RESEARCHREPORT

pork checkoff

PORK SAFETY

Title: Novel molecular approaches for the rapid detection of *Salmonella* from pork products and the pork processing environment- **NPB# 08-007**

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Industry Summary:

Novel rapid, sensitive, and simple detection assays that can be routinely used to prevent the transmission of *Salmonella* in the pork industry are in high-demand. Reverse-transcriptase Loop-mediated isothermal Amplification (RT-LAMP) assay is a novel molecular method that has advantages over real-time reverse-transcriptase Polymerase Chain Reaction (RT-PCR) in that it does not require expensive equipment such as a real-time PCR machine as the reaction occurs in a waterbath at one temperature and detection is by turbidity or fluorescence after 2 h. The objectives of this research were to develop and apply novel assays such as RT-LAMP and RT-PCR to pork products and the pork environment for the rapid and sensitive detection of *Salmonella* Typhimurium and for comparison to traditional cultural methods. Another objective was to apply the RT-LAMP assay to detect *Salmonella* from pork products obtained from grocery stores and from pork processing facilities to test the suitability of the novel assays for routine testing by the pork industry in real-world scenarios. Twenty-five gram pork chop, pork sausage, and ground pork samples were spiked with *S*. Typhimurium at high (10^8 to 10^6 CFU) and low (10^5 to 10^0 CFU) inocula levels. Carcass rinse samples (500 ml; that were concentrated through sequential filtration) and carcass swabs (100 cm^2) were spiked with only high inocula levels. Samples were stomached in 225 ml of Tetrathionate (TT) broth, portions were serially diluted and plated on Xylose Lysine Tergitol (XLT4) agar for traditional cultural detection and RNA was extracted and assayed by RT-PCR and RT-LAMP using previously described primers. Each experiment in duplicate was replicated at least twice.

The Trizol® RNA extraction method provided improved RNA quality over the Qiagen RNA extraction method and was used in these studies. The RT-PCR assay on pork products spiked with high inocula *S.* Typhimurium showed detection of 10^6 CFU/25g within one 8 h shift. Selective enrichment in TT broth for 10 h was necessary to obtain detection of 10^1 CFU/25g for pork chop and pork sausage, which required at least two 8 h work shifts. The previously described LAMP assay was developed into a RT-LAMP assay that gave detection sensitivities of 10^1 CFU/ml after gel electrophoresis for *S*. Typhimurium in pure culture and was better than RT-PCR by 1 log CFU. Spiked pork chop and pork sausage without enrichment gave detection sensitivities of 10^6 CFU/25g similar to traditional plating and RT-PCR. The lower inocula levels required selective enrichment in TT

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broth, to obtain detection limits of 10^2 CFU/25g by RT-LAMP which was one-log less sensitive than RT-PCR and traditional plating. However, this RT-LAMP method is faster than RT-PCR by ~2 h. For both assays, negative controls including sterile water, sterile TT broth, and un-inoculated samples did not show any positive results, eliminating cross-reactivity or false positives. Background flora and autoclaved *Salmonella* cultures inoculated on to pork samples did not show any positive results as well, again eliminating false positives, indicating the robustness of these assays. Pork products spiked with cold stressed cells to simulate conditions associated with storage and transport gave detection limits of ~10^2 CFU/25g, after pre-enrichment for 3 h in buffered peptone water and selective enrichment in TT broth for 10-12 h. Screening of 57 natural samples from pork processing facilities and grocery stores resulted in 9 positives by traditional cultural plating, 5 positives by RT-PCR and 10 positives RT-LAMP assays. These results indicate that RT-LAMP shows potential for application in routine testing of *Salmonella* from pork products that can obtain results within two 8 h work shifts, being faster and simpler than RT-PCR and traditional cultural assays, but less sensitive by 1-log CFU. Further work using fluorescence dyes is necessary to improve detection sensitivity that will also allow conversion of the RT-LAMP to a quantitative assay by using automated portable fluorometers. This will further simplify the assay for routine use by the pork industry.

A cost-effective, rapid, sensitive, *Salmonella* detection assay was developed for routine testing of pork commodities that can obtain results within two 8 h work shifts. Using this sensitive portable assay that will allow rapid detection will help to prevent contaminated products from being released in the market and contaminated products can also be isolated to prevent cross contamination of other pork commodities and food contact surfaces. The pork industry will be economically and socially benefited as early detection will prevent expensive recalls and associated litigation costs and will protect brand name. Also, any processing areas or equipment found contaminated with *Salmonella* can be attended to immediately, properly cleaned and improved mitigation strategies and HACCP plans can be implemented. Use of this novel rapid assay in routine testing and surveillance will therefore aid in the prevention of *Salmonella* transmission by the pork industry as well as pork-related *Salmonella* outbreaks in order to protect public health.

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Scientific Abstract:

Novel, rapid, and sensitive detection assays that can be routinely used to prevent the transmission of *Salmonella* in the pork industry remain in high-demand. Our objectives were to utilize novel assays such as reverse-transcriptase Loop-mediated Isothermal Amplification (RT-LAMP) and real-time reverse-transcriptase Polymerase Chain Reaction (RT-PCR) that have improved speed for comparison to traditional cultural methods for *Salmonella* Typhimurium detection from the pork environment. The RT-LAMP assay has advantages over RT-PCR in that it does not require expensive equipment such as a real-time PCR machine as the reaction occurs in a waterbath at one temperature and detection is by turbidity or fluorescence after 2 h of amplification. Both, RT-LAMP and RT-PCR assays are based on the chromosomally located *inv*A (invasion A) gene specific for the detection of *Salmonella* enterica species and are based on mRNA detection, rather than DNA, that have increased potential of detecting viable cells. These assays were optimized and applied to pork products and the pork environment. Twenty-five gram pork chop, pork sausage, and ground pork samples were spiked with *S.* Typhimurium at high (10^8 to 10^6 CFU) and low (10^5 to 10^0 CFU) inocula levels. Carcass rinse samples (500 ml; after concentration through sequential filtration) and carcass swabs (100 cm^2) were spiked with only high inocula levels. Samples were stomached in 225 ml of Tetrathionate broth (TTB) and portions were serially diluted and plated on XLT4 agar for traditional cultural assays. RNA was extracted from 10 ml samples using the TRIzol® method and Qiagen Mini-kit, treated with DNAse I to remove any carryover DNA, and assayed.

The Trizol® method provided better RNA quality and yields than the Qiagen method. The RT-PCR assay using the SYBR Green I one-step RT-PCR Invitrogen kit gave the expected amplification product of 347 bp with a melt temperature (Tm) of 87.5°C, with detection sensitivities of 10^2 CFU/ml for pure culture *S*. Typhimurium. Spiking of pork products with high inocula *S.* Typhimurium showed detection of 10^6 CFU/25g *S.* Typhimurium within one day. For the low inocula levels, selective enrichment in TTB for 10 h was necessary to obtain detection of 10^1CFU/25g for pork chop and pork sausage, which required 2 work shifts. To further ensure the absence of false negatives due to product inhibition, enzyme failure or instrument malfunction, each RT-PCR assay had an internal amplification control (IAC) that gave a product of 154 bp and a Tm of 82°C, with the same primers in the assay. Un-inoculated pork products and sterile water (negative controls) did not show the amplified product by real-time PCR or gel electrophoresis, as expected. Background flora and autoclaved *Salmonella* cultures did not show any positive results from inoculated pork samples. Pork products spiked with cold stressed cells to simulate conditions of storage and transport, required pre-enrichment in buffered peptone water for 3 h followed by selective enrichment in TTB for 10 h to detect up to 10^1CFU/ 25g.

