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Evaluation of PCR inhibitory effect of enrichment broths and comparison of DNA extraction methods for detection of *Salmonella* Enteritidis using real-time PCR assay

Ji-Yeon Hyeon[1], In-Gyun Hwang[3], Hyo-Sun Kwak[3], Chankyu Park[4], In-Soo Choi[2], Kun-Ho Seo[1,*]

[1]Department of Public Health, and [2]Department of Infectious Diseases, College of Veterinary Medicine, Konkuk University, Seoul 143-701, Korea
[3]Korea Food and Drug Administration, Seoul 122-704, Korea
[4]Department of Animal Biotechnology, College of Animal Bioscience and Technology, Konkuk University, Seoul 143-701, Korea

The best enrichment broth and DNA extraction scheme was determined for rapid and sensitive detection of *Salmonella* Enteritidis in steamed pork using real-time PCR. The inhibitory effect of commonly used *Salmonella* enrichment broths, Rappaport-Vassiliadis (RV) and Muller-Kauffmann tetrathionate with novobiocin (MKTTn), on real-time PCR was confirmed. The inhibition of PCR was statistically significant (p < 0.05) in RV and MKTTn, as compared with buffered peptone water (BPW) or phosphate-buffered saline. The inhibitory effect of the selective enrichment media was successfully removed by using a modified DNA extraction, PrepMan Ultra Reagent with an additional washing step or the DNeasy Tissue Kit. In three experiments, when applied to detection of *Salmonella* Enteritidis in steamed pork, the real-time PCR coupled with single 24 h enrichment with BPW performed better than double 48 h enrichment with BPW plus RV or MKTTn. The simple real-time PCR assay using BPW proved to be a rapid and sensitive test for detection of low concentrations of *Salmonella* Enteritidis in steamed pork samples as compared with the conventional culture method.

Keywords: enrichment broth, inhibition, real-time PCR, *Salmonella*

Introduction

Salmonellosis is one of the most widespread infectious diseases in the world and causes high incidences of gastrointestinal food poisoning [3,12]. The surveillance for the presence of *Salmonella* in food has therefore become routine all over the world for preventing foodborne illness [2]. The detection of pathogens in food is critical for the diagnosis of food poisoning and monitoring of food safety [15]. Real-time polymerase chain reaction (or real-time PCR) is currently used as a rapid and reliable tool for detection of *Salmonella* [2,3,9]. One major difficulty with PCR is the presence of compounds that inhibit the PCR reaction. These compounds can contaminate the DNA templates extracted from food samples or environmental samples such as air, soil, and water, and may in turn generate false-negative results [7,19]. Therefore, evaluation and elimination of PCR inhibitory compounds are important steps in the development of PCR and real-time PCR assays [1].

The PCR procedure is sufficiently sensitive such that, in theory, only a few template molecules are required to initiate the synthesis reactions [15,16]. However, an enrichment step is still required to detect small numbers of *Salmonella* in food samples. This step may consist of non-selective enrichment with buffered peptone water (BPW) and selective enrichment with Rappaport-Vassiliadis (RV) or tetrathionate [6]. These enrichment broths have been directly utilized for *Salmonella* DNA template preparation. However, limited research has been conducted to quantitatively evaluate the effects of the enrichment broths using conventional PCR assays and even less using a real-time PCR protocol. Therefore, identifying and eliminating the PCR inhibitory effects of the enrichment broths is key to enhancing the performance of PCR assays in detecting *Salmonella* in foods.

The aims of this study were to determine the inhibitory effects of enrichment media on detecting *Salmonella* spp. using real-time PCR and to compare the abilities of different DNA extraction methods to reduce the inhibitory effects. In addition, an optimum enrichment medium and DNA extraction method to detect *Salmonella* spp. in food

*Corresponding author
Tel: +82-2-450-4121; Fax: +82-2-450-3037
E-mail: bracstu3@konkuk.ac.kr

(steamed pork) using real-time PCR assays were evaluated.

Materials and Methods

Bacteria

Overnight cultures of *Salmonella enterica* subsp. *enterica* serotype Enteritidis (SE) from our laboratory stock, originally from the Food and Drug Administration (USA), were prepared by growing the stock culture in tryptic soy broth (TSB; Difco, USA) overnight at 37^{o}C. Viable SE counts were obtained by 10-fold serially diluting the overnight cultures in phosphate-buffered saline (PBS, pH 7.2) and plating 100 µL of the dilutions on nutrient agar (Difco, USA). The plates were incubated at 37^{o}C overnight and the SE counts then enumerated.

