QUANTITIVE DETECTION OF VIABLE *ESCHERICHIA COLI* O157:H7 IN HEAT TREATED GROUND MEAT USING THE COMBINATION OF PROPIDIUM MONOAZIDE AND REAL-TIME PCR ASSAY

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Abstract – In last decade, DNA-based molecular assays in particular real-time PCR (qPCR) became one of the most promising techniques for the detection and quantification of food-borne pathogens. However, that qPCR can not discriminate between dead and viable cells resulting in over-estimation of the target cell. More recently, the combination of qPCR assay with Propidium Monoazide (PMA) pretreatment was proposed to overcome false positive identification caused by dead cells. In this study, we applied PMA-qPCR method for quantitative detection of viable *E.coli* O157:H7 in heat-treated ground meats. Viability of heat-killed *E.coli* O157:H7 cells were determined by PMA-qPCR and compared with plate counts. A linear correlation was obtained up to 0.98 between PMA-qPCR and plate counts. The results showed that PMA-qPCR method may have a potential to determine the number of viable cells in heat treated foods or other environmental samples.

Key Words: dead and viable cells discrimination, *E.coli* O157:H7, PMA, qPCR

I. INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is an important foodborne pathogen that caused several distinct clinical diseases, namely hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP), some of which may result to death [1]. Normally, *E. coli* O157:H7 originate from the intestinal tract of many farm animals, and entire the food chain contaminated by the carcass and the other raw material is used for food products [2,3,4]. Since *E.coli* O157:H7 is inactivated by thermal processing, EHEC O157:H7 outbreaks generally caused by raw/under-cooked or cross-contaminated foods [4,5].

To evaluate the risk associated with *E. coli* O157:H7 in food chain increasingly demands to rapid and reliable detection methods. Culture-based traditional methods are generally characterized labour-intensive and time-consuming. Recently, culture-independent molecular methods in particular real-time PCR (qPCR) became the most favorite tool to rapid, sensitive and quantitative detection of *E.coli* O157:H7 in foods [6,7,8,9,10]. However, the major limitation of PCR-based techniques are their inability to differentiate live and dead cells, hence providing more false negative results that lead to overestimate target cells. In recent years, a valuable method depend on the sample pretreatment previous to the real-time PCR with nucleic acid intercalating chemicals, such as Ethidium Monoazide (EMA) or Propidium Monoazide (PMA) was proposed to overcome this problem. In this process, these chemicals penetrate only into damaged membrane of dead cells and strongly inhibit the PCR amplification by cross-linking to DNA. For this reason, PMA applications combined with qPCR have been applied to detect food-borne pathogens [10, 11,12,13]. The aim of this study was to develop and evaluate a rapid real-time PCR (qPCR) method combined with PMA for the quantitative detection of viable *E. coli* O157:H7 cells in sub-lethally and lethally heat-treated ground meat samples.

II. MATERIALS AND METHODS

Artificial contamination of meat samples with pathogenic bacteria and heat treatment applications

E.coli O157:H7 ATCC 43895 was used as a reference strain for inoculation of the ground meat samples. Before the heat treatment, 25 g

ground meat were weighted into glass bottles and sterilized by autoclave at 121 °C for 15 minutes. Sterilized meat samples were inoculated with suspensions of *E.coli* O157:H7 at the concentration ranged from 10^2cfu/ml to10^4 cfu/ml after inner temperature of the samples reached to the working temperatures (60 °C, 65 °C, 70 °C or 75 °C). Inoculated samples were subjected to heat treatment for 15 seconds, 1 minutes and 5 minutes separately and then added 250 ml 0.9% NaCl before transferred on ice.

PMA treatment

PMA treatment was performed in a 1.5 ml light-transparent micro-centrifuge tube using 0,625 µl of 20 mM solution of PMA (phenanthidium, 3-amino-8-azido-5[3-(diethylmethylammonio)]-6-phenyl dichloride, Biotium Inc, Hayward, USA) in 20% (v/v) dimethyl sulfoxide (Sigma-Aldrich, Canada) was added to 500µl proportion of sample suspension to final concentrations of 25µM. The tubes were exposed to intense visible light for 15 min by using a photo-activation system (Led-Active Blue, Ingenia Biosystems, Barcelona, Spain), after incubation in darkness at 40 °C for 30 min.

Real-Time PCR application

Bacterial DNA was isolated from meat samples using method previously described by Serrone and Nicoletti (2013) [14] with some minor modifications. qPCR TaqMan prob method was performed using the specific primers and prob set that targeted shiga toxin producing gene (stx1) [15]. Thermal cycler conditions were adjusted to denaturation at 94 °C for 15 s and primer and prob annealing at 57 °C for 60 s for 40 cycles.