Using previously described primers, the LAMP assay was developed into a RT-LAMP assay that gave detection sensitivities of 10^1CFU/ml after gel electrophoresis for *S*. Typhimurium in pure culture. Spiked pork chop and pork sausage without enrichment gave detection sensitivities of 10^6 CFU/25g similar to traditional plating and RT-PCR. However, even though this assay is faster, the lower inocula levels required selective enrichment for 10 h, to obtain detection limits of 10^2 CFU/25g which was 1-log less sensitive than RT-PCR and traditional plating. Negative controls, autoclaved cells and background flora from inoculated samples did not show any positive results, eliminating false positives. Samples spiked with cold stressed cells gave detection limits of ~10^2 CFU/25g, after pre-enrichment and selective enrichment. Screening of 57 natural samples from pork processing facilities and grocery stores resulted in 9 positives by traditional cultural plating, 5 positives by RT-PCR and 10 positives RT-LAMP assays. These results indicate that RT-LAMP has potential to be applied in routine testing schemes for the detection of *Salmonella* in pork products within two 8 h work shifts, being faster and simpler than RT-PCR and traditional cultural assays, but less sensitive by 1-log CFU. Further work using fluorescence dyes is necessary to improve detection sensitivity and to convert the RT-LAMP to a quantitative assay by using automated portable devices, which will further simplify this assay for routine testing by the pork industry.

Introduction: An overview of the researchable question and its importance to producers.

Salmonellosis is a growing global concern and is the second leading cause of bacterial foodborne illness in the U.S.(Foley et al., 2007). The U.S. CDC estimates that ~1.4 million cases of salmonellosis occur annually, that results in 17,000 hospitalizations and 585 deaths (Mead et al., 1999, Voetsch et al., 2004). Pigs and pork commodities are the major sources of human salmonellosis caused by *Salmonella* Typhimurium. Interestingly, the per capita consumption of pork has increased from 18.7 kg to 21.7 kg/yr since 1909 (~20% increase) (Buzby and Farah, 2006, Foley et al., 2007), thus increasing the risk of swine associated salmonellosis. Approximately 82,000 farms result in the sale of ~185 million hogs each year in the U.S. (Foley et al., 2007). This places a huge economic burden on the swine industry when *Salmonella* contamination occurs. The slaughter of *Salmonella*-infected pigs poses a high risk for contamination of carcass and other edible pork products, introducing *Salmonella* in the food chain (Vieira-Pinto et al., 2006). *Salmonella* prevalence varies with the type of the meat sample. The lowest positive *Salmonella* levels are found in pork chops (1 to 3.3%) and highest in ground pork (16%), possibly due to the commingling of tissues from multiple animals into ground pork (Zhao et al., 2006; Foley et al, 2007). The prevalence of *S. enterica* in cull sows pose a significant risk of pork contamination as most of the cull sow meat is sold as ground pork (Larsen et al., 2003).

The major social and economic impacts on the pork industry make prevention and control of this disease a high priority. In order to prevent and control disease spread, early and timely detection is not only necessary but crucial. Traditional cultural methods for *Salmonella* from food products are time-consuming (take >5 days), laborious and cumbersome to be of much benefit to the swine industry for outbreak prevention or for rapid product recall. Rapid methods such as polymerase chain reaction (PCR) for the detection of *Salmonella* (Rahn et al., 1992; Soumet et al., 1995; Malorny et al., 2003 and 2007; Hoorfar et al., 2000) require gel electrophoresis or post-PCR hybridization, followed by confirmation using southern hybridizations, nested PCR, restriction digestion or sequencing, which take additional time. Real-time PCR methods are faster than PCR as they eliminate gel electrophoresis and detect the product as it forms, using either double stranded DNA (dsDNA) binding dyes (SYBR® green I) or fluorescent probes. Yet, real-time PCR or reverse-transcriptase-PCR (RT-PCR) for detection in food systems needs more in-depth research due to low detection limits associated with inhibitors from the food matrix. Validation studies to eliminate non-specific binding in order to decrease false positives and false negative results become crucial.

Another major drawback with DNA-based PCR and real-time PCR methods are their inability to differentiate between live infectious and non-viable killed microorganisms. Several researchers have used RT-PCR, with mRNA as targets, to correlate viable microorganisms as opposed to PCR for the detection of *Salmonella* Enteritidis (Szabo and Mackey, 1999), and other foodborne pathogens. Our laboratory has developed a real-time RT-PCR for the detection of *S. enterica* serovars in pure culture (D'Souza et al., 2009; manuscript in press). This robust and specific assay primarily detects viable *S. enteric*a serovars with a sensitive detection limit of 10^1- 10^2 CFU/ml. However, it requires expensive equipment such as a real-time PCR machine (~18K). Loop-mediated isothermal amplication (LAMP) is a novel detection method that relies on an autocycling strand displacement DNA synthesis performed by *Bst* DNA polymerase large fragment at a single temperature of ~62°C in 60 min, requiring only a simple waterbath. It is more rapid and simple than PCR synthesizing large amounts of DNA (>45 ug DNA/50 ul reaction) or RNA (reverse-transcriptase-LAMP, RT-LAMP) that can be detected by turbidity, due to the formation of insoluble magnesium pyrophosphate, or by fluorescence (Nagamine et al., 2002). Thus, the need for expensive equipment such as PCR machines and gel electrophoresis is eliminated. Turbidity or fluorescence (by incorporating fluorescent dyes in the reaction mix) can be measured using simple portable turbidimeters and fluorometers. This novel rapid method has recently been used for foodborne bacterial and viral detection including the specific detection *Salmonella enterica* in pure culture (Hara-Kudo et al., 2005) and in naturally contaminated liquid eggs with detection limits around 10^2 CFU/ml (Ohtsuka et al., 2005).

Our research objectives were to utilize the described RT-PCR assay and convert the novel LAMP to an RT-LAMP assay for *Salmonella* Typhimurium detection from pork commodities for comparison in sensitivity and speed to traditional cultural methods, in order to be applicable for routine testing by the pork industry. This research will allow for a simple, cost-effective and portable assay for routine application in the field to become

available to swine producers and the pork industry for rapid *Salmonella* detection. This early rapid detection assay will be of tremendous benefit to the pork industry to prevent outbreaks by timely implementation of control and mitigation strategies and preventing product recalls.

Objectives: From the research proposal.

1. Develop and optimize the real-time RT-PCR assay for the rapid and sensitive detection of *Salmonella* Typhimurium from pork products and the pork processing environment.
2. Develop and optimize the novel RT-LAMP assay for the rapid and sensitive detection of *Salmonella* Typhimurium from pork products and the pork processing environment.
3. Compare the detection sensitivities and specificities of RT-LAMP assay to real-time RT-PCR and traditional cultural detection methods.
4. Apply the RT-LAMP assay to naturally contaminated pork products and pork environments.

Materials & Methods:

Bacterial cultures: *Salmonella enterica* serovar Typhimurium DT104, *Enterobacter aerogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Escherichia coli* 15597, *Enterococcus fecalis*, and *Lactobacillus plantarum* were obtained from the University of Tennessee culture collection. The test strains were cultured into trypticase soy broth at 37°C for 24 h. Cultures were transferred a minimum of two times at 24 h intervals prior to use. Overnight *S.* Typhimurium cultures were serially diluted in phosphate buffered saline (PBS; pH 7.2), spread plated on Xylose Lysine Tergitol 4 (XLT4) agar, incubated at 37°C for 24 h and enumerated. Portions of 24 h grown *S.* Typhimurium were also stored at -80°C, -20°C, and 4°C for 24 h prior to use for the detection of stressed cells. Serial dilutions in 0.1% peptone water containing high (10^8 to 10^6CFU/ml) and low (10^5 to 10^0 CFU/ml) levels of *S.* Typhimurium were used for inoculation. The overnight cultures of 7 test strains of *E. aerogenes*, *P. vulgaris*, *P. aeruginosa*, *Y. enterocolitica*, *E. coli*, *E. fecalis*, and *L. plantarum* were combined to yield a mixed culture of equal proportions of each test strain immediately prior to use as inocula of background flora. The serially diluted background flora mixtures were spread plated on trypticase soy agar (TSA) and enumerated after 24 h incubation at 37°C.

