Journal of Animal Research: v.13 n.05, p. 669-675. October 2023

DOI: 10.30954/2277-940X.05.2023.5

Selective Detection and Quantification of Viable *Bacillus cereus* by PMA qPCR from Ready-to-Eat Meat Products

S. Rathnapraba[1], B.J. Shathish Sharma[2], P. Kanagaraju[3]* and R. Narendrababu[4]

[1]Department of Veterinary Microbiology, Veterinary College Research Institute, Theni, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), INDIA
[2]Research Executive, Globion Pvt. Ltd., Hyderabad, INDIA
[3]Livestock Farm Complex, Veterinary College Research Institute, Theni, TANUVAS, INDIA
[4]Department of Meat Science and Technology, Madras Veterinary College, Chennai, TANUVAS, INDIA

**Corresponding author: P Kanagaraju, E-mail: kanraj2007@gmail.com*

Received: 24 July, 2023 **Revised:** 02 Sept., 2023 **Accepted:** 17 Sept., 2023

ABSTRACT

Bacillus cereus is one of the common causative organisms causing major foodborne outbreaks. Quantitative polymerase chain (qPCR) reaction could detect *Bacillus cereus* contamination in food samples, but both the viable and dead bacteria are detected. However, only viable bacterial pathogens might seriously jeopardize the safety of food. Therefore, in this study we used Propidium monoazide (PMA) based qPCR to detect and quantify the viable food borne bacterial pathogen, *Bacillus cereus* in ready to eat meat products. As a reference strain, Bacillus *cereus* (ATCC 117783) was used for preparation of viable and dead cells from artificially spiked meat samples. DNA was extracted from PMA treated and untreated samples, subjected to PMA based qPCR and conventional qPCR using fem A gene specific primers designed in the study. Further, meat samples (n=50) were subjected to qPCR and PMA based qPCR for viable bacterial pathogen detection. Among the 50 samples screened, 16 samples were positive for PMA qPCR with a detection range of > 10^3 CFU/ml, whereas in conventional qPCR, 27 samples were positive. The more positivity in conventional qPCR is due to amplification of both live and dead bacteria from the meat products screened. In comparison to qPCR, less than half of the samples were amplified by PMA qPCR indicating detection of only viable bacteria in the samples screened, thus eliminating false positive results. Thus, culture independent PMA based qPCR may be useful for rapid and selective detection of *Bacillus cereus* that could aid in reliable risk assessment in ready to eat meat products.

HIGHLIGHTS

- Detection of viable food borne *Bacillus cereus* from ready to eat meat products using Propidium monoazide based qPCR targeting fem A gene.
- PMA treated qPCR samples resulted in 32% positivity, at a range of >10^3 CFU/ml that are unfit for consumption.

Keywords: *Bacillus cereus,* Fem A gene, Propidium monoazide, Quantitative PCR, meat products

Foodborne illness is an ever-present and serious threat to public health globally. Among the various pathogens, viz., *Salmonella sp., Staphylococcus aureus, Campylobacter sp., Listeria sp., Escherichia coli,* and *Bacillus cereus,* have been incriminated for a large majority of foodborne illnesses. *Bacillus cereus,* being a Gram-positive, facultative anaerobic, spore-forming ubiquitous bacteria, causes spoilage in food and ready-to-eat food products. Generally, ready-to -eat meat products are consumed as such, highest incidence of *Bacillus cereus* has been found in ready-to-eat meat products and frozen meat (Mira and Abuzied, 2006). Hence, a rapid, reliable, and internationally accepted methods for detection of foodborne pathogens are of utmost importance for diagnostics and monitoring of

How to cite this article: Rathnapraba, S., Shathish Sharma, B.J., Kanagaraju, P. and Narendrababu, R. (2023). Selective Detection and Quantification of Viable *Bacillus cereus* by PMA qPCR from Ready-to-Eat Meat Products. *J. Anim. Res.,* **13**(05): 669-675.

