2008 RFP FINAL PROJECT REPORT, DUE APRIL 1, 2010

Project Title
A sensitive and specific molecular testing method for live *Salmonella* in produce

Project Period
January 1, 2009 through February 28, 2010

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Objectives
Objective 1: To design and optimize a LAMP assay that targets *Salmonella* strains.

Objective 2: To evaluate the sensitivity and specificity of the LAMP assay in detecting live *Salmonella*.

Objective 3: To apply the assay in the detection of live *Salmonella* in experimentally contaminated produce items (shredded lettuce, baby spinach, sliced tomato, sprouts, and cantaloupe cubes) of various stages of maturity.

RESEARCH OBJECTIVES

1) To design and optimize a LAMP assay that targets *Salmonella* strains

2) To evaluate the sensitivity and specificity of the LAMP assay in detecting live *Salmonella*

3) To apply the assay in the detection of live *Salmonella* in experimentally contaminated produce items (shredded lettuce, baby spinach, sliced tomato, sprouts, and cantaloupe cubes) of various stages of maturity

PROJECT OUTCOMES

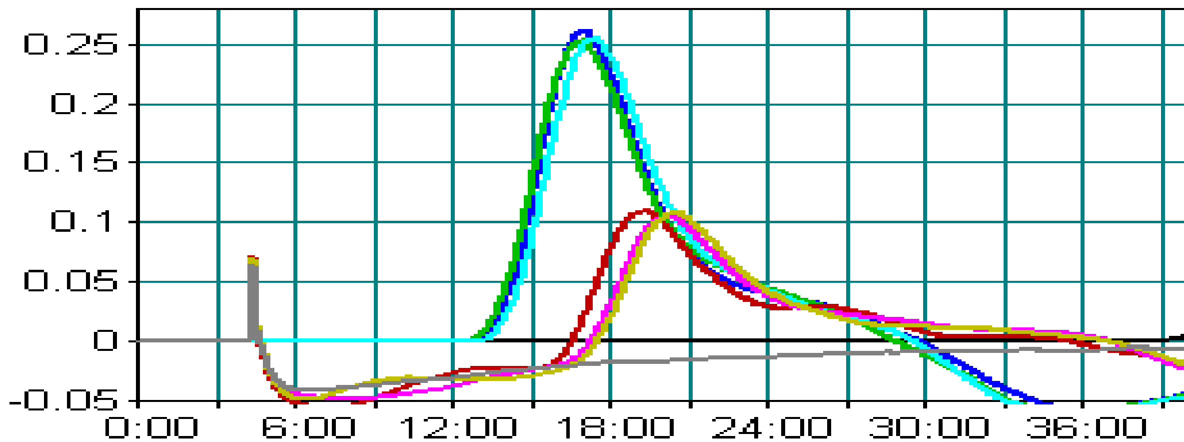
We organize the outcomes into two major sections, LAMP and PMA-LAMP. Under LAMP section, the assay design and evaluation outcomes were summarized, whereas under PMA-LAMP, the use of PMA coupled with LAMP for the live detection of *Salmonella* was summarized.

LAMP:

Assay design: We designed five sets of LAMP primers for *Salmonella* detection by targeting the *invA* gene using the PrimerExplorer software, namely Sal-1, Sal-4, Sal-8, Sal-9, and Sal-13. The LAMP reactions were run in a real-time turbidimeter and repeated 4 times. The speed, sensitivity, and quantitative capabilities of these primers were compared with a previously published LAMP primer set for *Salmonella invA* (Hara-Kudo, et al. 2005 FEMS Microbiology Letters, 253:155-161). Except for Sal-13, all LAMP primer sets performed well in terms of speed, giving a positive reaction within 30 min. The primer set Sal-8 we designed was the final primer chosen for the project because of its speed, sensitivity, reproducibility, and quantitative potential.

Deviation from the proposal: We had to use a real-time turbidimeter as a way to detect LAMP products instead of using color change as originally proposed in the proposal since the latter could not tell the speed of the reaction and also lack of quantitative capability. Since the purpose here was to differentiate the performance of these primers, a real-time turbidimeter was necessary.

