Final Report

Executive Summary Sheet

Project Title: Development of Real-time PCR methodology for the Rapid Enumeration of Low Numbers of *Salmonella* per Gram of Ground Beef Without Enrichment

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Objectives:

The goal of these proposed studies was to develop methodology to allow the Rti-PCR reaction to detect low numbers (5 cells/g) of *Salmonella* in ground beef of 7, 15, and 27% fat within 4 hrs. without enrichment.

Conclusions:

The use of β-cyclodextrin and milk protein coated activated carbon allowed the PCR to detect 3 seeded CFU (cells) of Salmonella/g without enrichment of samples, using ground beef containing 7, 15, and 27 % fat. The total assay time was 4.5 hr.

Deliverable:

This assay, specific for all *Salmonella* serotypes, will allow the meat processing industry to detect and quantify the presence of *Salmonella* well before shipment so as to prevent costly recalls. The additional costs for laboratory personnel and assay materials and equipment. should be more than offset by the reduction in indemnification premiums that should result from adoption of this assay.

Technical Abstract

This purpose of this study was to develop a rapid PCR assay for the detection of low numbers of CFU of *Salmonell*a in ground beef without pre-enrichment. The use of β-cyclodextrin and milk protein coated activated carbon (MP-CAC) allowed the PCR to detect low numbers of *Salmonella* seeded into ground beef without enrichment of samples. *invA* was used as target gene in the conventional PCR protocol. Stomached and seeded samples (25g) of ground beef containing 7.0, 15, and 27 % fat, were treated with 5.0, 10, and 15 % β-cyclodextrin respectively to remove fat followed by treatment with MP-CAC to remove PCR inhibitors. This methodology allowed the PCR detection of 3 CFU (cells) /g (equivalent to 75 CFU in a 25g sample). The total assay time was 4.5 hr.

Goals/Objectives

The goal of these proposed studies was to develop methodology to allow the Rti-PCR reaction to detect low numbers (5 cells/g) of *Salmonella* in ground beef of 7, 15, and 27% fat within 4 hrs. without enrichment.

Materials and methods

Bacterial strains and culture conditions

Salmonella enterica ser. Enteritidis strain ATCC BAA-708 was used throughout these studies. All media were obtained from Difco. Exponential growth phase cultures of *S. enterica* were obtained as previously described (Opet and Levin, 2013). Cells were serially diluted in Tryptic Soy Broth supplemented with 0.5% glucose (TSB^+, 4ºC) and kept on ice prior to use for inoculation of beef samples. Cell densities at 600nm in 1cm path length cuvets were determined by correlation with CFU counts on TSA^+. An absorbance of 0.50 at 600 nm for cell suspensions corresponded to 1.3×10^8 CFU/ml.

Preparation of ground beef samples

Ground beef containing different levels of fat (7.0, 15, and 27%) was purchased from local (Amherst, MA) retail sources. Portions of ground beef (25 g) were frozen in plastic zip-lock bags and stored at -20 ºC. Ground beef samples were cultured after freezing and thawing for *S. enterica* using standard methods outlined in the Bacteriological Analytical Manual (Andrews et al., 2011).

Preparation of MP-CAC

MP-CAC was prepared as previously described (Opet and Levin, 2013).

Preparation of β-cyclodextrin solution

β-cyclodextrin was purchased from United States Biological (cat. No. C8475-10, Swampscott, MA, USA). β-cyclodextrin, 10 or 20 g, was transferred into 1 liter sterile

flasks containing 100 ml of deionized water (dH_2O) and heated (~75 ^oC) with continuous agitation until the β-cyclodextrin was completely dissolved. The resulting 10% and 20% β-cyclodextrin stock solutions were cooled to ~ 55 ^oC immediately prior to use.

