RESEARCH IN PROCESS

Detection, Validation, and Assessment of Risks Implied by the Viable but Non-Culturable (VBNC) State of Enteric Bacterial Pathogens in Fresh Produce

SUMMARY

Comprehensive data about the prevalence of bacterial pathogens is essential for the development of accurate risk assessments and relevant quality control systems for fresh produce. None of the methods commonly used for the microbiological analysis of fresh produce can account for bacterial pathogens in dormant physiological states that preclude detection, notably the viable but non-culturable (VBNC) state. This project aims to develop a novel technique by the incorporation of loop mediated isothermal amplification (LAMP) with intercalating dye propidium monoazide (PMA) to detect both viable and VBNC *Salmonella* and Shiga toxigenic *E. coli* (STEC) in fresh produce. We first optimized the PMA-LAMP assay targeting viable *Salmonella* and STEC, separately. Second, the performance of PMA-LAMP for quantifying viable pathogens was determined in comparison to PMA-quantitative PCR (qPCR). Third, the assay was applied to quantify the experimentally induced VBNC cells. Our forthcoming studies include the detection and quantification of VBNC pathogen-spiked fresh produce by PMA-LAMP, the field and postharvest study and validation, and the semi-quantitative risk assessment using data from field trials. The resulting data, alongside with the acquired knowledge of risk implied by VBNC *Salmonella* and STEC on fresh produce will improve fresh produce safety and facilitate more reliable detection of risks implied by VBNC cells in fresh produce.

OBJECTIVES

1. Induce the VBNC state in relevant bacterial pathogens.
2. Assess intercalating dye performance and optimize treatment condition of intercalating dye.
3. Develop a LAMP assay, optimize assay conditions for both *Salmonella* and STEC, and verify assay performance *in vitro*.
4. Optimize conditions for the detection of pathogens in spiked produce (lettuce, tomato, cantaloupe and spinach) with PMA-LAMP assay and verify assay performance *in planta*.
5. Determine the survival of VBNC *Salmonella* and STEC on lettuce in the field and during simulated processing.
6. Perform semi-quantitative assessment of the risk implied by *Salmonella* and STEC in the VBNC state using data from field trials and simulated processing.

METHODS

Bacterial strains: Shiga toxigenic *E. coli* (STEC) O157:H7 strain EDL933 and *Salmonella enterica serovar* Enteritidis strain ATCC 43353 were used.

Induction of VBNC response: Bacterial cells in tryptic soy broth were grown until late log phase, washed three times with distilled water, and inoculated at a concentration of 108 CFU/mL to initiate VBNC induction. Several independent induction conditions were assessed, including starvation and low temperature (sterile distilled water, 4°C), osmotic stress (7% NaCl, 37°C), and oxidative stress (10 mM H_2O_2, 4°C).

Culturable cell counts: Culturable cells were monitored by the conventional plating assay (i.e., tryptic soy agar), and the data represent an average of three independent trials.

PMA-qPCR and PMA-LAMP: PMA was added to 500 µL bacterial culture to a final concentration of 100 µM and incubated for 5 min prior to light exposure for 2 min. DNA from non-treated and PMA-treated samples was extracted as described by Chan et al. (2011). SYBR green-based qPCR was performed using Applied Biosystems® 7500 real-time PCR system. LAMP reaction was carried out at 63°C for 50 min and terminated at 80°C for 5 min in an LA-320C real-time turbidimeter (Eiken Chemical Co., Tokyo, Japan). For both qPCR and LAMP, the targeted genes for the detection of *Salmonella* and STEC were *Salmonella bcfD* and STEC *wzy*, respectively.

Table 1. Comparison of the detection limits and quantification equations of LAMP, qPCR, PMA-LAMP and PMA-qPCR assays.