Spiked Samples: Twenty-five gram pork chop and pork sausage, obtained from local grocery stores, were aseptically transferred to a sterile container and surface washed twice with 10 ml of sterile distilled deionized (DDI) water, once with 5% trisodium phosphate (Fisher Scientific, NJ) (to eliminate background flora), rinsed with 10 ml of DDI water twice and dried under ultraviolet light in a BSL-2 hood for 5 min. Prepared samples were inoculated with 1 ml of the desired level of *S.* Typhimurium, stomached in sterile stomacher bags containing 224 ml of freshly prepared Tetrathionate broth (TTB) and either used directly for assay or incubated at 37°C for 10 h and then assayed. Background flora and stressed *S.* Typhimurium inoculated on to pork samples or natural samples (from groceries and processing plants), were stomached in sterile stomacher bags containing 224 ml of buffered peptone water (BPW) and incubated for non-selective pre-enrichment at 37°C for 3 to 4 h, followed by addition of 25 ml pre-enriched BPW into 225 ml of TTB and incubation at 37°C for 10-12 h. For detection comparison, portions of the TTB were used for enumeration on XLT4 agar and portions were used for nucleic acid extraction and molecular (RT-PCR and RT-LAMP) assays. Each experiment was replicated at least twice.

Carcass Rinse Water: For carcass rinse water, 500 ml samples were processed using centrifugation and sequential filtration in order to concentrate the sample prior to enrichment. After using various combinations for optimization of the concentration step, the samples were filtered through Whatman#4 (20-25 micron), then through Whatman# 1(11 micron) and finally through a 0.85 micron and/or 0.45 micron sterile filtration unit under vacuum, the filters were pre-enriched and selectively pre-enriched as described above and then used for detection and enumeration by traditional and molecular assays.

Controls: Overnight pure culture of *S*. Typhimurium and its serial dilutions were used as positive controls as well as samples spiked with overnight *S*. Typhimurium, when appropriate. Negative controls included un-inoculated pork chop or pork sausage samples that were treated to remove any background flora, autoclaved carcass rinses, sterile water, sterile peptone buffer and sterile TTB.

For Detection of Naturally Contaminated Samples: Carcass swabs, surface swabs, ground pork and carcass rinse water, were obtained from a pork processing facility in TN and tested by traditional cultural methods, RT-PCR and RT-LAMP assays as described above.

Nucleic acid extraction and DNAse I treatment: Nucleic acid was extracted from 1 ml un-inoculated sterile TTB (negative control), sterile buffered peptone water, un-inoculated treated samples (negative control), pre-enriched samples, background flora organisms, autoclaved cells, stressed cells, and overnight cultures of *S*. Typhimurium (positive control) using the TRIzol® extraction protocol (Invitrogen, Carlsbad, CA) and/or Qiagen RNeasy minikit (Qiagen, Valencia, CA) following the manufacturer's instructions. Extracted RNA was then treated with QIAshredder™ (Qiagen) column to improve the quality of nucleic acid. Each experiment was run in duplicate and replicated twice. RNA resuspended in RNAse-DNAse free water was used immediately or stored at -80°C until use. A DNAse I treatment (Ambion) following the manufacturer's instruction was carried out at 37°C for 30 min to degrade any possible carry-over DNA in nucleic acid samples. Nucleic acid samples with and without DNAse I treatment were used to compare the detection sensitivity by PCR and real-time RT-PCR.

Analysis of nucleic acid quality: Absorbance ratios of nucleic acid extracts were measured at A260/A280 and A260/A230 using the NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE) to determine potential protein contamination or salt/organic carry-over, respectively. Ratios >1.8 were considered optimal.

Preparation of the internal amplification control (IAC): The IAC was included in the rt-RT-PCR reaction to eliminate false negatives as described by D'Souza et al. (2009). The amplified DNAse I treated RNA product of 154 bp was diluted to the optimal concentration prior to use.

Real-time RT-PCR assay: Real-time RT-PCR was performed on the RNA extracts of the spiked pork samples following a previously described procedure (D'Souza et al., 2009). Fifty microliters reactions containing 5 µl RNA extracts in RNAse-DNAse free water, SYBR Green I Superscript III (SSIII) one-step RT-PCR kit reagents (Invitrogen), 0.02 µM of each *invA* primer (previously published by D'Souza et al., 2009), bovine serum albumin (BSA; 0.06 µg/µl) and IAC (1.9fg/ul) were used. Cycling conditions included RT at 50ºC/30 min, denaturation at 95ºC/5 min, followed by 45 cycles at 95°C/30 s, 58°C/30 s, 72°C/30 s, and a final extension at 72ºC/7 min in a BioRad iCycler (BioRad, Hercules, CA). Post-amplification melt temperature (Tm) analysis from 50ºC to 95ºC with 0.5ºC increments was conducted to determine specific *inv*A product (Tm= **87.5^{o}C**) and IAC product (Tm= 82°C). The iCycler detection software was used to determine threshold cycle (Ct) values, Tm, and the standard curve. Negative controls including RNAse-DNAse free water; nucleic acid extracts from un-inoculated TTB and peptone water; and un-inoculated pork samples to determine any possible cross-reactivity or contamination (false positives). Positive controls included nucleic acid extracts of overnight cultured *S.* Typhimurium and its serial dilutions. All experiments were run in duplicate and were replicated twice.

RT-LAMP Assay: A modified LAMP protocol of Hara-Kudo et al., (2005) was used and also converted to a reverse-transcriptase-LAMP (RT-LAMP) assay. A set of 6 previously described specific primers were used to target the *Salmonella inv*A gene for amplification, that did not show cross-reactivity against an array of foodborne pathogens and background flora (Hara-Kudo et al,, 2005). The assay used in this study had reaction mixtures that consisted of 0.04 µM of forward inner primer, 0.08 µM of reverse inner primer, 0.01 µM of each outer primer, 0.02 µM of each loop primer (Sigma-Genosys), 1 mM dNTP, 0.8 M betaine (Sigma), 10 mM $MgSO_4$, 8 U Bst polymerase large fragment (New England Biolabs, MA), 10X Thermopol Buffer (New England Biolabs), and 5 µl of nucleic acid extract (treated or un-treated with DNase I) per 50 ul reaction. For RT-LAMP assays, 3.75 U avian myeloblastosis virus (AMV)-RTase (Invitrogen) was added per 50 µl reaction. Negative controls including RNAse-DNAse free water and nucleic acid extracts from un-inoculated sterile TTB, sterile peptone water, and un-inoculated pork samples were used to determine any possible cross-reactivity or contamination (false positives). Positive controls included nucleic acid extracts from overnight cultures of *S.*

Typhimurium and its serial dilutions. The reaction mixture was incubated at 62°C for 90 min in a waterbath. All experiments were carried out in duplicate and replicated at least twice.
Analysis of rt-RT-PCR and RT-LAMP products: Ten microliter portions of the amplified products were analyzed by agarose gel electrophoresis using 2% agarose gels (Promega) in 1X Tris acetate-EDTA buffer (10 mM Tris-Acetate and 1 mM EDTA, Fisher BioReagents), stained with ethidium bromide (Bio-Rad), and visualized under UV transillumination using the Gel-Doc Camera and Quantity One program (Bio-Rad). To determine the size of the amplified products, a 100bp DNA ladder (Promega) was used as a marker.

Results: Report of research results by objective.

Objective 1: **Develop and optimize the real-time RT-PCR assay for the rapid and sensitive detection of *Salmonella* Typhimurium from pork products and the pork processing environment**.