Source of Support: None; **Conflict of Interest:** None

food hygiene in the food industry (Garrido *et al.*, 2013). Currently, *Bacillus cereus* is traditionally evaluated in food using culturing methods, which can be laborious and time-consuming and may also lead to misidentification or underestimation of the pathogen (Anita and Abdulla, 2015; Cattani *et al.,* 2016). Alternatively, molecular-based techniques have extensively been applied to detect foodborne pathogens, as reported by various researchers (Fernández-No *et al.,* 2011; Elizaquivel *et al.,* 2011; Martinez-Blanch, *et al.,* 2011; Garrido *et al.*, 2013). But the major limitation of an endpoint or real-time PCR-based assay is its inability to distinguish between DNA from viable and non-viable bacterial cells (Elizaquivel *et al.,* 2012). To overcome these issues, various researchers used Propidium monoazide (PMA) based qPCR, which was found to be more efficient in DNA amplification of viable cells, thereby inhibiting DNA amplification from dead cells (Nocker *et al*., 2007; Cattani *et al.,* 2013*;* Zhang *et al.,* 2014a; and Udomsil *et al.,* 2016). To the best of our knowledge, there is limited research on PMA q-PCR based selective quantification of *Bacillus cereus* contamination from ready-to-eat meat products targeting the Fem A gene. Hence, we developed and evaluated PMA-based q-PCR for the selective detection and quantification of viable *Bacillus cereus* bacteria from ready-to-eat meat products that would be a reliable rapid diagnostic tool in monitoring food contaminants.

MATERIALS AND METHODS

Bacterial Strain and culture conditions

The *Bacillus cereus* (ATCC 117783) reference strain was procured from Himedia Laboratories India Pvt. Ltd., Mumbai. The reference strain was cultured in Luria Broth (LB) and incubated at 37 °C in an aerobic atmosphere for 24 hrs. The viable *Bacillus cereus* cell count was determined by serial 10-fold dilutions and 100 µl of cell suspensions were plated on the Muller Hinton agar and incubated at 37 °C for 24 hrs.

Preparation of viable and dead cells

The colony forming unit of *Bacillus cereus* (ATCC 117783) is calculated by conventional culture method which resulted in 2.88×10^{11} CFU/ml. This well-known concentration of *Bacillus cereus* (ATCC 117783) was used for the preparation of viable and dead cells. For dead cells, 1 ml of overnight culture was collected in five separate centrifuged tubes: 1, 2, 3, 4, 5. From the 1st sample, 100 µl was aliquoted in to another 500 µl tube, followed by 200 µl, 300 µl, 400 µl and 500 µl from the 2nd, 3rd, 4th and 5th tubes respectively. The collected samples were kept in a heating block for 20 min at 85°C then the samples were transferred back in to their respective tubes and used for further studies.

Artificial contamination of samples for both PMA treatment and untreatment

Colony Forming Unit was calculated by a conventional culture method. The meat samples were collected aseptically and exposed to UV for 1 h. One gram of each sample was spiked artificially with 100 µl of overnight ATCC cultures with different dilutions based on their CFU and kept undisturbed for 30 min. To each 1g sample, 9 ml of BPW was added and homogenized using mortar and pestle. The rinsed solution was centrifuged at 800g for 10 min at 4°C to remove food debris, and the supernatant was incubated at 37°C for 6 hrs in a shaker incubator. These samples were further utilized for PMA treatment and the un-treatment process.

PMA treatment and its efficiency

PMA (BLU V viability kit (Cat No. 296015) was diluted as per the manufacturer's instructions to obtain a 2.5 mM concentration, and stored at 4°C. 500 µl of samples were transferred into fresh tubes and centrifuged at 1000 rpm for 3 min. The supernatant was discarded then 500 µl of elution buffer was added and mixed by vortexing. To this 10 µl of PMA was added and mixed well by inverting the tubes and incubated at dark for 10 min at room temperature. Further, the tubes were transferred into the photolyzer for the photo activation at 100 intensity for 20 min. and used in this study. The detection sensitivity and efficiency of the PMA treatment was studied by qPCR amplification after DNA extraction.

