LWT - Food Science and Technology 44 (2011) 1088–1097

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Real-time reverse-transcriptase PCR for *Salmonella* Typhimurium detection from lettuce and tomatoes

Nathan D. Miller, P.M. Davidson, Doris H. D'Souza*

University of Tennessee, Department of Food Science and Technology, 2605 River Drive Room, 102 FSPB, Knoxville, TN 37996, USA

ARTICLE INFO

Article history:
Received 21 January 2010
Received in revised form
5 August 2010
Accepted 6 August 2010

Keywords:
Salmonella
RT-PCR
Produce
Detection

ABSTRACT

Salmonella outbreaks linked to fresh produce stress the importance of rapid detection methods to help prevent disease. Real-time reverse-transcriptase-PCR (rt-RT-PCR) is based on the detection of mRNA (shorter half-life than DNA), showing promise of detecting viable pathogens while eliminating the need for gel electrophoresis. The research is aimed at applying rt-T-PCR to detect *Salmonella* from spiked lettuce and tomatoes within one day. Twenty-five grams of lettuce and ~100 g of tomatoes were inoculated with 1–8 log CFU of an overnight culture of *Salmonella* Typhimurium. Bacteria were recovered with 0.05 mol/L glycine–0.14 mol/L saline buffer (containing 0.05 g/100 ml Tween-20, 3 g/100 ml beef extract). For low inocula (5 log to 1 log CFU), a short pre-enrichment of 6 h in peptone buffer was carried out to improve assay sensitivity. Serial dilutions were spread plated on Xylose Lactose Tergitol 4 (XLT4) agar and incubated at 37 °C for 48 h and portions used for RNA extraction using the Qiagen RNeasy® Mini Kit. SYBR Green one step RT-PCR kit with *invA* gene primers and an internal amplification control was used for detection. Reaction conditions were 50 °C/40 min, then 94 °C/45 s, 58 °C/45 s, 72 °C/45 s for 45 cycles followed by melt temperature analysis. This rt-RT-PCR procedure could detect 4 log CFU/25 g *Salmonella* from lettuce and tomatoes (100 g) after pre-enrichment. Without pre-enrichment, *Salmonella* detection from lettuce was 6 log CFU/25 g and from tomatoes was ~6–7 log CFU/100 g. These results show that rt-RT-PCR can be used to detect *Salmonella* contamination in produce within 24 h.

1. Introduction

Fresh produce safety continues to be a growing global concern, with lettuce being implicated in several outbreaks associated with various serovars of *Salmonella* since the mid-90s. These lettuce outbreaks include *Salmonella* Heidelberg in 1993, and *Salmonella* Thompson and *Salmonella* Braenderup in 1994 (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). Among various other fresh produce related outbreaks, *Salmonella* was linked to tomatoes in 1990 (*Salmonella* Javiana) and 2006 (*Salmonella* Typhimurium; *Salmonella* Newport) (CDC, 2009; Sivapalasingam et al., 2004) as well as jalapeno peppers in 2008 (*Salmonella* Saintpaul) (CDC, 2008), along with other serotypes including *S.* Braenderup, *Salmonella* Montevideo, *Salmonella* Poona, *Salmonella*. Thompson, to name a few associated with produce and herbs (cilantro, etc) (D'Aoust and Maurer, 2007). Because currently reliable intervention methods for inactivating *Salmonella* in fresh produce are still being researched, detection systems play an important role in reducing the dissemination of contaminated products. It is therefore imperative to find more sensitive and rapid methods to detect *Salmonella* in fresh produce and the agricultural environment in which these products are grown.