Assay optimization: We optimized the LAMP reagent mix and reaction temperature one at a time by varying assay parameters including the concentrations of $MgCl_2$ (2 to 10 mM), betaine (0 to 1 M), deoxynucleotide triphosphate (0.4 to 2 mM), enzyme (2 to 10 U), outer (0.05 to 0.4 μM), inner (1.2 to 2 μM), and loop primer (0.2 to 1 μM), and assay temperature (60 to 65°C). The LAMP reactions were run in a real-time turbidimeter and repeated 4 times each. The optimized conditions were: 6 mM $MgCl_2$, 0 betaine, 1.2 mM deoxynucleotide triphosphate, 10 U enzyme, 0.1 μM of outer, 1.8 μM of inner, and 1 μM of loop primers, 2 μl of DNA template, and assay temperature of 63°C. Optimization was able to speed up the reaction by ~ 3 min and the signals were much stronger. Please see below for a graph showing the comparison between optimized and prototype conditions (each one was run in triplicate, and the earlier ones were the optimized conditions) . In addition, the optimized reagent mix eliminated the use of betaine.



Turbidity curve observed when running the LAMP assay under prototype (red, magenta, yellow) or optimized conditions (green, light blue, and dark blue).

Deviation from the proposal: Again, we had to use a real-time turbidimeter as a way to detect LAMP products instead of using color change as originally proposed in the proposal since the latter could not tell the speed of the reaction. Since the purpose here was to optimize the performance of LAMP reagent and reaction parameters, a real-time turbidimeter was necessary.

Assay sensitivity: The sensitivity of LAMP was tested using serially diluted *Salmonella enterica* Typhimurium LT-2 cells (10^7 CFU/ml to extinction). The LAMP reactions were run in a heater and observed for color change (color-LAMP) and repeated 4 times. The lower limit of detection was 13 cells, 100-fold more sensitive than PCR.

Assay specificity: The specificity of LAMP was tested using a panel of 25 *Salmonella* and 25 other related or unrelated bacteria genera, and LAMP was found to be highly specific, i.e., no false positive or false negative results were observed.

PMA-LAMP:

PMA was used to bind dead cell DNAs and prevent amplification from dead cells. PMA treatment conditions used were as follows: PMA (final concentration of 100 μM) was added into samples, incubated in the dark for 5 min, and then subjected to strong light exposure (650W) on ice for 5 min at a distance of 20 cm.

Three features of PMA-LAMP were evaluated: false positive rate for dead *Salmonella* cell detection, sensitivity of detecting live *Salmonella* in the background of dead *Salmonella*, and sensitivity of detecting live *Salmonella* in the background of dead *Salmonella* and other live bacteria.

False positive of PMA-LAMP for *Salmonella* detection in pure culture: The false positive detection of *Salmonella* was evaluated using serially diluted heat-killed *S. enterica* Typhimurium

LT-2 cells (10^8 CFU/ml to extinction). The experiments were repeated 3 times. After PMA treatment, DNA was extracted and run LAMP. The PMA-LAMP consistently gave negative results for dead cells levels ranging from 7.5×10^{-1} to 7.5×10^5 per reaction tube, but not for 7.5×10^6 per reaction tube.

<u>Sensitivity of detecting live *Salmonella* in the background of dead *Salmonella* in pure culture</u>: The sensitivity was obtained using serially diluted live *S. enterica* Typhimurium LT-2 cells (10^8 CFU/ml to extinction) mixed with 10^5 CFU/ml of heat-killed *Salmonella* cells. The experiments were repeated 3 times. After PMA treatment, DNA was extracted and run LAMP. By PMA-LAMP, the detection limit was 34 cells. In comparison, PCR had a detection limit of 3.4×10^2 cells, 10-fold less sensitive.

<u>Sensitivity of detecting live *Salmonella* in the background of dead *Salmonella* and other live bacteria in pure culture</u>: The sensitivity was obtained using serially diluted live *S. enterica* Typhimurium LT-2 cells (10^8 CFU/ml to extinction) mixed with 10^5 CFU/ml of heat-killed *Salmonella* cells and 10^5 CFU/ml of live *Escherichia coli*, *Citrobacter*, and *Shigella*. The experiments were repeated 3 times. After PMA treatment, DNA was extracted and run LAMP. By PMA-LAMP, the detection limit was 440 cells.

