EFFECTS OF MILD HEAT TREATMENTS ON NUMBERS OF SURVIVORS OF GENERIC AND VEROTOXIGENIC *ESCHERICHIA COLI* FROM BEEF ENRICHMENT CULTURES

Xianqin Yang, Madhu Badoni and Colin O. Gill

Agriculture and Agri-Food Canada, 6000 C&E Trail, Lacombe, Alberta, Canada, T4L 1W1.

Abstract – The purpose of this study was to determine whether reduction in numbers of total *Escherichia coli* is indicative of the reduction of verotoxigenic (VTEC) *E. coli* subjected to the same heat treatment. Swab samples were collected from hide-on beef carcasses and enriched for *E. coli* in *Escherichia coli* broth supplied with novobiocin. Suspensions containing cells in the stationary phase were mixed with equal volumes of Meat Juice medium and the mixtures were not heated or were heated to temperatures between 55 and 70 ºC. All preparations were treated with deoxycholate and propidium monoazide (PMA), and then DNA was extracted. Real-time PCR was performed using primers targeting the *uidA* gene for total *E. coli* and the *stx*$_1$ and *stx*$_2$ genes for VTEC. For samples that were not subjected to heat treatment, cycle threshold (Ct) values were from 14.00 to 17.20, 17.47 to 24.5 and 22.24 to 28.81 for the *uidA* gene, *stx*$_1$ gene, and *stx*$_2$ gene, respectively. Differences between changes in Ct values for *uidA* and *stx*$_1$ ranged from 0.17 to 0.79, and for *uidA* and *stx*$_2$ ranged from 0.03 to 0.67. The maximum change in the ratio of Ct values for *uidA* and *stx*$_1$ or *stx*$_2$ as a result of heating corresponded to a change of 0.3 log units, which is less than the 0.5 log generally considered to be microbiologically significant. Therefore, our findings indicate that the reduction of populations of total *E. coli* are indicative of reductions in the numbers of VTEC when they are subjected to heat treatments.

Key Words – Decontaminating treatments, Propidium monoazide and sodium deoxycholate, Viable real-time PCR.

I. INTRODUCTION

Generic *E. coli* has long been regarded as a reliable indicator for the possible presence and behavior of related enteric pathogens in water and foods. However, in recent years various workers have suggested or implied that generic *E. coli* is not satisfactory as an indicator for *E. coli* O157:H7. Consequently it has been considered necessary to conduct tests of decontaminating treatments for meat by detection or enumeration of organisms of that one serotype rather than generic *E. coli*. Regulatory authorities have recognized belatedly that VTEC of the O157 serotype are responsible for only some of the illness caused by VTEC. In addition to O157:H7, the USDA has deemed serotypes O26, O103, O45, O111, O121 and O145 as adulterants. Therefore, mandatory testing for these VTEC strains to start in June 2012, is being considered. Testing for, and demonstrating the effects of decontaminating treatments on each of the additional serotypes will be difficult, expensive and impractical for the meat industry. It would then be desirable to demonstrate that data obtained for generic *E. coli* would be adequate for indication of the effects of decontaminating treatments on VTEC. PCR-based methods have been developed to rapidly screen and quantify bacteria in food as an alternative to time-consuming culture techniques. However, these methods fail to discriminate between DNA from live and dead cells. Ways of discriminating between DNA from live and dead cells is necessary if PCR methods are to be used for rapid assessment of the microbiological condition of foods [1]. Recent research has demonstrated that, with the aid of the selective DNA intercalating dye propidium monoazide (PMA) and the membrane emulsificant sodium deoxycholate a real-time PCR method can be used to reliably enumerate viable *E. coli* after heat treatments when fractions of viable cells among live and dead cells are in the range of 1% to 100% [2]. The incorporation of selective agents for DNA from viable bacteria and real-time PCR is often is referred as viable real-time PCR. This study used a viable real-time PCR method to determine whether VTEC and generic *E. coli* were similarly inactivated by heat treatments.

II. MATERIALS AND METHODS

Samples were obtained by swabbing 100 cm^2 area of the surface of the butts of hide-on beef

carcasses using sterile cellulose acetate sponges (spec-sponge; VWR Canlab, Mississauga, Ontario, Canada) that each had been moistened with 7 ml 0.1% wt/vol peptone water containing 15% vol/vol glycerol. The sponges were stored at -80 °C until processed. Each sponge was enriched for *E. coli* in 20 ml *Escherichia coli* broth supplemented with 20 mg/L novobiocin, at 25 °C, overnight. Cells were pelleted by centrifugation at 9,000 x g for 10 min at 4 °C and resuspended in 10 ml Tris-HCl (10 mM, pH 7.0). The suspension was incubated at 37 °C for 2 h to place all cells in the stationary phase of growth. Then the cell suspension was mixed with an equal volume of Meat Juice medium [3] that was prepared at the same pH as the Tris buffer. A 1 ml portion of the mixture was dispensed to each of 10 2 ml Eppendorf tubes. Two tubes were kept in ice; the other 8 were heated at temperatures of 55, 60, 65 or 70 ºC in duplicate to obtain reductions in total *E. coli* numbers of about 2 to 3 log units. All tubes were immediately immersed in ice water after heat treatment. The cells in each tube were pelleted by centrifugation and resuspended in 240 ul 0.1% peptone water. All preparations were treated with deoxycholate and PMA, then DNA was extracted using DNeasy Blood&Tissue kit (Qiagen, Mississauga, Ontario, Canada), as described previously [2].

