PostDoc Journal
Vol. 1, No. 2, February 2013

Journal of Postdoctoral Research
www.postdocjournal.com

Rapid Detection of *Salmonella* in Bovine Lymph Nodes Using a Commercial Real-Time PCR System

Joshua Hill[1], Thomas S. Edrington[1*], Guy H. Loneragan[2], Sara E. Gragg[2], David J. Nisbet[1]

[1]United States Department of Agriculture, Agricultural Research Service, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit, 2881 F&B Road, College Station, Texas, 77845, USA
[2]International Center for Food Industry Excellence, Department of Animal and Food Sciences, Texas Tech University, Box 42141, Lubbock, Texas, 79409-2141, USA

*Email: tom.edrington@ars.usda.gov

Abstract

Rapid *Salmonella* detection is needed to help prevent the distribution of contaminated food products. Using traditional culture methods, *Salmonella* detection can take up to 3-5 days. Using an improved protocol and a commercial real-time PCR system, we have shortened the detection time to less than 24 hr with comparable sensitivity and specificity to traditional *Salmonella* culture methods.

Keywords: Salmonella, lymph node, cattle, detection, real-time PCR

Introduction

Salmonella infection in livestock can be transmitted to humans via associated products such as ground beef (Schneider et al. 2011) which may result in sporadic outbreaks of salmonellosis (Zansky et al. 2002). While generally a self-limiting illness, some cases require hospitalization or may even result in death (CDC 2011) with costs to society in the billions of dollars (Frenzen et al. 1999). Outbreaks of *Salmonella* infection also result in product recalls adding directly to industry costs. In beef processing facilities there has been great strides made to reduce both hide contamination (Serraino et al. 2012; Gill 2009; Carlson et al. 2008) and carcass contamination (Rekow et al. 2011) using post harvest interventions. Recently, there has been concern regarding *Salmonella* contained in bovine lymph nodes at the time of slaughter which can end up in ground beef. Initial studies indicate that *Salmonella* contaminated lymph nodes occur at a prevalence of 0.35% to 3.86% (Brichta-Harhay et al. 2012; Arthur et al. 2008). To date no pre- or post-harvest solution has been effective in dealing with *Salmonella* contained in non-mesenteric lymph nodes.

Traditional bacteriology methods for detecting *Salmonella* can be time consuming, requiring 3-5 days to complete and thus not suited to testing highly perishable goods. (Fricker 1987). Real-time PCR has been used successfully to detect *Salmonella* (Eyigor et al. 2002; Mainali et al. 2011; Suo et al. 2010) and is a relatively quick, robust method with many systems to choose from. We chose a commercially available platform (Pall GeneDisc, Pall Corp., Port Washington, NY USA) as it allows high-throughput at 480 results every hour from 96 samples (Pall n.d.) and is validated to meet Title 21 CFR 11 for electronic documentation (Pall n.d.). This system is based on real-time PCR amplification and detection, using proprietary master mixes that contain specific primer-probe combinations.

In this report, we used a commercial real-time PCR system as a starting point and designed a shortened protocol that allowed us to reliably detect the presence of *Salmonella* from cattle lymph nodes in less than 24hrs. This short turn-around time to detect *Salmonella* as compared to traditional culture methods may allow increased testing of highly perishable goods, thereby helping food production facilities quickly detect contaminated goods.

Materials and Methods

Reagents

The Pall GeneDisc system was used for all

experiments with either shiga toxin producing *Escherichia coli* (STEC) and *Salmonella* 12 sample or 6 sample disks. Molecular grade water was from Cellgrow (Corning, NY USA). Tetrathionate broth (Tet) and Rappaport-Vassiliadis (RV) broth were from Difco (Becton-Dickinson Sparks, MD USA). XLD agar, for isolation and differentiation of enteric pathogens, was from Oxoid (Thermo-Scientific, Basingstoke, Hampshire, England).

Ethics Statement

All experimental procedures performed in this study were in compliance with and approved by the United States Department of Agriculture, Agricultural Research Services, Food and Feed Safety Unit Animal Care and Use Committee. Animals were euthanized using Euthasol®, euthanasia solution (Delmarva Laboratories, Inc., Midlothian VA) and all efforts were made to minimize suffering.

