Comparison of Enumeration Methods for *Salmonella* spp. Contaminated in Agricultural Samples

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ABSTRACT

Salmonella spp. infections associated with vegetables crops have occurred with increasing frequency in recent years. Conventional methods (plate count) for detection of Salmonella spp. is time and labor consuming. Nowadays, the advanced molecular techniques especially real-time PCR (RT-PCR) is popular for estimating the quality and quantity of bacterial population. The aim of this study was to develop a method for detection of Salmonella spp. as guideline for rapid and accurate determination. The comparison of enumeration methods included BAM (Bacteriological Analytical Manual), plate count technique, and RT-PCR using pure cultures of S. typhimurium ATCC 13311 derived from the Department of Medical Science, Ministry of Public Health of Thailand. RT-PCR was the most accurate method to quantify the contamination of S. typhimurium ATCC 13311 (9.40 log CFU/mL) which was significantly comparable to that of plate count technique (8.94 log CFU/mL). These methods were also done in determination of agricultural samples. RT-PCR could detect contamination in all 100 samples whereas BAM method could detect only 25 samples. Moreover the RT-PCR has the advantage of fast (4:30 h), precise and occupied small space to work.

Key words: *Salmonella* spp., Real-time PCR, Agricultural samples

INTRODUCTION

Salmonella spp. is one of the most important foodborne human pathogens of animal origin (Altekuse et al., 1997). The bacterial pathogens can be transported from feed lots to field plots where vegetables are grown or through storm water after a heavy rainfall washing infected manure into the wells of the farming community and contaminating ground water and soil. In Thailand, 118,292 people were ill with diarrhea and 96,383 people from food poisoning. Two died in the Northeast provinces in 2009. Conventional methods that commonly used to detect bacterial pathogens are based on microbiological techniques. Even though these methods are still effective for the recognition of microorganism, they are rather time consuming requiring several days for completion. The development of other methods would allow a rapid and sensitive detection of *Salmonella* spp. in agricultural samples. Recently, several molecular methods have been described or reviewed for direct identification of *Salmonella* spp. in food and environmental samples. The real-time PCR (RT-PCR) technique can be both advantages for quality and quantity assessment. It does not require the post PCR analysis such as gel-electrophoresis (An et al., 2006) eliminates the risk of cross-contamination, reducing both the amount of work and analysis time (Omiccioli et al., 2009). Among the techniques base on rDNA which are suitable for quantifying bacteria, SYBG Green I RT-PCR provides a precise and sensitive method (Hein et al., 2001). This technique requires non-specific probe and classic PCR protocols can be easily adapted. The non-specific double-stranded DNA binding dye fluorescent and allows monitoring of the amplified product at each PCR cycle. Klerks et al. (2004) to compare different RT-PCR-based methods for detection of either *Salmonella* spp. or *E. coli* O157:H7 with respect to sensitivity, precision and accuracy. In addition, a general internal amplification control (IAC) is presented, allowing prevention of false negative results. The quantification threshold of the methods in which the IAC was included was determined at 1 pg of target DNA (equal to 200 CFU) per reaction. Qualitative detection was feasible down to 10 fg of target DNA per reaction using both methods in which the IAC was incorporated. Qualitative detection was feasible down to 10 fg of target DNA per reaction. Omiccioli et al. (2009) detected *Salmonella* spp., *Listeria monocytogenes* and *E. coli* O157 in milk by RT-PCR. The results indicated that this method represents the reduction of the turnaround time and workload.

The research was aimed to develop a rapid and accurate technique to quantify *Salmonella* spp. contamination in agricultural samples to substitute conventional methods.

MATERIALS AND METHODS

Enumeration of reference culture

A reference culture, *S. typhimurium* ATCC 13311 derived from Department of Medical Science, Ministry of Public Health of Thailand (Nonthaburi, Thailand) was used as positive control. It was enriched in Trypticase soy broth (TSB). The quantity of bacteria was 10-fold serially diluted and determined by plate count using Xylose lysine desoxycholate (XLD). Plates were incubated at 37±1°C for 24±2 h.