Traditional cultural methods such as selective and differential microbiological media can effectively detect and identify *Salmonella* in foods. However, traditional methodology takes up to 4 days to determine the absence of *Salmonella* and if a presumptive positive is obtained another 3 days to confirm the presence and serotype the bacteria (Mercanoglu & Griffiths, 2005). While researchers have devised much faster and more accurate molecular methods for detection of foodborne pathogens (Feng, 2007), their actual application to food systems has lagged because of the complex nature of the food matrix. Food contains many potential inhibitors that may cause molecular methods, such as the polymerase chain reaction (PCR) process, to fail and/or yield false results (Maurer, 2006). Hence, improved nucleic acid extraction methods which can result in purer and higher yields of nucleic acids are needed to overcome these issues. Additionally, there is the potential for cross-reactivity or non-specific product amplification due to interactions with components of the food matrix.

Cross-reactivity can be avoided and specificity of the PCR process can be increased by targeting specific virulence genes of a particular microorganism that are not present in other species. The *invA* gene

* Corresponding author. Tel.: +1 865 974 2753; fax: +1 865 974 7332.
E-mail address: ddsouza@utk.edu (D.H. D'Souza).

0023-6438/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.lwt.2010.08.003

that allows *Salmonella* to target epithelial cells of the intestinal wall (Rahn et al., 1992) is exclusively present on the chromosome of all invasive strains of *Salmonella* (Perera & Murray, 2008). The *inv*A gene is absent from other closely related organisms including *Escherichia coli* (Baumler, Tsolis, Ficht, & Adams, 1998) and other foodborne pathogens, making it a good target for the specific detection of *Salmonella* by molecular assays.

Typical DNA based molecular methods do not distinguish between live and dead cells. The suitability of using reverse-transcriptase-PCR (RT-PCR) for the determination of *Salmonella* viability has been investigated by several researchers by targeting the *inv*A mRNA (Fey et al., 2004; Gonzalez-Escalona et al., 2009; Jacobsen & Holben, 2007). Not only does RT-PCR allow for the potential detection of live infectious cells, it is useful to detect recent contamination as mRNA has a shorter half-life than DNA (Maurer, 2006). Rapid detection of *Salmonella* contamination in fresh produce by real-time RT-PCR (rt-RT-PCR) will not only assist with controlling dissemination of contaminated product but could assist in development of control strategies and as a validation tool/process for effective HACCP plans.

The objective of this research was to determine the sensitivity of a real-time RT-PCR method for the detection of *Salmonella* on lettuce and tomatoes and if the method could detect the pathogen within one day. The RT-PCR method developed evaluated the use of an SYBR Green I dye that is less expensive than currently used fluorescent probes and the previously described *invA* primers that are specific for *Salmonella* species (D'Souza, Critzer, & Golden, 2009). High (8 log to 6 log CFU/ml) and low (5–1 log CFU/ml) levels of inocula were used for spiking lettuce and tomatoes.

2. Materials and methods

2.1. Growth of Salmonella enterica serovar Typhimurium

Salmonella enterica serovar Typhimurium (DT104 2486; UT Cultural Collection) was grown in 10 ml of trypticase soy broth (TSB; Difco, Becton Dickinson and Company, Sparks, MD, USA) at 37 °C for 24 h, and used after transferring two consecutive times at 24 h intervals. Ten-fold serial dilutions of an overnight culture in 9 ml of peptone buffer (Oxoid LTD; Basingstoke Hampshire, England) per tube were spread plated on trypticase soy agar (TSA; Difco, Accumedia, Lansing, MI, USA) and xylose lactose tergitol 4 (XLT4) agar and incubated at 37 °C for 24–48 h and colonies were enumerated.

2.2. Preparation of lettuce and tomatoes

Fresh Iceberg lettuce (*Lactuca sativa)* and Roma tomatoes (*Solanum lycopersicum)* used in this study were purchased at a local grocery store. Prior to inoculation, the produce was washed for 1 min using tap-water and placed in an open sterile Petri dish in a BSL-2 hood to dry under ultraviolet light for 10 min at room temperature (25 °C). Lettuce (25 g) and tomatoes (~100 g) were individually inoculated with 0.1 ml high (6–8 logs) and low inocula (0–5 logs) of *Salmonella* Typhimurium and allowed to dry for 10 min at room temperature. Washed and dried uninoculated produce were used as controls and each experiment was replicated at least twice.