<u>Produce sample quality testing and preparation</u>: Three types of produce items, cantaloupe, spinach, and tomato were tested during three separate sampling trips. Produce samples were cut with sterile scissors or knives into small pieces, mixed with buffer peptone water (BPW), and homogenized.

Deviation from the proposal: We did not test shredded lettuce or sprouts as originally proposed in the proposal since historically, these items were not commonly involved in *Salmonella*-implicated produce outbreaks. Also, we did not consider produce stage of maturity since at the time (winter rather than summer) these experiments were performed, the produce samples were of relatively uniform maturity.

<u>False positive of PMA-LAMP for *Salmonella* detection in produce</u>: The false positive detection of *Salmonella* was evaluated using serially diluted heat-killed *S. enterica* Typhimurium LT-2 cells (10^8 CFU/ml to extinction) inoculated into homogenized produce samples. The experiments were repeated 3 times. After PMA treatment, DNA was extracted and run LAMP. The PMA-LAMP consistently gave negative results for dead cells levels ranging from (7.5×10^{-1} to 7.5×10^5 per reaction tube, but not for 7.5×10^6 per reaction tube.

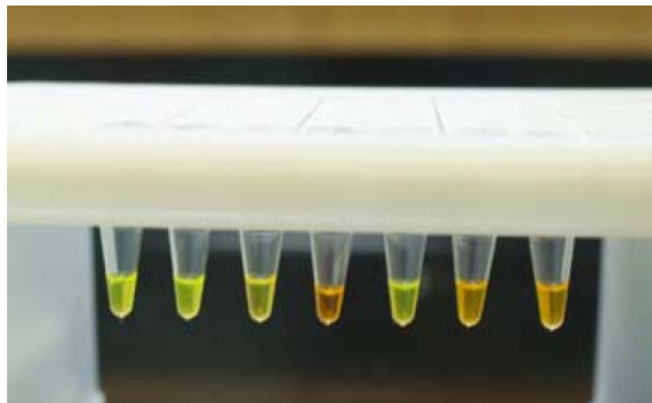
<u>Sensitivity of detecting live *Salmonella* in the background of dead *Salmonella* in produce</u>: The sensitivity was obtained using serially diluted live *S. enterica* Typhimurium LT-2 cells (10^8 CFU/ml to extinction) mixed with 10^5 CFU/ml of heat-killed *Salmonella* cells, and inoculated into homogenized produce samples. The experiments were repeated 3 times. After PMA treatment, DNA was extracted and run LAMP. By PMA-LAMP, the detection limit was 110 cells. In comparison, PCR had a detection limit of 1.1×10^3 cells, 10-fold less sensitive.

METHODS AND RESOURCES USED

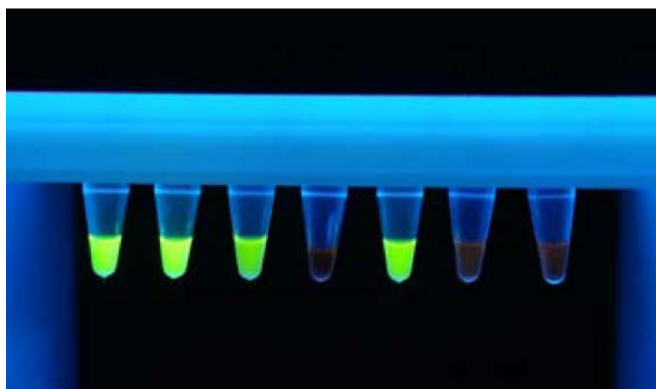
We first designed the primers and conducted primer comparison experiment to decide the best primer set to use. Secondly, we performed LAMP optimization to decide the optimum conditions to use.Thirdly, we evaluated the sensitivity and specificity of the primer set chosen. Afterwards, the optimized LAMP was combined with PMA for the live detection of *Salmonella* in pure culture and produce, and the false positive occurrence, sensitivity in the background of dead *Salmonella* cells, and sensitivity in the background of other bacterial cells were obtained.

The methods associated with each experiment have been briefly described in the above outcome section. Below is the set up of a piece of equipment used to run the color-LAMP and the LAMP products under normal air and UV light.

A dry heating block that maintains a constant temperature for performing the LAMP assay.



Positive samples (green to greenish-yellow color) and negative samples (orange color) under normal air.