DNA extraction

The PMA treated samples were centrifuged at 10000 rpm for 3 min, the supernatant was discarded. DNA was

extracted by boiling method, (Kwasaki *et al.,* 2005 and the concentration was determined by using a spectrometer (Eppendorf Bio Photometer Plus). The ratio of the absorbance at 260/ 280 nm was observed. Finally, the DNA was stored at -20°C for the following experiments.

Primers designed in the study for qPCR

The qPCR primers were designed using beacon designer software by targeting the fem A gene as Forward primer 5'TTCGGCTCCACCTGTTATG3' and reverse primer 5'TCGCATCTCCACCTAATACG 3' with an amplicon size of 200 bp. The primers were standardized by conventional gradient PCR using the *Bacillus cereus* ATCC reference strain DNA as a template. The PCR reaction was carried out in 20 µl reaction with 10 µl of Master mix (2X amplicon) 1 µl of each forward and reverse primer with 10 pico moles, and 1 µl of template DNA and 6 µl of nuclease free water. The reaction was carried out under the following conditions, 94 °C for 5 min, 94°C for 1 min. gradient temperature for 30 sec followed by 72°C for 30 sec and 72 °C for 5 minutes. The primers were optimized at 55°C and used for both qPCR and PMA qPCR.

Real time PCR 7500 (Applied Biosystems USA) was used for amplifying a total 10 µl of reaction mixture in duplicate containing 5µl of SYBR green master mix and 0.5 µl of each forward and reverse primers with 10 pico moles, 1 µl of template DNA and 3 µl of Nuclease free water. The real time PCR programme conditions, 94 °C for 5 min followed by 40 cycles 94°C for 30 sec 55 °C for 30 sec and 72 °C for 32 sec. was followed and the results were analysed.

Construction of standard curve

A standard curve was generated using ten-fold serial dilutions of viable *Bacillus cereus* (ATCC 117783) strain of a known colony - forming unit. After the PMA treatment, DNA was extracted, qPCR was performed. Similarly, for PMA untreated samples, DNA was extracted for qPCR analysis. The standard curve was plotted for the different concentrations against the Ct values obtained. Slope was obtained at the given range and the Ct values obtained by qPCR and PMA-qPCR were used to calculate the CFU/g present in each sample based on the standard curve analysis. The viable foodborne *Bacillus cereus* was calculated based on the linear regression equation obtained from the standard curve analysis.

Detection of viable *Bacillus cereus* in ready to eat meat and meat products by PMA qPCR

A total number of 50 meat products (including raw meat, khabab, cubes, kheema, sausage, nuggets, pakkoda, cutlet, ham, beacon, meat balls and patties) of chicken, mutton, beef and pork products. These samples were procured from different retail markets in and around Chennai. One gram of each sample was added to the 9 ml of buffered peptone water and homogenized in mortar and pestle. The rinsed solution was centrifuged at 800g for 10 min at 4°C to remove food debris, and the supernatant was incubated at 37°C for 6 hrs in a shaker incubator. Further, 2ml of samples were pelleted at 10000 g for 10 min, processed for PMA treatment as described earlier and subjected to PMA qPCR and qPCR.

RESULTS

PMA treatment and its efficiency

Final concentration of 2.5 mM PMA was used to stain the cells. DNA from the dead cells were not stained with the PMA will generate false positive result in realtime PCR with a Ct values which confirms the fact that the DNA persist after the cell death cannot able to differentiate between the live and dead cells in PCR and qPCR. By staining the cells with 2.5mM PMA will inhibit the amplification of dead cells.

To test the efficiency of the PMA treatment *Bacillus cereus* ATCC strain was heat treated (to kill) with the known CFU in different volumes, dead cells didn't show amplification signals where the 500 µl of dead cells shows 26. 9 Ct value and 200 µl of dead cells shows 23.64 Ct value, where in 10^{10} dilution shows 25.56 and 24.43 Ct value in PMA treated and untreated respectively. Similarly, in 10^{2} dilution shows 27 and 25.9 Ct values in both the treated and untreated culture. This confirms that the PMA could penetrate only the dead cell and inhibit the amplification. This shows that the Ct values generated from PMA treated viable cells shows higher than those generated from the untreated samples.