Traditional bacterial culture

Previously challenged cattle, with either *S. typhimurium*, *S. montevideo*, *S. newport* and *S. enteritidis*, had the following lymph nodes removed; popliteal, prefemoral, prescapular and mandibular. Lymph nodes were trimmed of excess fat, dipped in boiling water for 3 sec and placed in a whirl-pak filter bag containing 20 ml Tet. The lymph node was then pulverized with a rubber mallet and placed in a stomacher for 60 sec after which 1 ml was reserved for spiral plating (Autoplate 4000, Spiral Biotech, Advanced Instruments Inc. Norwood, MA USA) on XLD agar and 1 ml was removed and DNA prepared per the DNA preparation protocol, then an additional 80 ml Tet was added. Both the Tet broth and XLD agar were incubated for 12 to 16 hrs at 37°C. After the incubation, from the Tet broth; 1 ml was removed and DNA prepared per the DNA preparation protocol and 100 µl was removed and added to 5 ml RV. The inoculated RV was then incubated 12- 16 hrs at 42°C. After incubation the enriched RV was then streaked on XLD agar and incubated overnight at 37°C.

DNA preparation

The 1 ml of reserved Tet enriched and un-enriched or RV enriched culture was placed in a 15 ml conical tube. This was centrifuged for 5 min at 500 x g to pellet the tissue debris and 500 µl of the centrifuged supernatant was transferred to a microcentrifuge tube. This was centrifuged for 5 min at 11,000 x g. The supernatant was removed and 200 µl of molecular grade water added. This was then boiled in a water bath for 10 min.

DNA detection

In a microcentrifuge tube 20 µl of Pall GeneDisc supplied master mix, 18 µl molecular grade water and 2 µl sample DNA combined for a total of 40 µl. This was vortexed briefly to mix and then centrifuged briefly to collect liquid at the bottom of the tube. 36 µl of this mix was used for each sample sector on a 12 sample GeneDisc. The rest of the protocol is as directed by the Pall GeneDisc machine in regards to applying vacuum and mineral oil.

	Kappa Statistic of Agreement				
	Agreements[4]	Chance[5]	Kappa	SE of Kappa[6]	Strength[7]
Post-Tet vs. Culture[1]	54 (100%)	32.3 (59.88%)	1	0	Perfect
Post-Tet vs. Culture[2]	26 (100%)	13.7 (52.66%)	1	0	Perfect
Pre-Tet vs. Culture[3]	26 (61.90%)	19.1 (45.58%)	0.3	0.098	Fair

Table 1

1. Experiment 2; Tet enrichment vs Traditional Salmonella culture
2. Experiment 3; Tet enrichment vs Traditional Salmonella culture
3. Experiment 3; no Tet enrichment vs Traditional Salmonella culture
4. Number of observations (percent agreement)
5. Number of agreements expected by chance (percent)
6. Standard error of Kappa
7. Strength of agreement (poor, fair, moderate, good, very good, perfect)

	Predictive Values and Sensitivity				
	Samples	PCR +	PPV[4]	NPV[5]	Sensitivity[6]
Post-Tet vs. Culture[1]	54	15	100%	0	100%
Post-Tet vs. Culture[2]	26	16	100%	0	100%
Pre-Tet vs. Culture[3]	26	8	100%	55.6%	50%

Table 2

1. Experiment 2; Tet enrichment vs Traditional *Salmonella* culture
2. Experiment 3; Tet enrichment vs Traditional *Salmonella* culture
3. Experiment 3; no Tet enrichment vs Traditional *Salmonella* culture
4. Formula: True Positive / (True Positive + False Positive)
5. Formula: True Negative / (False Negative + True Negative)
6. Formula: True Positive / (True Positive + False Negative)

Statistics

Kappa statistic of agreement calculated by QuickCalcs, GraphPad software Inc. La Jolla, CA USA.