2.3. Recovery and extraction of bacteria from inoculated lettuce and tomatoes

Bacteria were removed from the produce by washing with 0.05 mol/L glycine–0.14 mol/L/saline buffer (pH 9.0) (glycine, Fisher Scientific, Fair Lawn, NJ, USA), glycine–saline buffer with 0.05 g/100 ml Tween-20 (Promega; Madison, WI, USA) or glycine-saline buffer with 0.1 g/100 ml Tween-20 and 3 g/100 ml beef extract. The various wash solutions were used to compare the efficiency of the removal of bacterial cells from the produce. Fifty-ml of each buffer was added to the lettuce and tomatoes in a sterile stomach bag. The tomatoes were gently hand rubbed for 1 min within the stomacher bag under aseptic conditions in the BSL-2 hood and stomached at 230 rpm for 30 s twice (Stomacher® 400 Seward; England). The lettuce samples were also stomached at 230 rpm for 30 s twice. Fifty-ml samples of rinse buffer were centrifuged at 8000*g* for 10 min at 4 °C (Sorvall Legend 23 R; Thermo Electron Corporation; Asheville, NC, USA) and the pellet was resuspended in 1 ml of 0.1 g/100 ml peptone buffer for enumeration by plating and also for isolating RNA.

2.4. Enumeration of bacteria

2.4.1. Traditional plating of Salmonella from produce

A short pre-enrichment process was carried out for produce inoculated with low inocula levels. Lettuce was stomached and tomatoes were gently hand rubbed and then stomached as described above in 225 ml of sterile peptone water (Oxoid) and incubated for 6 h at 37 °C. Following incubation, the entire rinse solution was centrifuged at 8000*g* for 10 min at 4 °C (Sorvall) and the cell pellet was resuspended in 1 ml of 0.1 g/100 ml peptone buffer. Both, the enriched and non-enriched samples were serially diluted and spread plated on XLT4 agar and incubated at 37 °C for 24–48 h to enumerate and detect typical black colonies of *Salmonella* as described earlier (Miller, Draughon, & D'Souza, 2010).

2.4.2. Addition of stressed and autoclaved cells to produce

Portions of 24 h grown *S.* Typhimurium were also cold stressed at −20 °C and 4 °C and heat-stressed at ~45 °C for 24 h and inoculated on produce as described above to determine the ability of this assay to detect stressed cells. Another portion was also heat inactivated in an autoclave at 121 °C for 15 min and inoculated on produce. Recovery was determined either by direct plating on XLT4, pre-enriching and plating on XLT4, or assaying by real-time RT-PCR.

2.5. Isolation of total RNA from bacteria

RNA from the samples was first stabilized by adding 1.2 ml of RNA later Stabilization Reagent (Qiagen) to 0.6 ml of each sample, incubated at room temperature for 10 min and vortexed several times as described before (Miller et al., 2010). The cells were centrifuged (Eppendorf 5417C; New York, NY) for 10 min at 8000*g* at 4 °C and the pellet was either stored at −80 °C or used directly for extraction.

The RNeasy Mini Purification Kit (Qiagen; Valencia, CA) was used to extract RNA from the pure culture of *Salmonella* Typhimurium, inoculated and enriched samples, and non-enriched lettuce and tomato samples, uninoculated lettuce and tomato (negative controls), and uninoculated sterile water and TSB. The manufacturer's instructions were followed as described earlier (Miller et al., 2010). RNA was eluted from the column using 40 μl of RNase-free water and stored at −80 °C until further analyses.

2.6. DNase I treatment

DNase I treatment was carried out using the TURBO DNA-free™ Kit (Ambion®; Applied Biosystems; Foster City, CA, USA) as previously described (Miller et al., 2010). The treated RNA sample was stored at −80 °C until use.