Standard curve analysis for PMA based qPCR absolute quantification

A standard curve was generated using ten-fold serial dilutions of viable *Bacillus cereus* (ATCC 117783) strain of known colony forming unit. The standard curve for DNA extracted from PMA treated and untreated samples showed a strong linear correlation (R^2 = 0.9986 and R^2 = 0.9904) in the range from 10^2 to 10^{10} colony forming units per reaction. A slope value of -0.306 and -0.183 for PMA treated and untreated was obtained within a reasonable theoretical range (Fig. 1). The standard curve analysis was also performed for known viable and dead cells concentration obtained by heat treatment, the correlation coefficient (R2) was found to be 0.9708 and the slope value was obtained at a range of 0.0115 as shown (Fig. 2). The viable foodborne *Bacillus cereus* was calculated based on the linear regression equation obtained from the standard curve analysis.

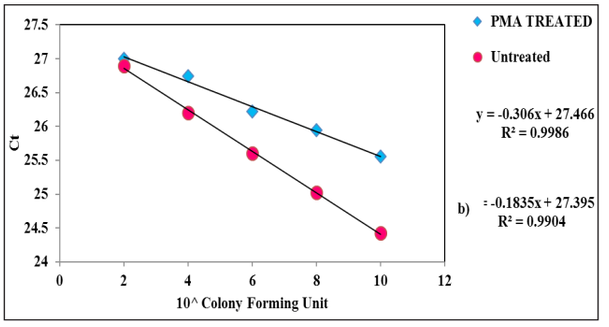

Fig. 1: Correlation of *Bacillus cereus* (ATCC 11783) counts obtained by qPCR (a) with PMA treatment and (b) without PMA treatment

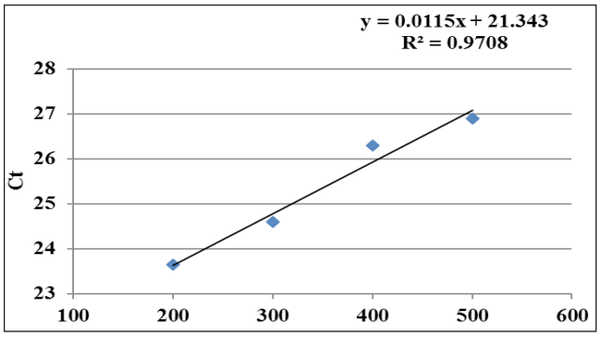

Fig 2: Correlation of *Bacillus cereus* (ATCC 11783) counts obtained by heat treated samples

Detection of viable *B. cereus* in meat products

A total number of 50 samples includes 25 chicken products,5 mutton products, 10 pork products and 10 beef products were screened for the presence of viable *Bacillus cereus* by treating the samples with the PMA. Among these 50 samples, includes 25 chicken, 5 mutton, 10 pork and 10 beef ready to eat products that were screened for the presence of viable *Bacillus cereus* by PMA qPCR. The real time melting curve and amplification plot of *Bacillus cereus* quantification were depicted in Fig. 3. Among the 50 samples screened, only 16 showed positivity by PMA qPCR with a count of $>10^3$ CFU/ml. The more positivity in the conventional qPCR may be due to amplification of both live and bacterial cells being amplified by qPCR thus eliminating false positive results.

DISCUSSION

Viable foodborne pathogens alone can retain pathogenicity and could impose major threat to food safety (Xiao *et al.,* 2015). Conventional PCR would amplify DNA from both dead and live bacteria whereas PMA based q PCR would amplify only viable cells and dead cells inhibit amplification. Propidium monoazide has been used for selective detection of viable *Escherichia coli* O157:H7, *Campylobacter and Bacillus cereus* from various food samples (Josefesen *et al.,* 2010, Elizaquivel *et al.,* 2012a and Zhang *et al.,* 2014a). PMA is a DNA- intercalating dye that penetrates dead cells with compromised cell integrity (Chang *et al.,* 2010). Upon intercalation into the DNA of the dead cells, photoinducible azide group inverts to highly reactive nitrene radicals and readily cross linked with a hydrocarbon near the binding site of DNA to form stable covalent nitrogen bond resulting in the cross-linked DNA strands preventing PCR amplification in dead cells (Nocker and Camper, 2009).

