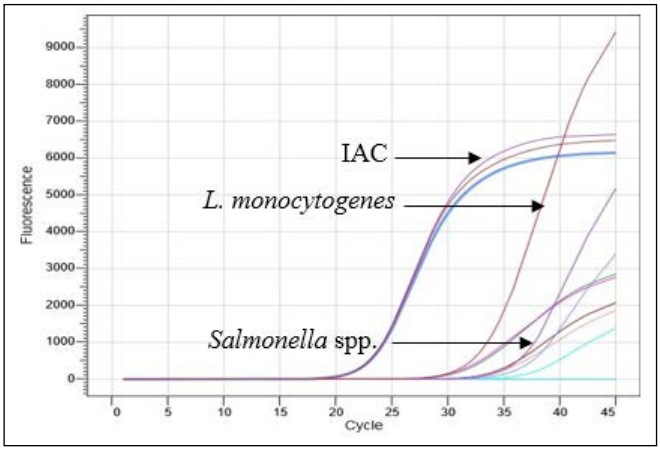
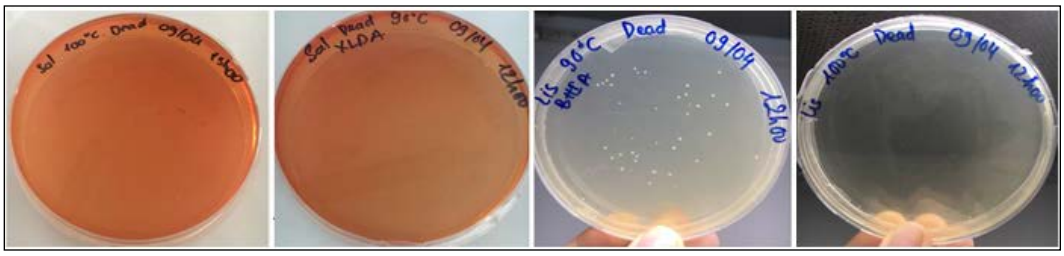


Figure 6. The amplification blot generated by real-time qPCR at the co-detection assay of artificial contaminated with *Salmonella* spp. and *Liseria monocytogenes* on the samples.



(A) (B) (C) (D)

Figure 7. The cultivation of heat inactivation of bacterial cells cultured based on conventional culture-based method on XLD Agar and BHI Agar. (A) 100°C-treated *Salmonella* spp.; (B) 90°C-treated *Salmonella* spp.; (C) 90°C-treated *Liseria monocytogenes*; and (D) 100°C-treated *Liseria monocytogenes.*

bacteria, dead bacteria (heat-killed cells), and mixtures of live and dead bacteria by real-time PCR (treated/untreated with PMA). As the results, both treated and untreated group gave the ΔCt< 1 in the group of live bacteria and mixtures of live and dead bacteria. In the group of dead bacteria, the treated and untreated group of the concentration of 50μM PMA gave the ΔCt (> 9: *Salmonella* spp.; >7: *Liseria monocytogenes*), which was larger than the ΔCt (>8: *Salmonella* spp.; >5: *Liseria monocytogenes*) given by the concentration of 25 μM PMA (Table 5). Thus, it could be concluded that the optimized concentration of PMA was 50μM, which could be used for distinguishing the dead and live bacteria.

Table 5. Results of the optimal concentration of PMA carried on three groups of bacteria

	0	25	50	ΔCt(1)	ΔCt(2)
Live					
Salmonella spp.	16.41	16.62	16.17	0.21	-0.24
Liseria monocytogenes	19.93	21.26	21.29	1,33	1.36
Dead					
Salmonella spp.	15.16	23.22	24.18	8.06	9.02
Liseria monocytogenes	21.26	26.63	28.63	5.37	7.37
Mix					
Salmonella spp.	15.88	15.57	16.82	-0.31	0.94
Liseria monocytogenes	22.39	22.69	23.38	0.30	0.99