The RT-PCR assay was applied for the detection of *Salmonella* from spiked raw pork and sausage at high (10^8 to 10^6 CFU) and low (10^5 to 10^0 CFU) inocula levels. Nucleic acid extraction (RNA) was carried out using two methods, the Qiagen RNeasy minikit and the TRIzol® method and RNA extraction efficiencies were compared. The TRIzol® method was found to be optimal and used for extractions from spiked pork products. Spiked pork chop and sausage samples without enrichment gave *Salmonella* detection limits of $\mathbf{10^6}$ CFU/25g (Figure 1) similar to traditional cultural plating (Table 1). Enrichment of low and high inocula levels were carried out on spiked 25 g raw pork and sausage samples to improve detection sensitivity. Initial pre-enrichment of 4 to 6 h in 225 ml tetrathionate broth gave detection limits of 10^5 CFU/25g pork samples, an improvement in detection sensitivity of **1 log** CFU/25g over un-enriched samples. To further improve assay sensitivity, enrichment times were increased to 10 h which significantly improved detection sensitivity to $\mathbf{10^1}$ CFU/25g for both pork and sausage samples (an increase in **5 log** detection over un-enriched samples) (Figure 2). The lowest inoculated level of 1 CFU/ml did not show detection by RT-PCR or traditional plating, however, the IAC was detected by RT-PCR indicating that false negatives were absent. Also, DNAse I treated nucleic acid extracts did not show any products by traditional PCR as expected, indicating that there was no DNA carryover. Negative controls (water, TTB, or peptone water), un-inoculated pork samples and pork products spiked with autoclaved *Salmonella* cells did not show any positive RT-PCR products. The maximum total assay time including enrichment was **two 8 hour** work shifts. Our results showed no detection when background flora was tested on both pork chops and pork sausages even after pre-enrichment followed by selective enrichment (data not shown), eliminating any possible cross-reactivity or false positives. Accordingly, our results indicate that objective 1 for the detection of *Salmonella* from spiked pork meat and pork sausage was successfully achieved.

In addition, in order to simulate conditions associated with storage and transport of pork products prior to sale, *S*. Typhimurium cells that were stressed in the cold at 4°C, -20°C, and -80°C for 24 h were also studied, to determine the robustness of this assay. After 3 h of non-selective pre-enrichment of pork products inoculated with stressed cells in BPW followed by a 10 h selective enrichment in TTB, the developed rt-RT-PCR assay could recover and detect *Salmonella* stressed at -80°C and 4°C up to $\mathbf{10^1}$ CFU/25g in both pork samples using high and low inocula levels after enrichment (Figure 3). However, *Salmonella* stressed at -20ºC was detected only up to 10^6 CFU/25g from both pork samples even after enrichment.

a) An **abstract** was **accepted and presented** as a poster at the Institute of Food Technologists annual meeting held in Anaheim, CA, June 2009. "Techathuvanan, C., F. A. Draughon, and **D. H. D'Souza**. Real-time Reverse-transcriptase-Polymerase Chain Reaction (RT-PCR) for the detection of *Salmonella* Typhimurium in pork. IFT poster, Anaheim, CA, 2009."

b) A **manuscript** is being ***submitted*** to the *Journal of Food Protection* for publication consideration.

Real-time RT-PCR for the detection of *Salmonella* Typhimurium from swine carcass rinses and carcass swabs were also studied as proposed during the final months of the project. We were dependent on the schedule of the processing plants to obtain these samples. Also, the concentration step of the large volume of carcass rinse samples for detection needed optimization, which was challenging. Our optimized procedure, included sequential filtration through filters of various pore sizes ranging from 25 micron to final 0.85 or 0.45 micron, followed by pre-enrichment and selective enrichment and detection by traditional plating and molecular assays.

High titers were studied and detection sensitivity of 10^7 CFU/500ml carcass rinse sample (~$\mathbf{10^4}$ CFU/ml) was obtained. Due to the time constraints with the project, lower inocula levels were not studied and need to be pursued further (*as anticipated in the original proposal, another 6 months would be beneficial for additional studies*). Carcass swabs were also studied only at the high inocula level that gave detection sensitivity of 10^7 CFU/ 100 cm^2 swab.

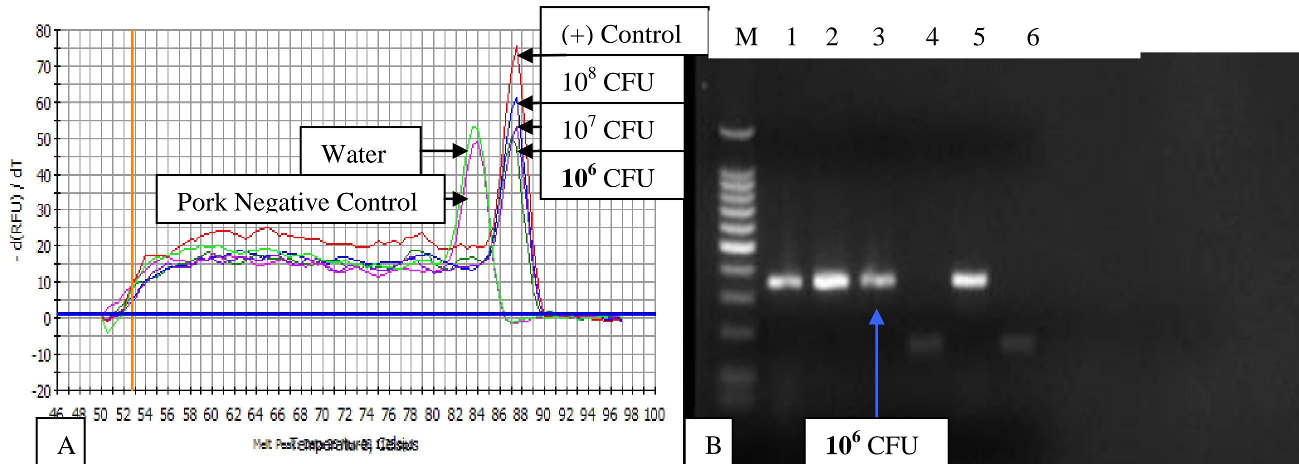

Figure 1. (A) Melt temperature curves of the RT-PCR products from un-enriched raw pork spiked with *Salmonella* showing specific peaks at 87.5°C with detection limits of 10^6 CFU/25g. The peaks from the un-inoculated pork control and the water control at 82°C shows the presence of IAC products. (B) Agarose gel electrophoresis of RT- PCR products from un-enriched spiked pork samples in TTB showing positive amplified 347 bp products. Lane M: 100 bp DNA Marker, Lane 1: 8 log CFU/25g; Lane 2: 7 log CFU/25g; Lane 3: 6 log CFU/25g; Lane 4: Negative un-inoculated Pork control; Lane 5: Positive *Salmonella* control; Lane 6: Water control.