In earlier studies, Rizzotti *et al*., 2015 utilized PMA q-PCR Ct values and standard curve analysis for quantification of microorganisms. In this study *Bacillus cereus* ATCC 117783 was used as positive control for constructing the standard curve for heat treatment in different dilutions used for quantifying the viable cells in meat and meat products. The standard curve presents the R^2 value 0.9986 and 0.9904 for both the PMA treated and untreated and 0.9708 in heat treated *Bacillus cereus* ATCC 117783 samples. There was a slight increase in Ct values in

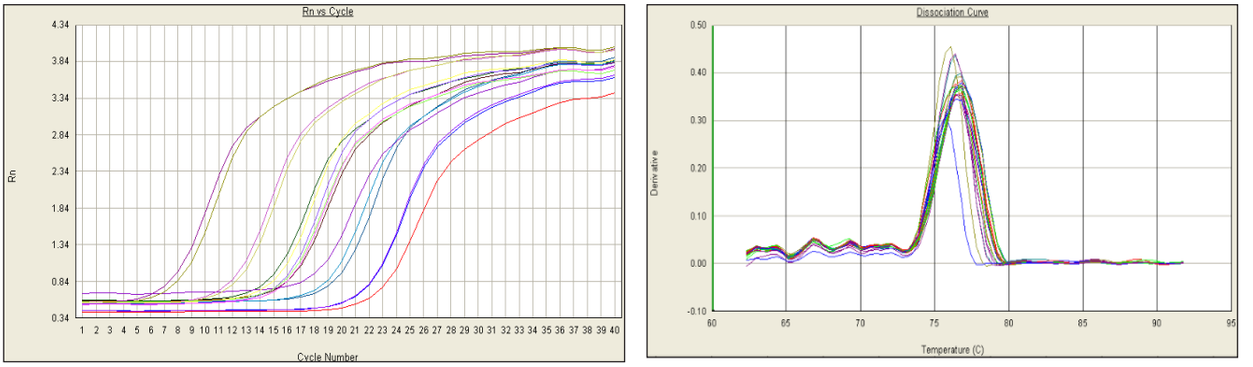

Fig. 3: Amplification plot and melt curve analyses of *Bacillus cereus*

PMA treated samples in comparison with untreated. The detection range of the assay was between 10^2 to 10^{10} as depicted earlier in Fig. 1, thus suggesting that PMA will quantify only viable bacteria and not the dead bacteria in PMA untreated samples, hence lesser Ct values in PMA untreated than in treated samples. In heat treated samples the standard curve was obtained with R^2 value 0.9708, where a greater number of viable cells obtained lower Ct value and low number of viable cells obtained higher Ct value because in q-PCR Ct values are inversely proportional to the amount of target nucleic acid in the sample. From these values it is demonstrated that PMA will completely inhibit the amplification of signals from dead cells. Similarly, reliable R^2 value higher than 0.95 was previously reported by Zhang and Fang, 2006 in detecting viable food borne pathogens.

On detection of viable bacteria from 50 meat products, only 16 samples (32 %) were positive, at the level of 10^3 CFU/ml in PMA treated samples that are unfit for consumer consumption and in conventional q-PCR, as expected 27 samples had more than 10^3 CFU/g resulting in 54% positivity. In the present study, the PMA based qPCR and conventional qPCR had the detection limit of $10^{0.96}$ CFU/g and 10^2 CFU/ml respectively for pure *Bacillus cereus* culture. *Bacillus cereus* contamination level below 10^3 CFU/ml in ready to eat foods is generally considered safe for the consumption (Martnez-Blanch *et al.,* 2011). Hence, the PMA qPCR positive 16 samples with more than 10^3 CFU/g viable bacterial contamination are considered unfit for consumption. The more positivity in conventional qPCR is due to amplification of both live and dead bacteria and PMA dye could distinguish live and dead bacterial cells thus amplifying only live cells there by eliminating false positive results. The results confirmed that PMA completely eliminate PCR signals from dead *Bacillus cereus* as to the previous report of Zhang *et al*. (2014). Thus, PMA treatment was a key step to exclude dead bacterial amplification in monitoring bacterial contamination in food.