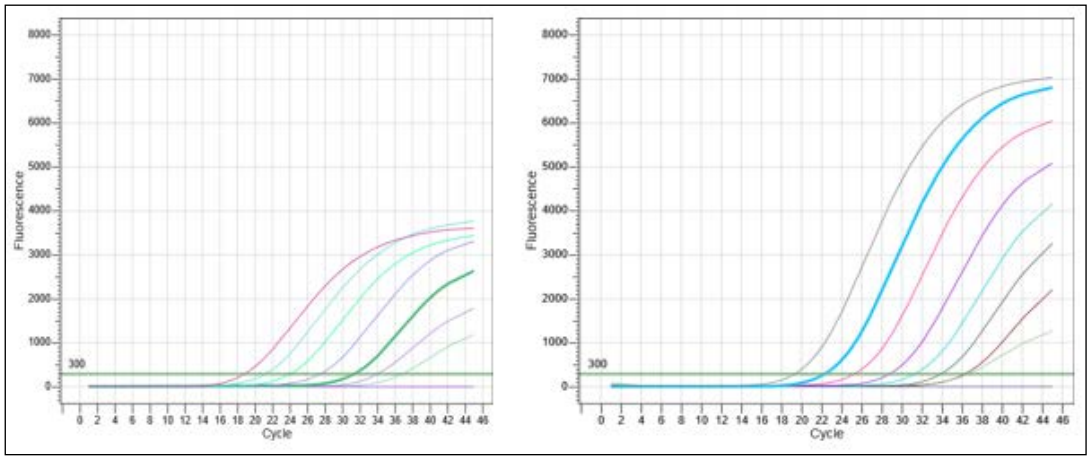
*Note: ΔCt(1) = Ct(25 μM)-Ct(0 μM); ΔCt(2) = Ct(50 μM)-Ct(0 μM)

4.3 Establishing PMA Real-Time PCR Assay

Results of PMA Real-time PCR assay on *Salmonella* spp., ATCC 19115 and *L. monocytogenes* ATCC 19115 was reported in Table 6 and Figure 8. Based on the value of ΔCt (ΔCt<1), it indicated that PMA did not effect on the genetic materials of live bacteria. The sensitivity of current protocol was 10^1 CFU/ml and PMA concentration was 50 mM. The sensitivity of the current protocol was higher than that of some previous studies[3,5,12].

4.4 Analysis of the Ability of PMA Real-Time PCR to Distinguish Live and Dead Bacteria

Concerning to the *Salmonella* spp. (live) and *L. monocytogenes* (Live), before and after PMA-treated sample gave the ΔCt value <1 Ct. these results indicated that PMA do not affect the live bacteria. In particular, no fluorescent signal was observed on the sample of *L. monocytogenes* with the concentration of 10^1 CFU/ml (Table 7 and Figure 9). In the sample of *Salmonella* spp. (Dead) and *L. monocytogenes* (Dead), before and after PMA-treated sample gave the ΔCt >8 and >6 (Table 7 and Figure 9). In the sample with the low concentration (< 10^4 CFU/ml), no fluorescent signals were after PMA-treated samples. These result indicated that PMA completely inhibited the Real-time PCR assay.



(A) (B)

Figure 8. The amplification blot generated by PMA real-time qPCR at gradient concentration from 10^7 CFU/mL to 10^1CFU/mL of (A) *Salmonella* spp.; and (B) *L. monocytogenes*.

Table 6. Results of real-time PCR on the sample of *Salmonella* spp., and *L. monocytogenes* with before and after treated with PMA

		10^7	10^6	10^5	10^4	10^3	10^2	10^1
Salmonella spp., (ΔCt<1)								
PMA (nM)	0	18.39	22.3	25.97	29.28	32.34	35.66	37.31
	50	19.16	21.54	24.41	27.87	31.7	34.36	37.14
L. monocytogenes, (ΔCt<1)								
PMA (mM)	0	18.13	20.94	24.29	28.45	31.99	34.12	37.17
	50	19.71	22.69	25.67	29.12	31.27	34.27	36.63

Table 7. Results of real-time PCR on the sample of *Salmonella* spp., (live, dead) and *L. monocytogenes* (live, dead) with before and after treated with PMA

		10^7	10^6	10^5	10^4	10^3	10^2	10^1
Salmonella spp., (live) (ΔCt<1)								
PMA (nM)	0	15.54	19.62	22.23	25.95	29.26	32.36	36.36
	50	17.40	20.95	24.08	27.55	31.08	34.49	37.85
Salmonella spp., (dead) (ΔCt>8-10)								
PMA (mM)	0	16.03	19.32	22.50	25.90	29.35	32.09	34.81
	50	30.97	27.41	30.73	34.94	(-)	(-)	(-)
L. monocytogenes (live) (ΔCt<1)								
PMA (mM)	0	17.01	20.59	23.69	27.02	29.85	32.68	35.24
	50	17.9	21.51	24.76	28.23	31.67	34.47	(-)
L. monocytogenes (live) (ΔCt>6-10)								
PMA (mM)	0	20.10	23.64	26.73	29.92	33.32	32.39	(-)
	50	30.66	35.53	34.20	35.58	(-)	(-)	(-)