Copy number determination

The PCR products of *E.coli* O157:H7 ATCC 43895 were cloned to the pC 2-1-TOPO vector (Invitrogen) and transferred into the *E.coli* DH5α-component cells according to the manufacturer's instructions. Plasmids were isolated from cloned *E.coli* cells by using plasmid Midiprep kit (Invitrogen) and DNA concentraion was determined by using nanodrop (ACTGene, UVS-99, USA). Serial dilutions (10^0 to10^7 copies/µl) of the plasmid DNA were used to generate the standard curve.

III. RESULTS AND DISCUSSION

In this experiment, copy number of target gene (stx1) was determined to detect the efficiency of PMA treatment to differentiate live and death cells. Additionally, the effects of heat treatment on *E.coli* cells in meat samples were analyzed by comparing the copy numbers generated from cycle threshold (C_T) values of qPCR with and without PMA treatment. Also, log *E.coli* values obtained from plate count were used to confirm this results. The copy numbers of target gene was estimated by linear regression analysis of the C_T values obtained by qPCR versus the amount of log *E.coli* counts supplied from the assay of each plasmid dilution (from 10^7 to 10^0 log cfu/ml) and the equation of standart curves to calculate the copy numbers was found as in brackets ($y = -0.277x + 10.95$; $R^2 = 0.996$)

The efficiency of real-time PCR with and without PMA treatment for quantifying of viable *E.coli* O157:H7 cells in heat treated ground meat samples was determined and compared with plate count results. For this reason, the C_T values derived from qPCR analysis (with or without PMA treatment of cells) were calculated as log of copy number gr^{-1} by using standard curves as previously described and also plate count results were converted from cfu gr^{-1} to log of cfu gr^{-1}. The results of the study indicated that when the temperatures of heat treatment increased, correlation of PMA-qPCR and plate count results was also increased. The correlation values between PMA-qCR and plate count were determined as 0.78, 0.85, 0.92 and 0.98 for 60, 65, 70 and 75°C heat-treatments respectively. The increasing in the lethal effect of the heat treatment caused an increase in cell damage, so penetration of PMA into the dead cells was higher. The correlation between qPCR without PMA treatment and plate count was also determined. The results were obtained at 60, 65, 70 and 75 °C as 0.75, 0.47, 0.25 and 0.028 respectively. These results show that, the amplification of DNA from heat-killed *E.coli* O157:H7 cells in ground meat samples can be effectively prevented by treatment with PMA prior the DNA isolation. Also, the real-time PCR combined with PMA can selectively detect

viable *E.coli* O157:H7 at as low as 2 log cfu/gr in meat samples.

IV. CONCLUSION

In conclusion, it can be suggested that PMA-qPCR analyses have a potential for accurately discrimination of live and viable cells in the food samples. The PMA pretreatment combined with qPCR assay seems to be a promising method to increase the accuracy and sensitivity of the detection of live or dead *E.coli* O157:H7 cells in foods.

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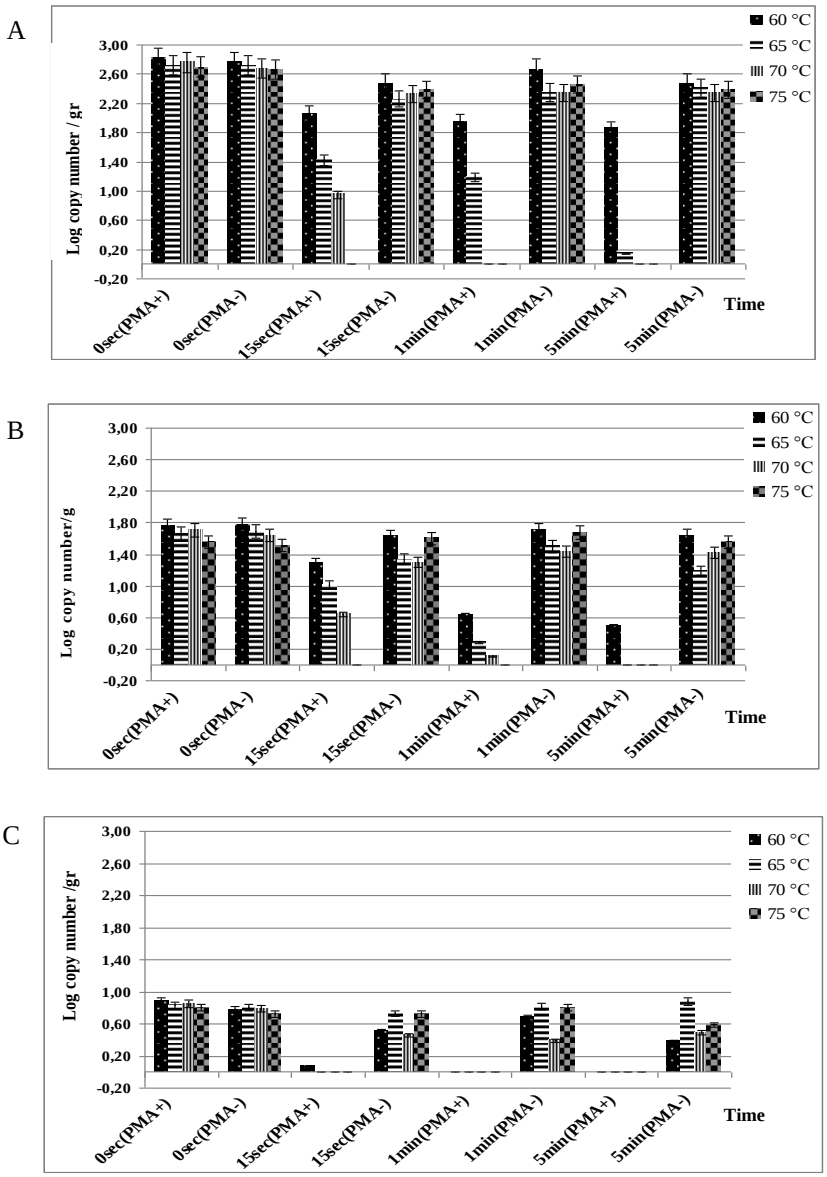