In the study to test the efficacy of PMA dye to inhibit the DNA amplification of dead cells, cells were subjected to heat treatment with different known volume of cells and mixture of viable and non-viable cells were treated with PMA before q-PCR was performed. We evaluated PMA dye performance using DNA extracted from heat treated pure culture of *Bacillus cereus* ATCC11783. PMA was able to differentiate the viable and non-viable cells where a lesser number of non-viable cells represents lesser Ct value and vice versa as depicted in Fig. 2. On detection sensitivity assay, it had a low detection range of live cells $10^{0.9}$ CFU/ml of *Bacillus cereus* in spiked meat samples by PMA qPCR. Thus, the present approach was able to differentiate the live and dead bacteria and also for rapid detection of toxic genes of *Bacillus cereus*. This assay could be a rapid diagnostic aid to assess the safety of food, hence detection limit was mentioned above which can able to detect the viable cells at too low concentrations. Hence, sensitivity of the approach was used to detect minimum level of contaminations considered as unsafe limit in food samples. In this study, we optimized the PMA combined q-PCR using the designed specific primers for selective detection of viable *Bacillus cereus* in meat and meat products, and PMA treatment eliminated the false - positive results by quantifying only viable bacterial cells

and would aid in reliable risk assessment in ready to eat meat products.

ACKNOWLEDGEMENTS

The authors greatly acknowledge Department of Biotechnology, New Delhi for funding and Tamil Nadu Veterinary and Animal Sciences University, Chennai – 51 for providing all the infrastructure facilities to carry out this work.

REFERENCES

Anita, T. and Abdulla, S. 2015. *Bacillus cereus* food poisioning: International and Indian perspective. *J. Food Sci Technol.,* **52**(5): 2500-2511.

Cattani, F., Ferreira, C.A.S. and Oliveira, S.D. 2013. The detection of viable vegetative cells of *Bacillus sporothermodurans* using propidium monoazide with semi-nested PCR. *Food Microbiol.*, **34**: 196-201

Cattani, F, Barth, V.C. Jr., Nasário, J.S.R., Ferreira, C.A.S. and Oliveira, S.D. 2016. Detection and quantification of viable *Bacillus cereus* group species in milk by propidium monoazide quantitative real-time PCR. *J. Dairy. Sci.,* **99**(4): 2617-2624.

Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F. and Watanabe, H. 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of the viable *Legionella* cells. *Jpn. J. Infect. Dis.,* **63**: 119-123.

Elizaquível, P., Gabaldón, J.A. and Aznar, R. 2011. Quantification of *Salmonella spp., Listeria monocytogenes* and *Escherichia coli O157:H7* in non-spiked food products and evaluation of real-time PCR as a diagnostic tool in routine food analysis. *Food Contrl.,* **22**: 158-164.

Elizaquivel, P., Sanchez, G. and Anzar, R. 2012. Quantitative detection of viable foodborne *E.coli* 0157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combing propidium monoazide and real-time PCR. *Food Contrl.,* **25**: 704-708.

Elizaquivel, P., Sanchez, G., Selma, M.V. and Anzar, R. 2012a. Application of propidium monoazide-qPCR to evaluate the ultrasonic inactivation of *Escherichia coli* 0157:H7 in fresh cut vegetables wash water. *Food Microbiol.,* **30***:* 316-320

Fernández-No, I.C., Guarddon, M., Böhme, K., Cepeda, A., Calo-Mata, P. and Barros-Velázquez, J. 2011. Detection and quantification of spoilage and pathogenic *Bacillus cereus, Bacillus subtilis* and *Bacillus licheniformis* by real-time PCR. *Food Microbiol.,* **28**: 605-610.