Conventional method

One mL of *S. typhimurium* ATCC 13311 cultured broth was mixed to 10 mL Tetrathionate broth base broth (TT) and 0.1 mL to 10 mL Rappapor-vassiliadis medium (RV). Both media were incubated at 42±1°C for 24±2 h. Isolation of *Salmonella* spp. in both media were done by streaking a loopful (10 µL) of samples on Bismust sulfite agar (BS), XLD agar and Hektoen enteric agar (HE). These agar plates were incubated at 35±1°C for 24±2 h. The colony morphology of bacteria grown on selective media was determined by their color; brown, gray or black with sometimes a metallic sheen performing on BS agar, pink with or without black centers or black colony on XLD agar, blue-green to blue colony with or without black centers or black on HE agar.

RT-PCR method

DNA extraction

An aliquot of 1 mL of 10^{-1} from each pure culture was transferred to 1.5 mL microcentifuge tube. After centrifugation at 12,000 rpm, 4°C for 5 min the supernatant was discarded and the pellet was washed twice with 1M NaCl. Five hundred µL of Tri-EDTA buffer (TE) was pipetted into the microcentrifuge tube, vortex to resuspend and centrifuge at 12,000 rpm for 5 min at 4°C. The supernatant was discarded and 500 µL TE buffer with 0.1% Tween20 were added, and heated at 90°C for 10 min. The mixed suspension was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was collected and transferred into a new tube. This supernatant was used as template DNA.

Standard curve

The standard curve was modified from An et al. (2006): *S. typhimurium* ATCC 13311 was enriched in TSB broth, incubated at 35±2°C for 18±2 h. The freshly prepared *S. typhimurium* ATCC 13311 was harvested and serially diluted in 10-fold with distilled water (10^{-1} to 10^{-9}). Aliquot of 1 mL from the 10^{-5} to 10^{-9} of each serial dilution was spread plate in BS, XLD and HE agar and made triplicate. The bacterial quantity was enumerated after plates were incubated at 37±2°C for 24±2 h. Its DNA extracted from serial dilution of 10^{-5} to 10^{-9} were followed the DNA extraction in DNA extraction step.

Optimization of RT-PCR condition

Preparing the PCR conditions, the template DNA was (2 µL) mixed with 10 µL of 1X DyNAmoTM HS SYBR Green qPCR kit. One µL of 0.5 µM of each primer for ttr-6 (forward) and ttr-4 (reverse) (Malorny et al., 2004). The negative control preparation was as same as the samples but without primers and template DNA. The RT-PCR tubes were covered with flat cap strips, and they were centrifuged briefly to bring all reagents to the bottom. Amplification reaction was performed using the following program with CHROMO4 RT-PCR instrument: 10 min at 95°C; 40 cycles of (10 s at 94°C, 30 s at 60°C, 30 s at 72°C, followed by a 5 min extension at 72°C and a hold at 4°C). Data analysis was performed using CHROMO4 RT-PCR detection system software.

Enumeration of *Salmonella* spp. contaminated in agricultural samples

Sample collection

Samples were collected from four provinces in Thailand; Kanchanaburi, Phitsanulok, Nakhon Sawan and Chiang Mai. The choice was based on the fact that these areas are major producers of vegetables for both domestic consumption and export. In these four provinces, samples were collected from wholesale and retail markets, shelves, plantations and farms, and Center of Agricultural Research (100 samples). The samples of soil, water, organic fertilizers, and feces amounting to 32, 17, 9 and 16 samples, were additionally collected respectively.

Serial dilution preparation

Vegetable samples were cut to 1 cm but other samples were not cut because they were already small. These samples were divided to 25 g, put into the stomached bag (Stomached 400 mL) containning 225 mL TSB and left about 5 to 10 min before detection. For water samples, 1 mL was used to mixed in 9 mL of Butterfield's phosphate buffer (BPB) to be 10^{-1}. The mixture was shaken for 10 min.

Statistical analysis

All data counts were converted to log10 count before statistical analysis. All data were analyzed by statistix 8.0 (Tallahassee, FL, USA). Data were analyzed by analysis of variance (ANOVA) using a completely randomized design. The differences between the treatments were examined by Fishers Least significant difference (LSD) test at a significance level of 95%. The correlation coefficient for methods was analyzed using Pearson correlation coefficient.