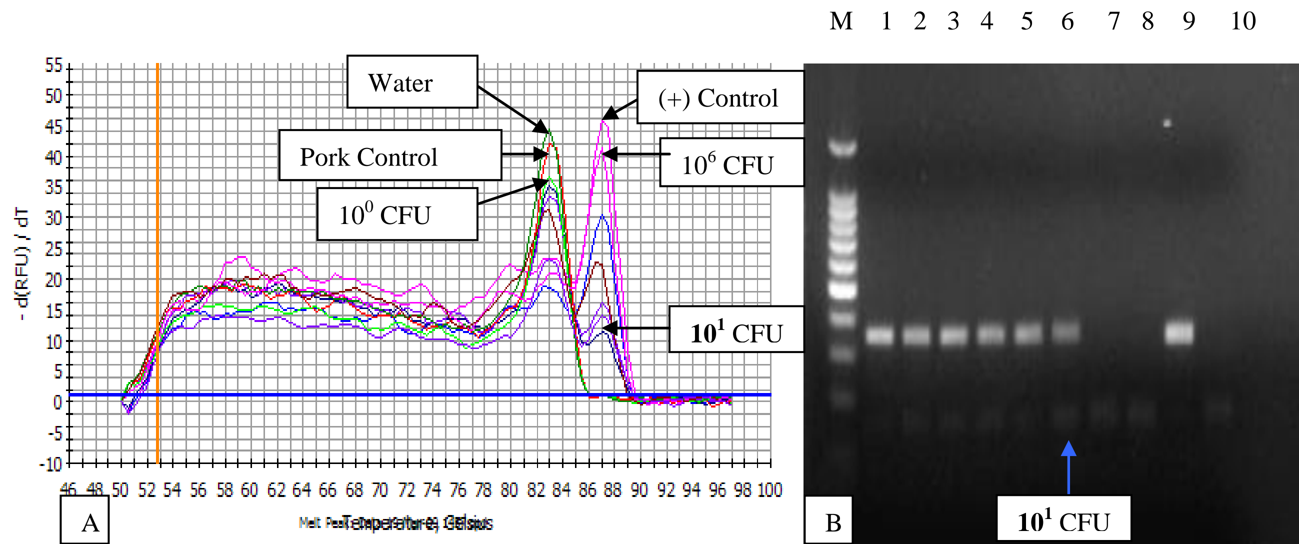

Figure 2.(A) Melt temperature curves of the RT-PCR products from 10 h enriched raw pork spiked with *Salmonella* Typhimurium at 37°C in TTB showing specific speaks at 87.5°C with detection limits of 10^1 CFU/25g. The peaks from the negative samples, the un-inoculated pork control and the water control at 82°C shows the presence of IAC products. (B) Agarose gel electrophoresis of RT- PCR products (347 bp *inv*A product and 154 bp IAC product) from pork samples spiked with *S*. Typhimurium and enriched in TTB at 37°C for 10 h. Lane M: 100 bp DNA Marker, Lane 1: 6 log CFU/25g; Lane 2: 5 log CFU/25g; Lane 3: 4 log CFU/25g; Lane 4: 3 log CFU/25g; Lane 5 : 2 log CFU/25g; Lane 6: 1 log CFU/25g; Lane 7: 0 log CFU/25g; Lane 8: Negative un-inoculated Pork control; Lane 9: Positive *Salmonella* control; Lane 10: Water control.

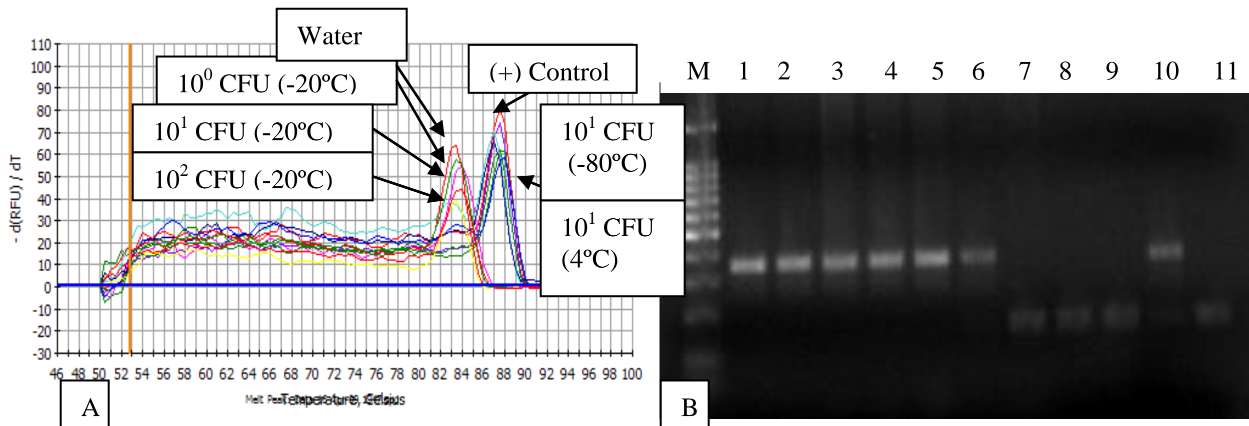

Figure 3.(A) Melt temperature curves of the RT-PCR products from raw pork spiked with *Salmonella* Typhimurium stressed at at -80ºC, -20ºC, and 4ºC after 3 h pre-enrichment in BPW and 12 h enrichment in TTB at 37°C showing specific speaks at 87.5^{o}C. The peaks from the water control at 82°C shows the presence of IAC products. (B) Agarose gel electrophoresis of RT- PCR products (347 bp *inv*A product and 154 bp IAC product) from pork samples spiked with low inocula level of stressed *S*. Typhimurium and enriched in BPW for 3 h and in TTB for 12 h at 37°C. Lane M: 100 bp DNA Marker, Lanes 1-3: 3 to 1 log CFU/25g of *Salmonella* stressed at -80ºC; Lanes 4-6: 3 to 1 log CFU/25g of *Salmonella* stressed at 4ºC; Lanes 7-9: 3 to 1 log CFU/25g of *Salmonella* stressed at -20ºC; Lane 10: Positive *Salmonella* control; Lane 11: Water control.

Objective 2: **Develop and optimize the novel RT-LAMP assay for the rapid and sensitive detection of *Salmonella* Typhimurium from pork products and the pork processing environment.**

Reverse-**T**ranscriptase **L**oop-mediated isothermal **AMP**lification (RT-LAMP) was successfully optimized for the rapid detection of *S*. Typhimurium in pure culture with a detection sensitivity of $10^{\mathbf{1}}$ CFU/ml within 90 min of amplification, followed by detection using agarose gel electrophoresis within 60 min (Figure 4) showing the expected ladder of products. Total assay time was approximately 3 h after RNA extraction from pure culture. This method was applied to raw pork and pork sausage spiked with high and low inocula levels. Detection limits without enrichment were $\mathbf{10^6}$ CFU/25g for both samples (Figure 5). Enrichment in tetrathionate broth for 6 h gave detection up to$10^{\mathbf{4}}$ CFU/25g pork. Increasing enrichment times to 10 h, improved detection sensitivity to $\mathbf{10^2}$ CFU/25g for both, raw pork and sausage (Figure 6). Autoclaved *Salmonella* cells spiked on pork products did not show any positive results by the RT-LAMP assay. Negative controls included sterile water, sterile TTB, and un-inoculated pork products that did not show any positive RT-LAMP products as expected, indicating the absence of false positives. The RT-LAMP assay did not show any positive detection when background flora was tested in both pork chops and pork sausages even after pre-enrichment followed by selective enrichment (data not shown), eliminating any possible cross-reactivity.

In order to determine the robustness of the assay for routine detection, conditions associated with storage and transport were simulated and pork products were spiked with cold and freezed stressed *Salmonella* cells. The RT-LAMP assay showed the detection limit of $\mathbf{10^2}$ CFU/25 g for all tested 24 h freeze-and cold-stressed *Salmonella* (at -80°C, -20°C, and 4°C) after 3 h pre-enrichment in BPW and 12 h enrichment in TTB in pork chop samples. For pork sausages, $\mathbf{10^1}$ CFU/25 g of cold- and freeze-stressed *Salmonella* could be detected after the same enrichment procedures (Figure 7).

a) The results of this **study were peer-reviewed, accepted and presented** as a poster at the American Society for Microbiology, Annual meeting held in Philadelphia, PA, May, 2009. "Techathuvanan, C., F. A. Draughon, and **D. H. D'Souza**. Reverse-transcriptase Loop-mediated Isothermal Amplification (RT-LAMP) for the detection of *Salmonella* Typhimurium in pork. ASM poster presentation, Philadelphia, PA, May, 2009."

b) **A manuscript has been submitted** to the *Journal of Food Science*, was peer-reviewed, and a revised manuscript is currently under review.