Fig. 1.The effects of heat treatment on log copy number of *E.coli* O157:H7 cells, determined by qPCR assays with and without PMA treatment. Initial inoculation concentration of *E.coli* O157:H7 in meat samples at 4 log cfu/gr (A), at 3 log cfu/gr (B), at 2 log cfu/gr (C) . The bars show the means values and the error bars show the standart deviations (n=2)

REFERENCES

1. Griffin, P.M., Tauxe, R.V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E.coli* and, the associated hemolytic uremic syndrome. Epidemiologic Reviews 13: 60-98.

2. Bouvet, J., Bavai, C., Rossel, R., Le Roux, A., Montet, M. P., Ray-Gueniot. & Vernozy-Rozand, C. (2001). Prevalence of verotoxin-producing *Escherichia coli* and *E. coli* O157:H7 in pig carcasses from three French slaughterhouses. International Journal of Food Microbiology 71: 249−255.

3. Zheng, H., Jing, H., Wang, H., Xia, S., Hu, W., Cui, S. & Xu, J. (2005). Stx2vha is dominant genotype of Shiga toxin-producing *Escherichia coli* O157:H7 isolated from patient and domestic animals in three regions of China. Microbiology and Immunology 49: 1019−1026.

4. Gordillo R., Rodríguez A., Werning, M.L., Bermúdez, E., Rodríguez, M. (2014). Quantification of viable *Escherichia coli* O157:H7 in meat products by duplex real-time PCR assays. Meat Science 96: 964-970.

5. Sheen, S., Hwang, C. A. (2010). Mathematical modeling the cross-contamination of *Escherichia coli* O157:H7 on the surface of ready-to-eat meat product while slicing. Food Microbiology 27: 37−43.

6. Gordillo, R., Còrdoba, J.J., Andrade, M.J., Luque, M.I., Rodrìguez, M. (2011). Development of PCR assays for detection of *Escherichia coli* O157:H7 in meat products. Meat Science 88: 767-773.

7. Spano, G., Beneduce, L., Terzi, V., Stanca, A.M., Massa, S. (2005). Real-time PCR for the detection of *Escherichia coli* O157:H7 in dairy and cattle wastewater. Letters in Applied Microbiology 40: 164-171.

8. Elizaquìvel, P., Sánchez, G., Aznar, R. (2012). Quantitative detection of viable foodborne *E.coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables propidium monoazide and real-time PCR. Food Control 25: 704-708.

9. Nocker, A., Camper, A.K. (2009). Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiological Letters 291 : 137-142.

10. Liang, N., Dong, J., Li, Y. (2011). Detection of viable Salmonella in lettuce by propidium monoazide real-time PCR. Journal of Food Science 76 (4): 234-237.

11. Nocker, A., Cheung, C.Y., Camper, A.K. (2006). Comparision of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. Journal of Microbiological Methods 67: 310-320.

12. Nkuipou-Kenfack, E., Engel, H., Fakih, S., Nocker, A. (2013). Improving efficiency of viability-PCR for selective detection of live cells. Journal of Microbiological Methods 93:20-24.

13. Martinon, A., Cronin U.P., Quealy, J., Stapleton, A., Wilkinson, M.G. (2012). Swab sample preparation and viable real-time PCR methodologies fort the recovery of *Escherichia coli*, *Staphylococcus aureus* or *Listeria monocytogenes* from artifically contaminated food processing surfaces. Food Control 24: 86-94.

14. Serrone, P.D., Nicoletti, M. (2013). Antimicrobial activity of a neem cake extract in a broth model meat system. International Journal of Environmental Research and Public Health 10 (8): 3282-3295.

15. Sharma V.K., Dean-Nystrom E.A. (2003). Detection of enterohemorrhagic *Escherichia coli* O157:H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. Veterinary Microbiology 93: 247–260.