Positive samples (bright fluorescent green) and negative samples (dark, non-fluorescent) under UV.

UNEXPECTED OUTCOMES AND MANAGEMENT

Due to the large quantity of DNA amplified by the LAMP technique, we experienced carry-over contaminations, i.e., positive results from negative control samples. To solve the problem, we maintained clean benches, used filter-tips, separated work areas of pre- and post-LAMP manipulations, and limited the number of times post-LAMP tubes were opened. These procedures seemed to have solved the problem well.

COLLABORATIVE EFFORTS

Dr. John Beaulieu, Co-PI of the project, is a plant physiologist with the Southern Regional Research Center of USDA/ARS. Dr. Beaulieu has participated in the experimental design, produce purchase, and quality evaluation of produce samples. Additionally, Dr. Beaulieu has been serving on the graduate committee of an M.S. student who primarily worked on this project.

SUFFICIENCY OF THE FUNDS

Due to the PI's oversight, the funds requested fell short of what had been needed to fully implement the project. Specifically, funds for two graduate research assistants would be necessary instead of one, particularly given that the funding period was relatively short.

In addition, a real-time turbidimeter was deemed an essential piece of equipment for the project, which could not be obtained using the grant money.

BUDGET MODIFICATIONS

Proposed funds in Travel ($4,500) and Publication ($1,000) have been moved to Materials and Supplies. Explanations are below.

> For Travel, we originally requested funds to present research findings at one professional meeting, such as the International Association for Food Protection annual meeting. However, although most meetings take place between May and August, the abstract submissions generally happen in January or February, which is at the very beginning of the funding period. At that time, the project has not yet generated enough

data for an abstract submission. We did submit an abstract in January 2010 to the American Society for Microbiology general meeting and the abstract has been accepted for a poster presentation.

Similarly for Publication, although we are planning to submit a manuscript based on the study, due to the time required for manuscript preparation and review, the paper will not be actually published by the end of 2009.

Therefore, those funds have been transferred to Materials and Supplies to absorb costs in that category, particularly the propidium monoazide reagent required for live cell detection.

BREAKDOWN OF THE GRANT FUNDS SPENT

The grant funds were spent on graduate students and supplies with the following breakdown:

Graduate students ($17,666.65)
Two graduate students worked on the project during the funding period. The graduate students participated in the experimental design, conducted research, analyzed data, and prepared abstract and reports.

Supplies ($22,500)
The supplies purchased helped complete the project by providing necessary materials, reagents, and consumables for the experiments.

Total spent ($40,166.65)/Total awarded ($40,500)

PUBLICATIONS AND PRESENTATIONS

There have been no publications. A manuscript is under preparation. The citation will be: Chen, S., F. Wang, J. C. Beaulieu, W. Prinyawiwatkul, and B. Ge. 2010. Detection of live *Salmonella* cells in produce by coupling propidium monoazide with loop-mediated isothermal amplification (PMA-LAMP).

A poster presentation is scheduled to occur at the American Society for Microbiology 110th General Meeting in San Diego, California, May 23-27, 2010. The abstract is entitled “Live *Salmonella* Detection by Coupling Propidium Monoazide with Loop-Mediated Isothermal Amplification (PMA-LAMP).” An electronic copy of the accepted abstract by ASM accompanies this report on page 7.

SUGGESTIONS

A few suggestions to CPS:

1) Funding period of 1 year is short, especially when the funds did not come in till 4 months past the project initiation time.
2) It is preferable that the funding starts on July 1.

Friday, March 05, 2010

Dear Dr. Chen:

Congratulations! On behalf of the American Society for Microbiology, your abstract number **3891**, entitled "Live *Salmonella* Detection by Coupling Propidium Monoazide with Loop-Mediated Isothermal Amplification (PMA-LAMP)" has been accepted for poster presentation at the General Meeting in San Diego, California, May 23-27, 2010.

We are in the process of finalizing the date, time and location of your presentation. Further details will be emailed to you no later than Friday, March 19, 2010.

You may register for the General Meeting online by clicking http://gm.asm.org/. Advance pre-registration is available through March 28, 2010. We look forward to seeing you in San Diego!

Sincerely,

Jeff F. Miller
Chair, General Meeting Program Committee

Margaret McFall-Ngai
Vice Chair, General Meeting Program Committee

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