Techathuvanan, C., F. A. Draughon, and **D. H. D'Souza**. 2009. Reverse-transcriptase Loop-mediated Isothermal Amplification (RT-LAMP) for the detection of *Salmonella* Typhimurium in pork. ***J. Food Science (revised manuscript under review).***

RT-LAMP for the detection of *Salmonella* Typhimurium from swine carcass rinses and carcass swabs were also studied as proposed during the final months of the project. As mentioned in the results section of objective 1, we were dependent on the schedule of the processing plants to obtain these samples. Also, the concentration step of the large volume of carcass rinse samples for detection needed to be optimized, which was challenging. Our optimized procedure, included sequential filtration through filters of various pore sizes, followed by pre-enrichment and selective enrichment and detection. High titers were studied and detection sensitivity of $\mathbf{10^7}$ CFU/500ml carcass rinse sample (~$\mathbf{10^4}$ CFU/ml) was obtained. Due to the time constraints with the project (as anticipated another 6 months is needed for further studies), lower inocula levels were not studied and need to be optimized and pursued further. Carcass swabs were also studied only at the high inocula level that gave detection sensitivity of 10^7 CFU/100 cm^2. A significant amount of time in June, July, and August was spent on preparation of posters and oral presentations, manuscript writing, revisions of manuscripts, and travel to attend conferences.

Further development and optimization of the assay using YO-PRO-1 iodide dye (fluorescent dye) is needed to convert this assay into a real-time system eliminating the need for gel electrophoresis to decrease assay time by 60 min. This research needs to be further pursued to decrease detection time and improve detection sensitivity using portable fluorometers or turbidimeters. As we envisioned in our original proposal, this would require another 6 months for optimization and application to the pork environment for screening as well as quantification purposes and ***hope that the Pork Checkoff (National Pork Board) will consider providing funding in the near future to continue this project****.*

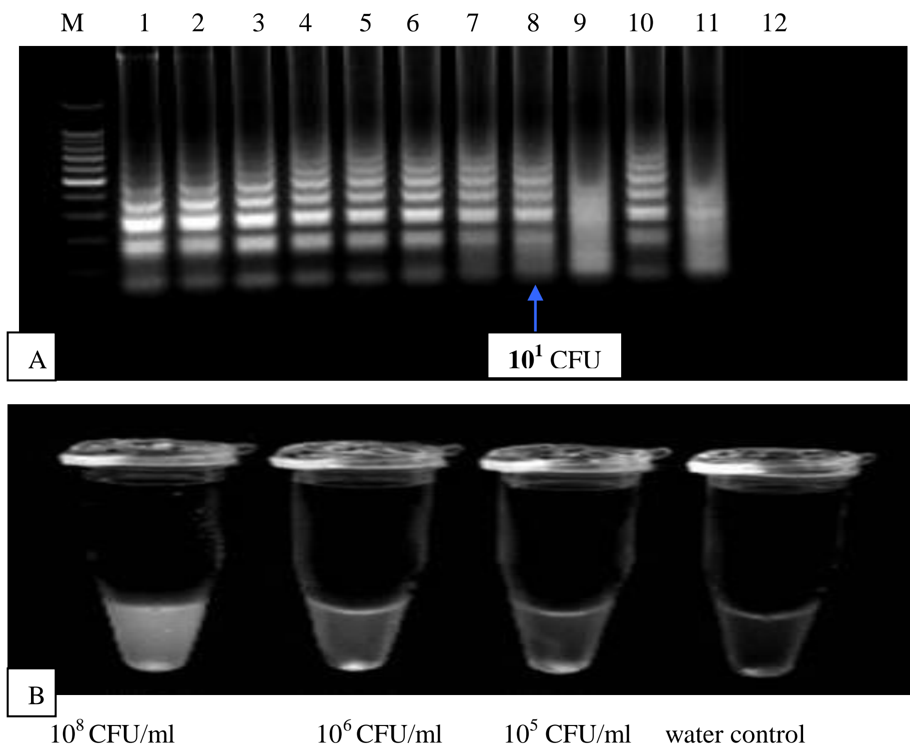

Figure 4. (A)Agarose gel electrophoresis of RT-LAMP products from nucleic acid extracts of pure culture *Salmonella* Typhimurium in TTB. M: 100 bp Marker, Lanes 1 to 9: RNA extracts from serially diluted cells (10^8 to10^0 CFU/ml); Lane 10: Positive *Salmonella* control; Lane 11: Negative un-inoculated TTB control; Lane 12: Negative water control. (B) Visual turbidity detection of LAMP products from nucleic acid extracts of pure culture *Salmonella* Typhimurium in TTB.

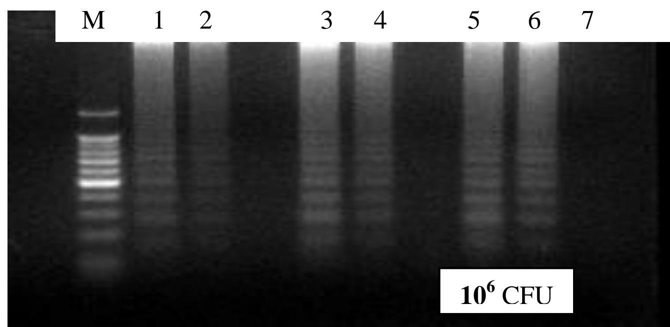

Figure 5. Agarose gel electrophoresis of RT-LAMP products from un-enriched pork samples spiked with *Salmonella* Typhimurium indicating detection limits. M: 100 bp Marker, Lanes 1-2: 8 log CFU/ml; Lanes 3-4: 7 log CFU/ml; Lanes 5-6: 6 log CFU/ml; Lane 7: Negative water control.

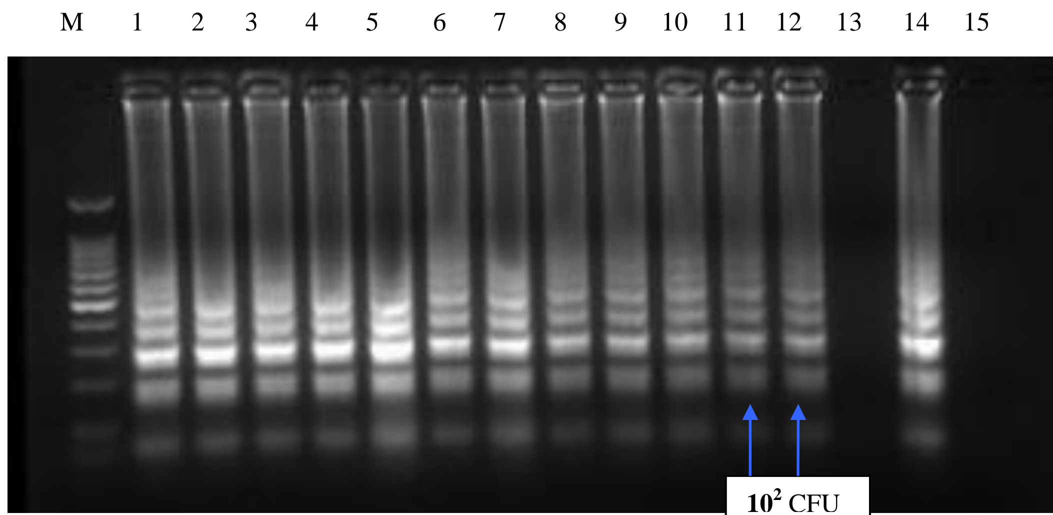

Figure 6. Agarose gel electrophoresis of RT-LAMP products indicating detection limits of sausage samples spiked with *Salmonella* and enriched in TTB at 37°C for 10 h. Lane M: 100 bp DNA Marker, Lanes 1-2: 7 log CFU/25g; Lanes 3-4; 6 log CFU/25g; Lanes 5-6: 5 log CFU/25g; Lanes 7-8: 4 log CFU/25g; Lanes 9-10: 3 log CFU/25g; Lanes 11-12: 2 log CFU/25g; Lane 13: Negative un-inoculated pork control; Lane 14: Positive *Salmonella* control; Lane 15: water control.