Removal of fat by β-cyclodextrin

Ground beef (25 g) was placed into Whirl-Pak® filter bags (Fort Atkinson, WI. USA) with 75.0 ml of 0.05M phosphate buffered saline (PBS, pH7.0) at 37 ^{o} C. Mid-log phase cells of *S. enterica* (section 2.1) were serially diluted in TSB^+ to yield 7.5×10^1, 2.5×10^2, 7.5×10^2, 2.5×10^3, 7.5×10^3, CFU/ml. A portion (1.0 ml) of each cell suspension was seeded into a series of the above filter bags containing ground beef and buffer (resulting in 3, 10, 30, 100, and 300 CFU/g). The filter bags were placed into a stomacher (Stomacher 400 lab blender, type BA 7021, serial # 27758, EN) and stomached for 30 sec at normal speed (~37 ^{o} C). The filter bags were then put into a water bath at 37 ^{o}C for 10 min.

β-cyclodextrin solutions (100 ml of 10%, 100 ml of 20%, and 200 ml of 20%) were added to the stomached ground beef samples for the removal of fat (7.0, 15, and 27%, respectively). The seeded filter bags were then placed into the stomacher and stomached for 2 min at normal speed (~37 ^{o} C) to mix. The ground beef masticates were completely transferred into 250 ml centrifuge bottles and vigorously shaken. The samples were chilled on ice for 10 min. The preparations were then centrifuged at 1,200 rpm (210 x g) for 6 min to pellet large particles of tissue and the majority of the β-cyclodextrin-fat complex. Resulting supernatants were passed through a 50 ml sterile plastic syringe barrel containing fine glass wool (0.5g firmly packed) at the bottom. The filtrate was then centrifuged at 11,000 rpm (17,600 x g) in 250 ml plastic centrifuge bottles for 6 min to pellet bacterial cells. The resulting pellets were resuspended in 30.0 ml 0.05M PBS (pH 7.0), at room temperature (20-25^{o}C) for subsequent treatment with MP-CAC.

Removal of PCR inhibitors with the use of MP-CAC

Dry MP-CAC (4.6 ± 0.05g) was transferred to a sterile 250 ml beaker and washed twice with 20 ml of sterile 0.05M PBS. The cell suspension (30 ml in PBS) was mixed with the washed MP-CAC, and bentonite (0.1g) added to further assist in the removal of PCR inhibitors. The preparations were then held at room temperature (20-25^{o}C) with rotary agitation (150 rpm) for 15 min. The treated solutions were passed through a 50 ml sterile plastic syringe barrel lined with fine glass wool (0.2g). The samples were then eluted with sterile PBS until 30 ml was collected in a sterile graduated cylinder. The 30 ml solutions were centrifuged at 12,000 rpm (16,100 x g) for 6 min (15^{o}C) to pellet the targeted cells.

Cell lysis and DNA preparation

The pellet containing target cells was resuspended in 0.5 ml of a solution containing 100 μl (3 mg/ml) BSA (cat. no. BP-1605-1, Fisher BioTech), 100 μl (0.1 mg/ml) salmon sperm DNA (cat. no. 14377, USB Corp.), 300 μl dH_2O and an equal amount (0.5 ml) of double strength TZ lysing solution (Abolmaaty et al. 2000). The tubes were then heated in a boiling water bath for 10 min to lyse the cells. The cell lysates were cooled to room temperature and centrifuged at 13,400 rpm (12,100 x g) for 5 min. The supernatant was carefully transferred to a fresh microcentrifuge tube.

Conventional PCR

The primer sequences used were Salm. F 5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′ and Salm. R- 5′-TCA TCG CAC CGT CAA AGG AAC C-3′ (Rahn et al., 1992). The primers were synthesized by Sigma-Aldrich (The Woodlands, TX, USA). These primers amplify a 284-bp fragment of the *invA* gene encoding for the primary cell invasion gene specific to *Salmonella*. Premixed ready strips for PCR (FastMix Frenche PCR i-Taq, cat. no. 25411, iNtRON Biotechnology Inc., Sangdaewon-Dong, Korea) were used to perform the reactions. The manufacturer's instructions were followed by adding 1.0 μl (10μM) of each primer, 2 μl sample DNA (section 2.7), and 16 μl of sterile dH_2O (20 μl total reaction volume). The PCR was performed with a TC-3000x thermocycler (Techne, Burlington, NJ, USA) using a modified version from the protocol of Riyaz-Ul-Hassan et al (2004) with initial denaturation at 94.0ºC for 3 min, followed by 35 cycles of denaturation for 5s (94.0ºC), annealing at 60.0ºC for 15s, and extension at 72.0ºC for 30s. This was followed by a final extension for 4 min (72.0 ^{o} C). Samples were held at 4.0ºC until gel electrophoresis was performed. All PCR assays were accompanied by a negative control containing sterile dH_2O in place of target DNA. In addition, 2μl of 5 x 10^4 CFU/ml of a pure culture of *S. enterica* ser. Enteritidis was used as a positive control for normalization of the relative fluorescent intensity of amplified bands.