RESULTS AND DISCUSSION

Enumeration of a reference culture, *S. typhimurium* ATCC 1311 with plate count, BAM and RT-PCR, indicated that there was no significant difference between plate count and RT-PCR ($P>0.05$) with the value of 8.94±0.00 log CFU/mL and 9.40±0.32 log CFU/mL, respectively. In the case of BAM method, generally indicates the occurring of microbe as positive or negative only (Table 1). In agricultural samples, RT-PCR could detect the quantity of contamination of all samples while BAM method could determine only 25 from 100 samples (data not shown). The results of comparison of working steps and consuming time between BAM and RT-PCR methods showed that BAM consumed 49 h whereas RT-PCR using only 4:30 h. In contrast, the budget for 1 sample of BAM was 338.73 Bahts which was cheaper than that of RT-PCR (724.01 Bahts) (Table 2). Although, the short duration of the detection finished within 49 h but in fact this method requires 3 days to yield a confirmed negative result and over 5 days for a confirmed positive result (Ferretti et al., 2001). At present, there were several methods for detection of *Salmonella* spp. such as ISO 6579 (07/2002), U 47/100(07/2002), AIASKA method, and DIN EN 12824 (German and European standard), these methods require selective media which take longer time more than RT-PCR. Lepper et al. (2002) had developed method of detection of *Salmonella* spp. in food based on enrichment and isolation on selective media. AOAC International has approved a rapid automatic method, using the VIDAS® Immuno-Concentration *Salmonella*, which captures *Salmonella* cells for subsequent detection by the VIDAS immunofluorescent assay. The VIDAS ICS method was shown to be conventional and considerably faster than the BAM procedure for detection, but it could not quantify the pathogen and take longer time than RT-PCR. Many studies have used RT-PCR for detection of *Salmonella* in several samples. More recent studies have reported the successful application of developed RT-PCR assays for the detection of *Salmonella* in naturally-contaminated samples (Catarame et al., 2006). They developed and successfully applied a RT-PCR method for the Light Cycler instrument in the detection of *Salmonella* in naturally contaminated meat samples within a much shorter time than the standard culture method, i.e., within 27-28 h. Liming and Bhagwat (2004) applied RT-PCR to detected *Salmonella* species. They could detect as few as 1-4 CFU per reaction. A comparison of two commercially available kits utilizing MB-PCR (iQ-Check, Bio-Rad laboratory) and conventional (AOAC)-approved PCR was performed on artificially inoculated product. As few as 4 CFU/25g of product were detected after 16 h of enrichment in buffered peptone broth.

Table 1. Comparison of *S. typhimuriun* ATCC 1311 population in plate count, BAM and RT-PCR cultivation techniques.

Test methods	*S. typhimurium* ATCC 1311[1]
Plate count	8.94±0.00 (log CFU/mL)
BAM	detected
RT-PCR	9.40±0.32 (log CFU/mL)
F-test	ns
C.V.	2.43

[1] Each value represents mean ± standard deviation of three replications. Means with the same letter within column are not significantly different at the $P \leq 0.05$ by least significant difference. The “ns” is no significance.

Table 2. Working steps and time consuming of *Salmonella* spp. detected by BAM and RT-PCR methods in one sample with triplicate.

Test methods	Steps	Time (h)	Total (h)	Budget (Baht)
Conventional	Preparation sample	1		
	Enrichment	24		
	Isolation	24	49	338.73
RT-PCR	Preparation sample	1		
	DNA extraction	0.3		
	RT-PCR test	3	4.3	724.01

Catarame et al. (2006) compared BAM and RT-PCR for the detection of *Salmonella* in Irish beef, chicken, pork and turkey. They found that developed 16s rRNA gene-based RT-PCR assay demonstrated comparable specificity and sensitivity to the currently used standard culture method but was considerably more rapid. This method dramatically reduces the time required to detect *Salmonella* in agricultural samples when comparison with the standard culture method (BAM). In addition, some selective agents employed are expensive, toxic and unpleasant to use. The elimination of a pre-enrichment step increases the overall speed of the detection method. For this study, time saving could be of benefit when information on the presence of *Salmonella* in export vegetables could be reported rapidly because fresh vegetables could easily get rotten.

CONCLUSION

RT-PCR method could also be used to quantify the *Salmonella* spp. contaminated in agricultural samples but the BAM method showed no potential for enumeration. The advantages of RT-PCR is enable to reduce the reduction of materials, area, labor, time, and device. Additionally, it could be used to manage for many samples in one day for laboratory service.

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