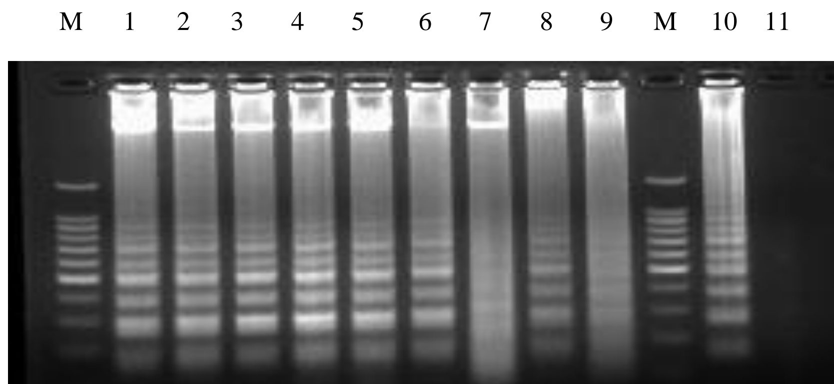

Figure 7. Agarose gel electrophoresis of RT-LAMP products indicating detection limits of sausage samples spiked with stressed *Salmonella* and enriched in BPW for 3 h and TTB for 12 h at 37°C. Lane M: 100 bp DNA Marker, Lanes 1-2: 2 to 1 log CFU/25g of -80°C *Salmonella*; Lanes 3-4: 2 to 1 log CFU/25g of -20°C *Salmonella*; Lanes 5-6: 2 to1 log CFU/25g of 4°C *Salmonella*; Lane 7: Negative un-inoculated pork control; Lane 8: 2 log CFU/25g of optimally grown *Salmonella* as positive control; Lane 9: Negative un-inoculated TTB control; Lane 10: Positive *Salmonella* control; Lane 11: water control.

Objective 3: **Compare the detection sensitivities and specificities of RT-LAMP assay to real-time RT-PCR and traditional cultural detection methods**.

Detection sensitivities and specificities of the RT-LAMP assay were compared to real-time RT-PCR and traditional cultural detection methods for *Salmonella* from spiked raw pork, sausage, ground pork and carcass rinse water samples. RT-LAMP was similar in specificity to the RT-PCR assay in that they both did not show cross-reactivity against a cocktail of background microflora spiked on pork products and was capable of only specifically detecting *Salmonella* from these products. Negative controls, un-inoculated pork products, sterile water, pre-enrichment and enrichment media, did not show any positive results by both these methods, similar to traditional plating. The RT-PCR assay showed similar detection sensitivity to the traditional methods on both un-enriched and enriched spiked pork products with detection limits of $\mathbf{10^6}$ CFU/25 g and $\mathbf{10^1}$ CFU/25g, respectively (Table 1). On the other hand, the RT-LAMP assay which was faster than RT-PCR, showed a one-log lower detection sensitivity for low inocula enriched pork products (detection limits were $\mathbf{10^2}$ CFU/25 g), but was similar in detection sensitivity to RT-PCR and traditional methods, for the high inocula un-enriched samples. Similar detection sensitivities and specificities by all three methods of 10^7CFU/500ml or 10^7 CFU/100cm^2 for spiked carcass rinse water and carcass swabs, respectively, were obtained with high inocula.

a) These comparison results from the raw pork and sausage studies **were presented as an oral technical presentation** at the International Association of Food Protection (IAFP) Annual Meeting held in Grapevine, TX, July 2009. "Techathuvanan, C., F. A. Draughon, and **D. H. D'Souza**. Comparison of Reverse-transcriptase Loop-mediated Isothermal Amplification (RT-LAMP) to RT-PCR and Cultural Methods for the Detection of *Salmonella* Typhimurium in Pork. IAFP oral presentation, Grapevine, TX, July 14, 2009".

b) A *manuscript* on the comparison of these assays, including data from spiked rinse water and carcass swabs, is in preparation and will be submitted to either the *Journal of Food Science* or *Journal of Food Protection* for publication consideration.

Table 1. Comparison of *Salmonella* Typhimurium detection limits using pure culture and spiked pork samples by traditional cultural methods, RT-PCR, and RT-LAMP assays.

	Lowest Inoculated Detection Limit (CFU/ml or CFU/25g)		
Sample	**Traditional Method**	**RT-PCR Assay**	**RT-LAMP Assay**
Salmonella pure culture	10^0	10^2	10^1
Un-enriched			
Pork chop (high inocula)	10^6	10^6	10^6
Pork sausage (high inocula)	10^6	10^6	10^6
10 h enriched			
Pork chop (low inocula)	10^1	10^1	10^2
Pork sausage (low inocula)	10^1	10^1	10^2
Ground Pork (high inocula)	10^6	10^6	10^6
Carcass Rinse[a](high inocula)	10^7	10^7	10^7

***high inocula = 10^8 to 10^6 CFU; low inocula = 10^5 to 10^0 CFU; [a]Carcass rinse=500 ml sample**

Objective 4: Apply the RT-LAMP assay to naturally contaminated pork products and pork environments.

In order to determine the application of the newly developed RT-LAMP assay for routine testing in the pork environment, samples of pork chops and ground pork that were from at least two different lots were purchased from several local grocery stores (total of 14 samples) and screened for the presence of *Salmonella* by traditional cultural methods, RT-PCR, and RT-LAMP assays after pre-enrichment in BPW for 3 h and selective enrichment in TTB for 10 h. The RT-LAMP assay showed positive results from 1 pork chop and 1 ground pork sample (obtained from different stores), while the RT-PCR assay failed to detect any positive samples. The assays were run in duplicate twice along with appropriate positive and negative controls as described in objective 1 and 2.

In addition, samples were obtained from the pork processing plant that included 13 surface swab, 14 carcass swab, 6 pork sausage, and 10 carcass rinse water samples, and were analyzed for the presence of *Salmonella*. Our results showed that 2 surface swab samples were positive by RT-LAMP, but only 1 by RT-PCR and 4 by traditional plating. From the 14 pork carcass swab samples, 4 were positive by RT-LAMP and 3 were positive by RT-PCR and 4 by traditional plating. None of the pork sausage samples tested positive for *Salmonella* by either traditional cultural assays, RT-PCR or RT-LAMP. Carcass rinse water samples are being processed and thus far, 2 out of the 10 tested samples have shown to be positive by RT-LAMP assay (Table 2) and 1 out of 10 has tested positive by the RT-PCR assay. The colonies obtained by traditional plating from these natural samples were isolated and confirmed using biochemical tests such as TSI (Triple Sugar Iron) and citrate slants for confirmation of the presence of *Salmonella*.

a) Results from this study *will be submitted* as part of the manuscript for comparing detection sensitivities between traditional and molecular assays as described in objective 3 and/or as a future presentation.

Table 2. Detection of *Salmonella* Typhimurium from natural pork products and pork processing environment by traditional cultural methods, RT-PCR and RT-LAMP assays.

	Number of *Salmonella* positive samples (per 25g)		
Sample (number of tested samples)	**Traditional Method**	**RT-PCR Assay**	**RT-LAMP Assay**
Pork chop from grocery stores (8)	0	0	1
Ground pork from grocery stores (6)	0	0	1
Swabbed* pork processing surfaces (13)	4	1	2
Swabbed* pork carcasses (14)	4	3	4
Pork sausages from local plant (6)	0	0	0
Pork carcass rinse water 500 ml (10)	1	1	2

*Swab samples were from a 100 cm^2 surface

Discussion: Research results and a summary of the results that is of immediate or future benefit to pork producers.