Sample type	Method	Detection limit (CFU/reaction)	Quantification equation	Linear R^2
Salmonella enterica serovar Enteritidis ATCC 43353	LAMP	2	y = -1.6667x + 23.307	0.948
	qPCR	2	y = -3.4895x + 36.925	0.998
	PMA-LAMP	20	y = -3.13x + 33.909	0.988
	PMA-qPCR	20	y = -3.5156x + 39.337	0.997
E. coli O157:H7 EDL 933	LAMP	20	y = -2.9733x +29.621	0.943
	qPCR	20	y = -4.1x + 39.372	0.998
	PMA-LAMP	200	y = -1.29x + 28.837	0.854
	PMA-qPCR	20—200	y = -3.4071x + 39.848	0.993

RESULTS TO DATE

Induction of VBNC response. VBNC cells were successfully induced during exposure to prolonged starvation and cold stresses or osmotic stress. Survival curves for *S.* Enteritidis and STEC are shown in Figure 1. Although the survival of bacterial cells kept at low temperature and distilled water significantly decreased (Figure 1A), the high salt condition (7% NaCl) turned out to be more effective for VBNC induction (Figure 1B).

Sensitivity and quantitative capability of LAMP. The detection limit of *Salmonella bcfD* -based LAMP assay was 2 CFU/reaction, and that of STEC *wzy*-based LAMP was 20 CFU/reaction. Similar detection limits were observed using qPCR (Table 1).

Specificity of PMA-LAMP for viable *Salmonella* cells. PMA-LAMP assay was conducted with 10-fold serially diluted dead bacteria cells. Negative results were achieved for dead *Salmonella* cells ranging from 2×100 to 2×10^4 CFU/mL, and for dead STEC cells ranging from 2×100 to 2×10^5 CFU/mL. The false-positive results for dead *Salmonella* cells occurred at 2×10^5 CFU/mL, and at 2×10^6 CFU/mL for dead STEC cells.

Sensitivity and quantitative capability of PMA-LAMP. In the presence of 2×10^4 CFU/mL dead *Salmonella* cells or 2×10^5 CFU/mL dead STEC cells, the detection limits of PMA-LAMP were 20 CFU/reaction of viable *Salmonella* cells and 200 CFU/reaction of viable STEC cells. The sensitivity of PMA-LAMP was similar to that of PMA-qPCR (Table 1). The typical amplification graphs and standard curves are shown in Figure 2.

Quantification of viable cells using PMA-LAMP. The viable cells in the VBNC bacterial cultures were determined using PMA-LAMP and PMA-qPCR, separately. Similar results were observed with the two quantification methods (Figure 3).

BENEFITS TO THE INDUSTRY

This research project may significantly contribute to the on-going efforts to develop rapid, sensitive, reliable and cost-effective analytical methods for the detection of foodborne pathogens in the fresh produce chain. A key benefit of the proposed LAMP-intercalating dye assay will be the capacity to detect pathogens that evade detection using methods currently used by the majority of laboratories, thereby improving the accuracy of analysis. The beneficiaries will include growers, harvesters and processors. The developed methods will also estimate the prevalence of VBNC enteric bacteria and provide new knowledge to achieve an improved understanding of the ecology of pathogens in fresh produce chains.

Figure 1. Survival curves showing culturable cell counts during exposure to prolonged starvation and cold stresses (A) or osmotic stress (B). Three independent experiments were conducted. Error bars indicate standard deviations. (A) Bacterial cells from the exponential phase were incubated at 4°C for up to 45 days, including *E. coli* O157:H7 strain EDL 933 cultivated in tryptic soy broth (TSB, blue) and distilled water (orange), and *Salmonella enterica* serovar Enteritidis strain ATCC 43353 cultivated in TSB (grey) and distilled water (yellow). (B) *Salmonella enterica* serovar Enteritidis strain ATCC 43353 (green) and *E. coli* O157:H7 strain EDL 933 (red) cells were separately inoculated in 7% NaCl and incubated at 37°C for up to three weeks. Three independent experiments were conducted. Error bars indicate standard deviations.