The primers URL-301 (5'-TGT TAC GTC CTG TAG AAA GCC C-3') and URR-432 (5'-AAA ACT GCC TGG CAC AGC AAT T-3') that target the gene encoding β-glucuronidase [4] were used for real-time PCR to determine the surviving populations of total *E. coli*. The primers stx1F (5'ATAAATCGCCATTCGTTGACTAC-3') and stx1R (5'-AGAACGCCCACTGAGATCATC-3') that target the gene encoding Shiga toxin 1 (stx_1) and the primers stx2F (5'-GGCACTGTCTGAAACTGCTCC-3') and stx2R (5'-TCGCCAGTTATCTGACATTCTG-3') that target the gene encoding Shiga toxin 2 (stx_2) were used to determine the surviving populations of VTEC [5]. The *uidA* gene is present in all *E. coli*, while stx_1 and/or stx_2 are present in all VTEC but not in non-VTEC *E. coli*. Real-time PCR was performed for each of the three primer sets individually. Real-time PCR mixtures contained 12.5 µl of 2 x Brilliant II SYBR green master mix (Stratagene, La Jolla, CA, USA), 300 nM the forward and reverse primers, and 5 µl of each DNA preparation. The volume of each reaction mixture was adjusted to 25 µl with sterile DNA-free Milli-Q water. Real-time PCRs were performed using a Stratagene Mx3005P QPCR system, with one cycle of initial denaturation of template DNA and activation of Taq DNA polymerase at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Data acquisition was performed using MxPro™ software (Stratagene).

Cycle threshold (Ct) values for the *uidA*, stx_1 and stx_2 genes for DNA prepared from non-heated samples were subtracted from Ct values for DNA prepared from the corresponding heated samples to obtain differences, which were analyzed statistically using a paired t-test in Minitab.

III. RESULTS AND DISCUSSION

For samples that were not subjected to heat treatment, Ct values were from 14.00 to 17.20, 17.47 to 24.50 and 22.24 to 28.81 for the *uidA* gene, stx_1 gene, and stx_2 gene, respectively, in enriched samples, which indicate approximate numbers of 7 to 8 and 3 to 7 log units of generic *E. coli* and VTEC *E. coli*, respectively. With preparations heated at 55 ºC, Ct values for all genes increased by between 4.3 and 5.0 Ct; while with heating to higher temperatures Ct values increased by between 6.5 and 7.9 Ct (Table 1).

Table 1 Differences in Ct values determined by real-time PCR for total *E. coli* (*uidA*) and VTEC (stx_1, stx_2) after heat-treatments

Temperature (°C)	**Means for differences (Ct)**			**Differences in means (Ct)**	
	uidA	stx_1	stx_2	*uidA*-stx_1	*uidA*-stx_2
55	5.01	4.32	4.49	0.68	0.52
60	7.81	7.30	7.78	0.51	0.03
65	7.90	7.11	7.23	0.79	0.67
70	6.50	6.67	6.55	0.17	0.05

Differences between changes in Ct values for *uidA* and stx_1 ranged 0.17 to 0.79, and for *uidA* and stx_2 ranged 0.03 to 0.67. Previous work had shown that a 1 log difference in numbers of surviving *E. coli* gave a difference in Ct values of 2.5. Thus, the maximum change in the ratio of Ct values for *uidA* and stx_1 or stx_2 as a result of heating corresponded to a change of 0.3 log units. Differences in bacterial numbers <0.5 log units are regarded as trivial. Thus, the findings indicate that mild pasteurizing heating similarly inactivates VTEC and non-VTEC *E. coli*.

IV. CONCLUSION

VTEC were found in all samples after enrichment and heating inactivated VTEC and non-VTEC to similar extents. Consequently, the effects of heating treatments on beef borne *E. coli* generally can be regarded as indicative of the effects of the treatments on VTEC.

ACKNOWLEDGEMENTS

The authors would like to thank the Alberta Livestock and Meat Agency for providing funding and Miss. Suraksha Rajagopal and Manasi Rajagopal for their technical support.

REFERENCES

1. Nocker, A. & Camper, A. K. (2009). Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiology Letters 291**:** 137-142.
2. Yang, X., Badoni, M. & Gill, C. O. (2011). Use of propidium monoazide and quantitative PCR for differentiation of viable *Escherichia coli* from *E. coli* killed by mild or pasteurizing heat treatments. Food Microbiology 28**:** 1478-1482.
3. Yang, X., Balamurugan, S. & Gill, C. O. (2009). Substrate utilization by *Clostridium estertheticum* cultivated in Meat Juice medium. International Journal of Food Microbiology 128**:** 501-505.
4. Bej, A. K., DiCesare, J. L., Haff, L. & Atlas, R. M. (1991). Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. Applied and Environmental Microbiology 57**:** 1013-1017.
5. Paton, A. W. & Paton, J. C. (1998). Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}. Journal of Clinical Microbiology 36**:** 598-602.