Gel electrophoresis

Agarose (cat. no. BP164-100, Fisher Biotech, Fort Lawn, NJ, USA) gels (1%, 50 ml) was prepared using 0.05M sodium borate buffer (SB, pH 8.0) (Brody and Kern, 2004). RedSafe™ DNA stain (2.5μl, cat. no. 21141. iNtRON Biotechnology Inc., Sangdaewon-Dong, Korea) was added to the gel prior to casting as instructed by the manufacturer. Gels were cast and run in an Owl cast chamber (Model B1A, Owl Separation Systems, Portsmouth, NH, USA). After 30 min at 75 volts, the gels were visualized with an ultraviolet transilluminator (302nm, model no. TR-302, Spectroline Corp., New York, NY, USA) for detection of green fluorescent bands. Stained gels were photographed with a digital camera (PowerShot G10, Canon Inc., Japan).

Analysis of the relative fluorescent intensity of the amplified DNA bands was performed using the public domain NIH Image Analysis program ImageJ. The relative fluorescent intensity of each band was plotted against the CFU/g of ground beef using a semi-log plot from which the DNA in each PCR assay was derived.

Results

Preliminary results (not presented) indicated that without treatment with β–cyclodextrin and MP-CAC the lowest level of PCR detection was 5.0 x 10^5, 5.0 x 10^6, and 5.0 x 10^6 CFU/g of ground beef containing 7.0, 15, and 25% fat respectively.

Varying numbers of CFU of *Salmonella* seeded into processed ground beef of 7.0, 15, 27% fat, were subjected to the PCR following treatment with β-cyclodextrin and MP-CAC. Standard curves (semi-log plots) were then generated by plotting the means of triplicate assays and standard deviations of relative fluorescence values of amplicon bands vs. the number of CFU/g of ground beef. The use of β–cyclodextrin followed by treatment with

MP-CAC resulted in a sensitivity of detection of 3.0 CFU/g with ground beef containing 7.0, 15, and 27 % fat (Fig. 1), which was equivalent to 75 CFU per 25 g sample of ground beef assayed.