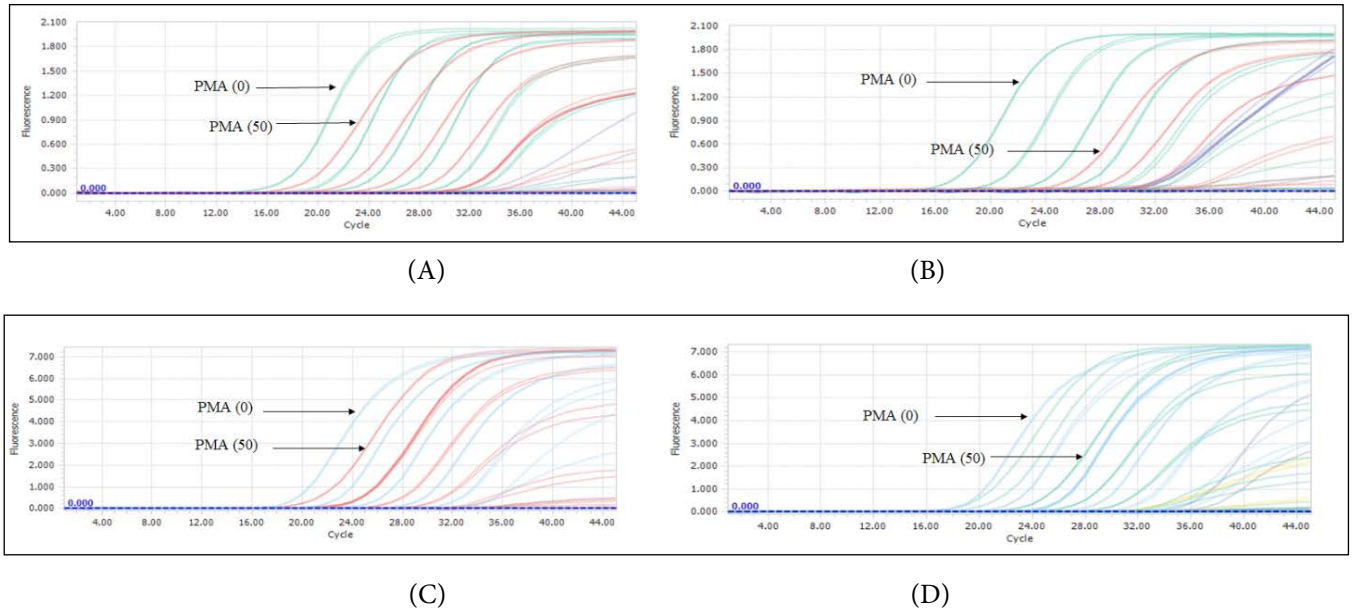


Figure 9. The amplification blot generated by PMA Real-time qPCR at gradient concentration from 10^7 CFU/mL to 10^1CFU/mL of (A) *Salmonella* spp. (live); (B) *Salmonella* spp. (dead); (C) *L. monocytogenes* (live); and (D) *L. monocytogenes* (dead).

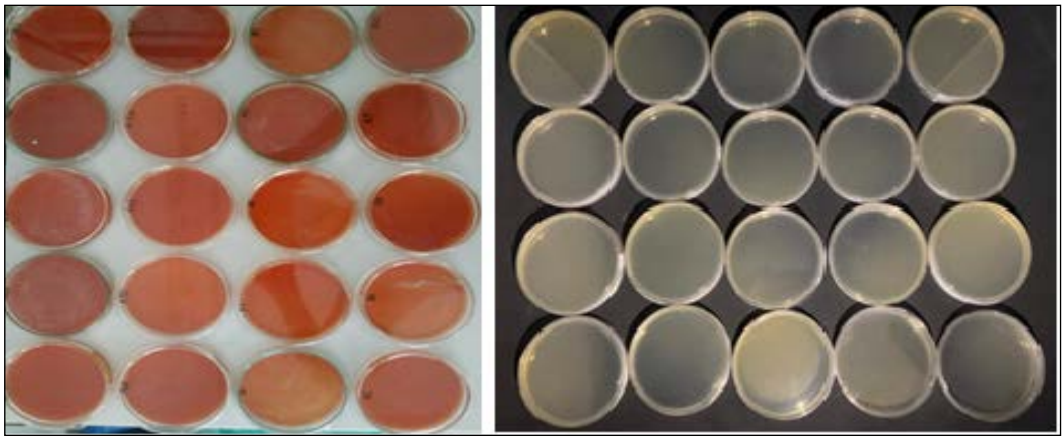


Figure 10. No colonies were observed on twenty local milk samples.