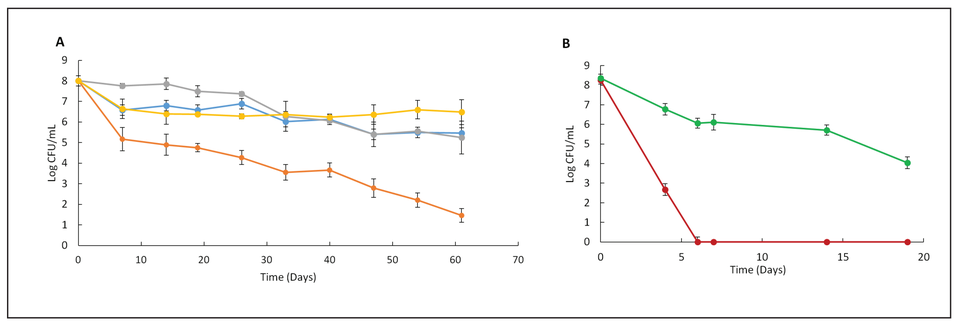


Figure 2. Comparison of the sensitivity and quantitative capabilities of PMA-LAMP and PMA-qPCR assays. *Salmonella enterica* serovar Enteritidis strain ATCC 43353 was tested in the presence of 2×10^4 CFU/mL of dead *Salmonella* cells. (A) Representative PMA-LAMP amplification graph. Five curves, from the left to the right, correspond to 10-fold serial dilutions of *S.* Enteritidis cells from 2×10^5 to 2×10^1CFU/mL. The green line represents the negative control where water was used instead of gDNA. (B) Standard curve generated from three independent PMA-LAMP tests. (C) Representative PMA-qPCR amplification graph. Five curves, from the left to the right, correspond to 10-fold serial dilutions of *S.* Enteritidis cells from 2×105 to 2×10^1CFU/mL. The green line represents the negative control. (D) Standard curve based on three independent PMA-qPCR tests.

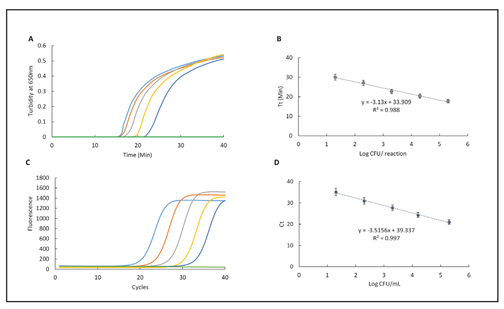
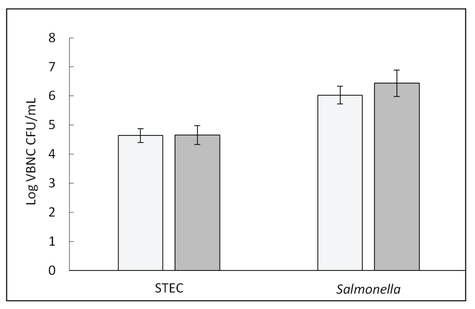


Figure 3. Quantification of VBNC cells using PMA-LAMP in comparison to PMA-qPCR. Viable bacterial cells were determined by PMA-LAMP and PMA-qPCR according to the standard curves. Culturable bacterial cells were determined using the conventional plating assay. The number of VBNC cells was calculated by subtraction of the numbers of viable cells to the culturable cells. The white bar represents PMA-qPCR and the grey bar represents PMA-LAMP. Three independent experiments were conducted. Error bars indicate standard deviations.



CONTACT

Dr. Xiaonan Lu
University of British Columbia
Food, Nutrition and Health Program
2205 East Mall
Vancouver, BC V6T 1Z4, Canada
T: 604-822-2551
E: xiaonan.lu@ubc.ca

AUTHORS

Lu Han[1], Lina Ma[1], Vivien Nie[1], Dr. Pascal Delaquis[2], Dr. Susan Bach[2], Dr. Jeff Farber[3], Dr. Xiaonan Lu[1]

ACKNOWLEDGEMENTS

1. Food, Nutrition and Health Program, University of British Columbia, Vancouver, BC V6T 1Z4
2. Agriculture and Agri-Food Canada, Summerland Research and Development Center, Postal Box 5000, Summerland, BC VoH 1Zo
3. Department of Food Science, University of Guelph, Guelph, ON N1G 2W1

LENGTH OF FUNDING

January 1, 2017 – December 31, 2018

CENTER *for* PRODUCE SAFETY