Our results with the developed RT-LAMP assay and applied RT-PCR assay indicate that molecular methods are suitable for use as rapid diagnostic tests for the detection of *Salmonella enterica* serovars from pork products and the pork environment. These assays are significantly faster (requiring only two 8 h work shifts including enrichment) than traditional cultural methods that can take up to 1 week. The RT-PCR assay using the SYBR Green I one-step RT-PCR Invitrogen kit gave the expected amplification product of 347 bp with a melt temperature (Tm) of 87.5^{o}C with detection sensitivities of 10^2 CFU/ml for pure culture *S*. Typhimurium. When pork products were spiked with high inocula *S*. Typhimurium, detection limits of 10^6 CFU/25g *Salmonella* were obtained within one day. For the low inocula levels, selective enrichment in TTB for 10 h was necessary to obtain detection of 10^1 CFU/25g for pork chop and pork sausage, which required two 8 h work shifts. To further ensure the absence of false negatives due to product inhibition, enzyme failure or instrument malfunction, each RT-PCR assay had an internal amplification control (IAC) that gave a product of 154 bp and a Tm of 82°C, with the same primers in the assay. Negative controls that included un-inoculated pork products, TTB, BPW, and sterile water did not show target amplified product by real-time RT-PCR or gel electrophoresis as expected, indicating the absence of false positives. Background flora did not show any positive results from inoculated pork samples, eliminating false positives further validating the robustness of this assay. Pork products spiked with autoclaved *Salmonella* cells did not show any positive RT-PCR products, showing the potential of this assay to detect only live viable cells. Pork products were spiked with cold stressed cells to simulate conditions of storage and transport that required pre-enrichment and selective enrichment to allow detection at levels up to 10^1CFU/25g. In addition, carcass rinse water and carcass swabs using samples obtained from a pork processing facility, show promise for detection of *Salmonella* by this assay. So far, only high inocula levels have been studied for carcass rinse water and carcass swabs due to scheduling and technical difficulties with concentration of water samples which were eventually overcome as well as time limitations with the project (as expected an additional 6 months would help to further advance the project). Our results showed detection sensitivities of 10^7CFU/500 ml carcass rinse water and 10^7 CFU/100 cm^2 carcass swab. This RT-PCR assay, though rapid, specific, and sensitive with comparable results to traditional cultural methods in detection sensitivity and specificity, does require expensive equipment such as a real-time PCR thermocycler (~18K) that could potentially be a limiting factor for routine use by the pork industry.

On the other hand, the newly adapted RT-LAMP assay showed a detection limit of 10^1 CFU/ml after gel electrophoresis for overnight cultures of *S*. Typhimurium, which was 1-log CFU better in detection sensitivity than RT-PCR. RT-LAMP assays on spiked pork chop and pork sausage without enrichment gave detection sensitivities of 10^6 CFU/25g similar to traditional plating and RT-PCR. The lower inocula levels required selective enrichment in TTB to obtain detection limits of 10^2 CFU/25g, which was 1-log less sensitive than RT-PCR and traditional plating. However, this assay is faster than RT-PCR by about 2 hours and does not require expensive equipment such as thermocyclers, but only a simple waterbath. Negative controls such as un-inoculated pork products, sterile TTB, and sterile water did not show any false positive results by this assay. Background flora and autoclaved cells did not show any positive results from inoculated pork samples, again eliminating false positives. Carcass rinse water and carcass swabs obtained from a pork processing facility show promise for detection of *Salmonella* by RT-LAMP assay as well. Again, only high inocula levels have been studied using the RT-LAMP assay for carcass rinse water and carcass swabs due to the technical difficulties with concentration of water samples which were eventually overcome and time limitations with the project. Carcass rinse water and carcass swabs gave detection limits of 10^7CFU/500 ml and 10^7CFU/100 cm^2, respectively. Pork products spiked with cold stressed cells were used to simulate conditions of storage and transport of pork products that gave detection limits of ~10^2 CFU/25g, after pre-enrichment and selective enrichment. This indicated that a robust assay was developed and could be used as a routine screening tool for the presence of *Salmonella* in the environment.

For pure culture *S*. Typhimurium, traditional plating showed the highest sensitivity and could detect up to 1 CFU/ml overnight culture, followed by RT-LAMP that could detect 10^1 CFU/ml, and RT-PCR that could detect 10^2 CFU/ml. In comparison to traditional plating, RT-PCR and RT-LAMP gave similar detection sensitivities (10^6 CFU/25g) and specificities for high inocula samples of pork chop and pork sausage. The RT-PCR assay showed similar sensitivities to traditional plating for low inocula levels. However, for the low inocula levels that required

enrichment, the RT-LAMP gave similar specificities but showed lower detection limits (10^2 CFU/25g) than RT-PCR or traditional plating (10^1 CFU/g) by 1-log CFU. This could be due to inhibitors from the food matrix themselves or from the enrichment media, that affect the amplification steps resulting in decreased amplification and detection. Alternatively, yields of RNA extraction may not be optimum, affecting the amplification process and causing decreased yields. Future work should be aimed at removal of inhibitors from the food matrix or enrichment media and improving RNA yield to improve detection sensitivity, while simultaneously increasing speed of detection to about 1 day (or one 8 h work shift).

Screening of 57 natural samples from pork processing facilities and grocery stores resulted in 9 positives by traditional cultural plating, 5 positives by RT-PCR and 10 positives RT-LAMP assays, using pre-enrichment in BPW for 3 h, followed by selective enrichment in TTB for 10 h. As stressed cells inoculated on pork products as well as natural samples, gave positive results after pre-enrichment and selective enrichment, the recommendation for routine analysis is to use pre-enrichment in buffered peptone water for ~3 h followed by selective enrichment in TTB for ~10 h and then assaying by molecular methods. The newly developed RT-LAMP assay shows potential for routine application in the pork industry, as it is simple to use, rapid (within 3 h after enrichment steps), requiring only a simple waterbath and a portable turbidimeter or fluorometer to enable automation and ease of interpretation of results.

Currently, the detection sensitivity of enriched pork samples using the RT-LAMP assay is 10^2 CFU/25 g with obtainable results within two 8 hour working shifts. However, there is still scope to improve sensitivity by incorporating fluorescent dyes. Alternatively sensitivity can be improved by increasing enrichment times, which defeats the purpose of increasing speed of the assay. Fluorescence is known to improve detection by at least one order of magnitude (1-log CFU) over traditional chemical or colorimetric reactions (Gao et al., 2009). Further work involving incorporation of fluorescence dyes, such as SYBR Green I or YO-PRO-1 iodide dye (an intercalating dye that binds all double stranded DNA non-specifically) or molecular beacon probes (that bind only to specific target nucleic acids) to the LAMP assay can be carried out to observe amplification of the target gene in a real-time manner. A simple hand-held fluorometer can monitor the increase in fluorescence as the reaction proceeds. Though the initial cost for equipment is required, it can eliminate the cost of gel electrophoresis and speed up the detection time by at least 1 h. This would result in improved detection of small amount of amplicons in a manner far superior than the detection limit of the natural human eye or by using turbidimeters. Also, a quantitative assay that can detect the exact amounts of *S*. Typhimurium in a sample can be developed by using automated instruments such as turbidimeters or fluorometers based on standard curves using known amounts of *Salmonella*.

BENEFIT TO THE PORK INDUSTRY: In summary, this novel RT-LAMP assay based on mRNA detection using *inv*A gene primers is specific for the detection of *Salmonella enterica* serovars that is applicable to pork products and the pork environment for routine diagnostics. The total detection time including enrichment is a maximum of two 8 hour work shifts requiring only a simple waterbath and portable turbidimeters or fluorometers for ease of analysis. The results are rapid, being obtainable within one day, and sensitive with current detection limits of 10^2 CFU/25g, that may have improved detection sensitivities by at least 1-log CFU by incorporation of fluorescent dyes. Based on the results of this study, cost-effective, rapid, sensitive and portable *Salmonella* detection assays will become available for routine testing of pork commodities. Rapid detection will prevent contaminated product from being released in the market. In addition, contaminated products can be isolated to prevent cross contamination of other pork commodities and food contact surfaces, thus preventing expensive recalls and associated litigation costs and will protect brand name. Also, any processing areas or equipment found contaminated with *Salmonella* can be immediately and effectively cleaned and improved mitigation strategies and HACCP plans can be implemented. Furthermore, if this study is extended to allow monitoring and surveying of additional local grocery stores and natural samples, it will provide data that can be used in future risk modeling assessment (for hazard identifications and tracking of sources of contamination).

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