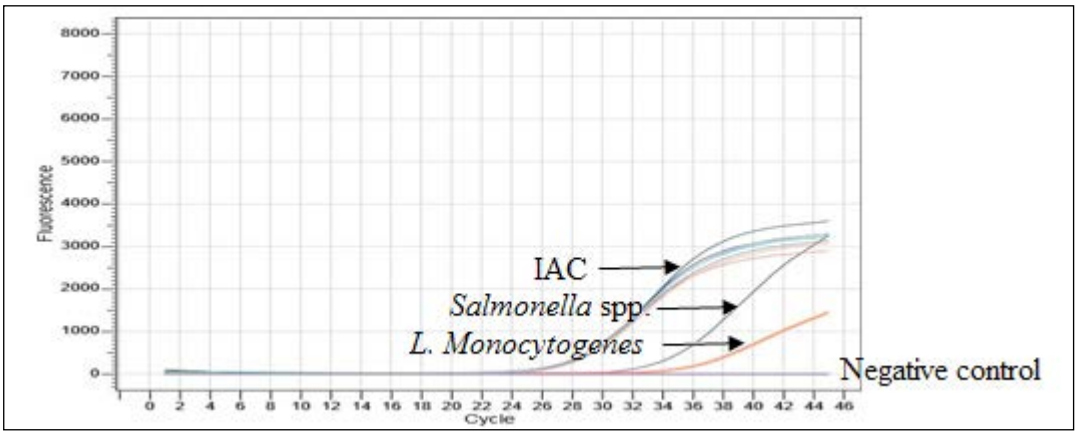


Figure 11. The amplification blot generated by PMA real-time qPCR at local samples.

Implementation on the artificial contaminated pasteurized milk, the results indicated that established PMA Real-time PCR could detect the *Salmonella* spp. Within the concentration of 10^1 CFU/ml and *L. monocytogenes* within the concentration of 10^2 CFU/ml. These results were the sensitivity of current assay was similar to the study of Li *et al.* (2013), and higher than the study of Liang *et al.* (2011)[5] and Yang *et al.* (2012)[12].

4.5 PMA Real-Time PCR Assay on Local Milk Samples

At first, twenty milk samples were collected in the local market then the samples were cultured on XLD Agar and BHI Agar to confirm whether or not contaminated with *Salmonella* spp. and *L. monocytogenes*. As the results, no colonies were observed on 20 local milk samples (Figure 10). It indicated that all of 20 samples did not contaminate with *Salmonella* spp. and *L. monocytogenes*.

Then, the PMA Real-time PCR assay was applied to detect the contamination of twenty local milk samples. As the results, the fluorescent signals were observed in the positive group, and no the fluorescent signals were observed in the positive group. Concerning to twenty local samples, no signals were records, meanwhile the signals of IAC were recorded at the Ct of 25 (Figure 11). Thus, it indicated that no contamination of *Salmonella* spp. and *L. monocytogenes* were detected in local samples. The results of PMA Real-time PCR were similar to its performed by conventional culture-based methods.

According to economic viability, this in-house multiple kit for co-detection of *Salmonella* spp. and *Listeria monocytogenes* on milk product will be at the price of 3.0 USD per test. Therefore, it facilitates the application

for the detection of these pathogens in the dairy products in developing countries.

5. Conclusion

We successfully established the protocol of PMA (50 μM) Real-time PCR for co-detection of *Salmonella* spp. and *Listeria monocytogenes* on milk product. The sensitivity of established protocol was 10^1 CFU/ml for detection of *Salmonella* spp., and 10^2 CFU/ml for detection of *Listeria monocytogenes*. The current PMA Real-time PCR protocol was applied to detect the contamination of twenty local milk samples. As the result, no sample was co-contaminated with *Salmonella* spp. and *Listeria monocytogenes*. These results were similar to its performed by conventional culture-based methods. In summary, the current established PMA (50 μM) Real-time PCR could be applied for the co-detection of *Salmonella* spp. and *Listeria monocytogenes* on milk and dairy food.

6. Declaration of Interest

None

7. Reference

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