Results

The ability to detect *Salmonella* contamination in food products has always been a concern. The ability to do it quickly and accurately has been of paramount importance. Our objective was to determine if the commercial real-time PCR system could detect *Salmonella* contamination of peripheral lymph nodes faster than traditional *Salmonella* culture methods. In the first experiment we wanted to determine the sensitivity of the commercial PCR system. From an overnight tryptic soy broth culture of *S. typhimurium* 10 fold dilutions were made and either spiral plated on XLD agar for enumeration or DNA prepared. The fewest number of *S. typhimurium* that were detected by the commercial real-time PCR system was 1.95 ± 1.77 S.D. (n = 3) per ml. Next, we examined the components of the traditional *Salmonella* culture, Tet or RV selective enrichment broths for inhibition of *Salmonella* detection and determined if certain serovars of *Salmonella* are able to be detected by the commercial real-time PCR system. To do this, we spiked 1ml Tet and RV broth with 1 µl of an overnight tryptic soy broth culture of common *Salmonella* serovars (*S. typhimurium*, *S. montevideo*, *S. newport* and *S. enteritidis*) previously cultured from cattle and then used our protocol to prepare the samples. All of the spiked samples were positive for *Salmonella* by the commercial PCR system.

In the second experiment, 54 non-mesenteric lymph nodes were collected from cattle at necropsy and analyzed in tandem with traditional *Salmonella* culture methods and with the commercial real-time PCR system. Samples were tested by the commercial real-time PCR system after Tet-RV enrichment. Of the 54 lymph nodes tested, 15 were confirmed positive for *Salmonella* after the traditional Tet-RV enrichment. Those same 15 were also positive for *Salmonella* by the commercial real-time PCR system after the first Tet enrichment and after the full Tet-RV enrichment.

Since we were able to detect *Salmonella* after the first Tet enrichment we wanted to determine if this enrichment step was necessary, further decreasing the detection time. In a third experiment another 26 non-mesenteric lymph nodes were collected and again analyzed in tandem with traditional *Salmonella* culture methods and with the commercial real-time PCR system before and after the Tet enrichment. Of the 26 lymph nodes, 8 were positive for *Salmonella* before and an additional 8 were positive after the Tet enrichment, by the commercial real-time PCR system. The same lymph nodes positive by the commercial real-time PCR system were also positive for *Salmonella* after traditional Tet-RV enrichment.

Discussion

Since this method is based on real-time PCR it is limited by the failings of all PCR systems, namely interference of inhibitors carried over from the tissue being sampled. The Pall GeneDisc system has built into the master mix and detection software positive and negative controls to help discover if PCR inhibitors are present. If the positive control does not produce a significant signal above background this could be a sign that PCR inhibitors are present. The original sample would then be diluted and re-run to help counter act the affect of the PCR inhibitors. Therefore, the ability of the operator to exclude tissue pieces from the DNA preparation starting material is important. This limitation may be overcome using a whole genome DNA extraction kit, but was not tested in this report. The sensitivity of the Pall GeneDisc, at approximately 2 cfu/ml, is on par with other real-time PCR assays for the detection of pathogens such as the Rickettsia group (Stenos et al. 2005) and Escherichia coli (Mohammadi et al. 2003)

Employing the Kappa statistic for the magnitude of agreement between two observations (Table 1) and applying it to commercial PCR post-Tet enrichment vs. traditional *Salmonella* culture, the result is 1, meaning there is perfect agreement between the two observations and not a chance agreement. In experiment 3 comparing the commercial PCR pre-Tet enrichment vs. traditional *Salmonella* culture, the result is 0.3, and the strength of the agreement is fair. If the positive predictive value (PPV) analysis (Table 2) to compare a gold standard test (tradition bacterial culture) to a new test assay (commercial real-time PCR system) is used and applied to experiment 2 and 3 (Tet enriched starting points), the PPV = 100%, NPV = 0% and sensitivity is 100%, meaning the real-time PCR system correctly identified *Salmonella* in 100% of the lymph nodes that were positive by tradition bacterial culture. Applying PPV to experiment 3 (no Tet enrichment), the PPV = 100%, NPV = 55.6% and the sensitivity is 50%, meaning the real-time PCR system was only 50% correct in identifying *Salmonella* in the lymph nodes that were positive by tradition bacterial culture. The Kappa statistic and sensitivity results would indicate that for the correct detection of *Salmonella* an initial Tet enrichment is necessary. Even with the Tet enrichment step, the detection of *Salmonella* in the lymph nodes was possible within 24 hr. The ability of this system to reliably determine the presence or absence of *Salmonella*, a full 72 hrs faster than traditional culture methods, may allow for food production facilities to sample perishable products more frequently and reduce the incidence of distributing contaminated foods.