Forghani, F., Langaee, T., Eskandari, M., Seo, K.H., Chung, M.J. and Oh, D.H. 2015. Rapid detection of viable *Bacillus cereus* emetic and enterotoxic strains in food by coupling propidium monoazide and multiplex PCR (PMA-mPCR). *Food Contrl.,* **55**: 151-157.

Garrido, A., Chapela, M.J., Román, B., Fajardo, P., Lago, J., Vieites, J.M. and Cabado, A.G. 2013. A new multiplex real-time PCR developed method or *Salmonella* sp. and *Listeria monocytogenes* detection in food and environmental samples. *Food Contrl*., **30**: 76-85.

Josefsen, M.H., Lofstrom, C., Hansen, T.B., Christensen, L.S., Olsen, J.E. and Hoorfar, J. 2010. Rapid quantification of viable *Campylobacter* in chicken carcasses using real-time PCR and propidium monoazide treatment as a tool for quantitative risk assessment. *Appl. Environ. Microbiol.,* **76**: 5097-5104.

Kwasaki, S., Horikoshi, N., Okada, K., Takeshita, T., Sameshima.T. and Kawamoto, S. 2005. Multiplex PCR for simultaneous detection of *Salmonella sp*., *Listeria monocytogenes,* and *Escherichia coli* in meat *samples. J. Food. Prot.,* **68**(3): 551-556.

Mackay, L.M, 2004. Real time PCR in the microbiology laboratory. *Clinical Microbiol. Infect.,* **10**: 190-212.

Martinez-Blanch, J.F., Sanchez, G., Garay, E. and Aznar, R. 2011. Detection and quantification of viable *Bacillus cereus* in food by RT–qPCR. *Euro. Food Res. Technol.,* **232**(6): 951-955.

Mira, E.K.I. and Abuzied, S.M.A. 2006. Prevalence of *B. cereus* and its enterotoxin in some cooked and half cooked chicken products. *Assiut Vet. Med. J.,* **52**(109): 70–78.

Nocker, A., Sossa, K.E. and Camper, A.K. 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *J. Microbiol. Methods*., **70**(2): 252-260.

Nocker, A. and Camper, A.K. 2009. Novel approaches towards preferential detection of viable cells using nucleic acid amplifications techniques. *FEMS Microbiol.Lett*., **291**: 137-142.

Rizzotti, L., Levav, N., Fracchetti, F., Felis, G.E. and Torriani, S. 2015. Effect of UV-C treatment on the microbial population of white and red wines, as revealed by conventional plating and PMA-qPCR methods. *Food Contrl.,* **47**: 407-412.

Udomsil, N., Chen, S., Rodrong, S. and Yongsawatdigul, J. 2016. Quantification of viable bacterial starter cultures of *Virgibacillus sp* and *Tetragenococcus halophilus* in fish sauce fermentation by real time quantitative PCR. *Food Microbiol.,* **57**: 54-62.

Xiao, L., Zhang, Z., Sun, X., Pan, Y. and Zaho, Y. 2015. Development of a quantitative real-time PCR assay for viable

Salmonella spp. without enrichment. *Food Contrl.,* **57**: 185-189.

Zhang, T. and Fang, H.H.P. 2006. Applications of real-time polymerase chain reaction for quantification of microorganism in environmental samples. *Appl. Microbiol. Biotechnol.,* **70**: 281-289.

Zhang, Z., Liu, W, Xu, H., Aguilar, Z.P., Shah, N.P. and Wei, H. 2014. Propidium monoazide combined with real-time PCR for selective detection of viable *Staphylococcus aureus* in milk powder and meat products. *J. Dairy. Sci*., **98**: 1-9.

Zhang, Z., Wang, L., Xu, H., Aguilar, Z.P., Liu, C., Gan, B., Xiong, Y., Lai, W., Xu, F. and Wei, H. 2014a. Detection of non-emetic and emetic *Bacillus cereus* by propidium monoazide multiplex PCR (PMA - mPCR) with internal amplification control. *Food Control.,* **35**: 401-406.