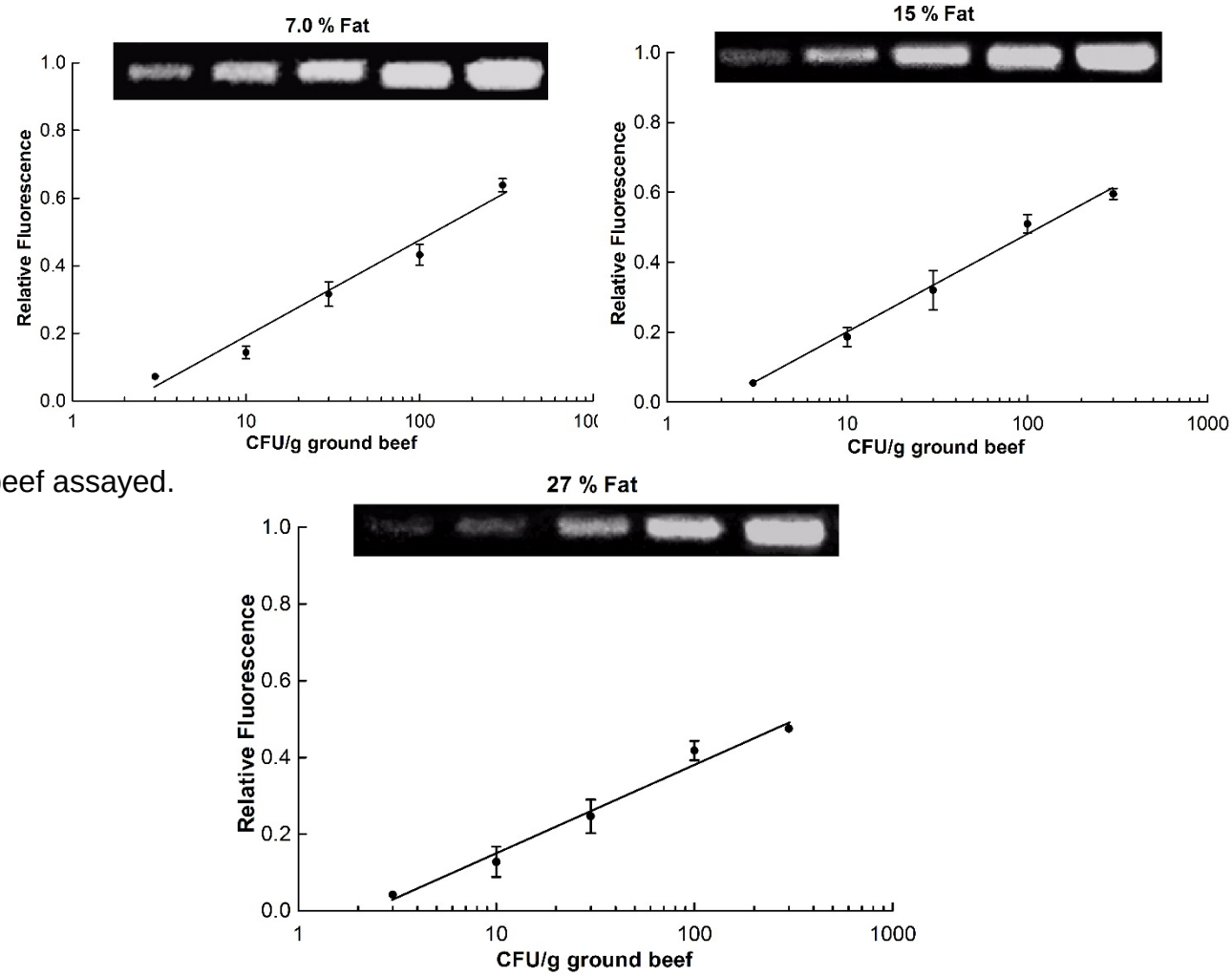

Fig. 1. Relative fluorescence of *invA* amplicon derived from seeding of ground beef. *S. enterica* was seeded into 25 g of ground beef (3, 10, 30, 100, and 300 CFU/g) containing 7.0, 15, and 27% fat. Each point plotted was derived from the mean and standard deviation of triplicate samples processed in duplicate. The correlation coefficients (R^2) were 0.97, 0.99, and 0.98 for the semi-log plots of 7.0, 15, and 27% fat respectively. DNA bands are typical of an individual PCR assay.

Conclusions

The use of β-cyclodextrin and milk protein coated activated carbon allowed the PCR to detect 3 seeded CFU (cells) of *Salmonella*/g without enrichment of samples, using ground beef containing 7, 15, and 27 % fat. The total assay time was 4.5 hr.

Presentations and Publications

Opet, N., Levin, R. 2013. Efficacy of coating activated carbon with milk proteins to prevent binding of bacterial cells from foods for PCR detection. J. Microbiol. Meth. 94:69-72.

Opet, N., Levin, R. 2013. Use of β-cyclodextrin and activated carbon for quantification of *Salmonella enterica* ser. enteritidis from ground beef by conventional PCR without Enrichment". Submitted to Applied Bacteriology. Presently under review.

Recommendations for Future Research

This research involving conventional PCR should be transitioned into real-time PCR to reduce the total time of assay and to obtain a sensitivity of 1 CFU/g or below. In addition, studies should be undertaken to replace β-cyclodextrin with starch for binding and separation of fat to reduce the assay cost. In addition, the assay should be expanded to include other *Salmonella* serotypes other than Enteritidis and effort made to reduce the total assay time to as close to 3 hrs. as possible.