Acknowledgements

This project was funded by the Beef Checkoff and the USDA National Institute of Food and Agriculture's National Integrated Food Safety Initiative Award No.: 2011-51110-31081. No additional external funding was received for this study. The authors gratefully acknowledge the excellent technical assistance of Kate Andrews, Reiley Street and Jim Snodgrass.

References

1. Arthur, T.M. et al., 2008. Prevalence and characterization of *Salmonella* in bovine lymph nodes potentially destined for use in ground beef. Journal of food protection, 71(8), pp.1685–8.

2. Brichta-Harhay, D.M. et al., 2012. Microbiological analysis of bovine lymph nodes for the detection of *Salmonella* enterica. Journal of food protection, 75(5), pp.854–8.

3. Carlson, B.A. et al., 2008. Comparison of Antimicrobial Efficacy of Multiple Beef Hide Decontamination Strategies To Reduce Levels of Escherichia coli O157:H7 and *Salmonella*. Journal of food protection, 71(11), p.5.

4. CDC, 2011. Surveillance for Foodborne Disease Outbreaks. MMWR., 60(35), pp.1197–1202.

5. Eyigor, A., Carli, K.T. & Unal, C.B., 2002. Implementation of real-time PCR to tetrathionate broth enrichment step of *Salmonella* detection in poultry. Letters in applied microbiology, 34(1), pp.37–41.

6. Frenzen, P.D. et al., 1999. *Salmonella* Cost Estimate Update Using FoodNet Data. Food Safety, 22(2), pp.10–15.

7. Fricker, C.R., 1987. The isolation of *salmonella*s and campylobacters. Journal of Applied

Microbiology, 63(2), pp.99–116.

8. Gill, C.O., 2009. Effects on the microbiological condition of product of decontaminating treatments routinely applied to carcasses at beef packing plants. Journal of food protection, 72(8), pp.1790–801.

9. Mainali, C., McFall, M.E. & King, R.K., 2011. Validation of a real-time polymerase chain reaction assay for the detection of *Salmonella* in crops of broiler chickens. Poultry science, 90(3), pp.660–4.

10. Mohammadi, T. et al., 2003. Optimization of real-time PCR assay for rapid and sensitive detection of eubacterial 16S ribosomal DNA in platelet concentrates. Journal of clinical microbiology, 41(10), pp.4796–8.

11. Pall, GeneDisc® Cycler. Available at: http://www.pall.com/main/Biopharmaceuticals/Product.page?id=55051 [Accessed December 27, 2012a].

12. Pall, GeneDisc® Rapid Microbiology System - Food Testing Applications. Available at: http://www.pall.com/main/Biopharmaceuticals/GeneDisc-Rapid-Microbiology-System-Food-52281.page [Accessed December 27, 2012b].

13. Rekow, C.L. et al., 2011. Implementation of targeted interventions to control Escherichia coli O157:H7 in a commercial abattoir. Meat science, 87(4), pp.361–5.

14. Schneider, J.L. et al., 2011. Multistate outbreak of multidrug-resistant *Salmonella* newport infections associated with ground beef, October to December 2007. Journal of food protection, 74(8), pp.1315–9.

15. Serraino, A. et al., 2012. Visual evaluation of cattle cleanliness and correlation to carcass microbial contamination during slaughtering. Meat science, 90(2), pp.502–6.

16. Stenos, J., Graves, S.R. & Unsworth, N.B., 2005. A Highly Sensitive and Specific Real-Time PCR Assay for the Detection of Spotted Fever and Typhus Group Rickettsiae. Am J Trop Med Hyg, 73(6), pp.1083–1085.

17. Suo, B. et al., 2010. A Multiplex Real-Time Polymerase Chain Reaction for Simultaneous Detection of *Salmonella* spp., Escherichia coli O157, and Listeria monocytogenes in Meat Products. Foodborne Pathog Dis., 7(6).

18. Zansky, S. et al., 2002. Outbreak of Multidrug-Resistant *Salmonella* Newport—United States, January-April 2002. JAMA, 288(8), pp.545–548.