DNA preparation

DNA templates for real-time PCR were extracted from 1 mL of each sample in centrifuge tubes for all experiments using PrepMan Ultra Reagent (Applied Biosystems, USA), except for an experiment to compare the DNA extraction methods. Tubes containing samples were centrifuged at 16,000 × g for 3 min, and the supernatants were aspirated and discarded. The cell pellets were resuspended in 200 µL PrepMan Ultra Reagent. Samples were boiled for 10 min and cooled at room temperature for 2 min. After centrifugation at 16,000 × g for 3 min, the supernatants were collected in new tubes for use as DNA templates.

Real-time PCR

For real-time PCR reactions, the extracted DNA (5 µL) was added into 20 µL of PCR mixture, except for an experiment in which culture media was directly added into the PCR mixture (45 µL). The mixtures contained Taqman Universal PCR Master Mix (12.5 µL; Applied Biosystems, USA), forward primer (2.5 µL, 300 nM), reverse primer (2.5 µL, 900 nM), and probe (2.5 µL, 200 nM). The sequences for the *Salmonella*-specific oligonucleotide primers and probe were designed to amplify a 94-bp segment of the *ttr* gene (GenBank accession no. AF 282268) and are described in Table 1 [9].

The optimized real-time PCR protocol for SE developed by Primer Express (Applied Biosystems, USA) was used, specifying two holding periods, one at 50^{o}C for 2 min and another at 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 60 sec. The threshold cycle (Ct), which is the intersection between each fluorescence curve and a threshold line, was calculated using ABI 7500 Software (Applied Biosystems, USA).

Addition of culture media into PCR mixture

DNA was extracted from 1 mL of an overnight culture of SE (10^9 CFU/mL) in TSB using PrepMan Ultra Reagent. Each enrichment medium, i.e. BPW (Difco, USA), RV (bioMériuex, France), Muller-Kauffmann tetrathionate with novobiocin (MKTTn; bioMériuex, France), and PBS as the control, was added in amounts of 1, 2, 3, or 5 µL, respectively, into the PCR mixture (45 µL). PCR mixtures contained DNA template extracted from SE cells (5 µL), Taqman Universal PCR Master Mix (25 µL), forward primer (5 µL, 300 nM), reverse primer (5 µL, 900 nM), and probe (5 µL, 200 nM). In this way, we were able to assess the level of the inhibitory effect of each enrichment broth on the PCR reaction. The final reaction volumes of all samples with each medium were adjusted to 50 µL by adding sterile distilled water accordingly to each PCR tube, and real-time PCR was performed for all samples as described previously. Two trials of the same experiment were performed with quadruplicate samples.

Dilution of overnight culture with media

Overnight cultures (0.1 mL) of SE in TSB were diluted with 0.9 mL of the enrichment broths BPW, RV, or MKTTn or with 0.9 mL PBS which served as the control. These diluents were prepared to evaluate the enrichment broths with regard to the sensitivity of the real-time PCR assay for the same number of SE cells in each medium. DNA templates were extracted from all samples using PrepMan Ultra Reagent, followed by real-time PCR. Three trials of the same experiment were performed with quadruplicate samples.

Comparison of DNA extraction methods

Overnight cultures of SE were grown in BPW, RV, and MKTTn broth at 37^{o}C. DNA templates for the real-time PCR assays were extracted from 1 mL of each sample in centrifuge tubes. Three different DNA extraction methods, PrepMan Ultra Reagent, PrepMan Ultra Reagent w/additional washing step and the DNeasy Tissue Kit (Qiagen, England), were compared for each of the three enrichment broths.

The PrepMan Ultra Reagent method was performed as described in the DNA preperation. The PrepMan Ultra

Table 1. Primers and probes sequences used for *Salmonella*-specific real-time PCR assay

Designation	Sequence	Position	Reference
ttr-6 (forward)	CTC ACC AGG AGA TTA CAA CAT GG	4287-4309	
ttr-4 (reverse)	AGC TCA GAC CAA AAG TGA CCA TC	4359-4381	[9]
ttr-5 (Probe)	Cy3-CAC CGA CGG CGA GAC CGA CTT T-BHQ3	4336-4356	

Reagent protocol with an additional washing step was performed as follows. After centrifugation of the overnight culture at 16,000 × g for 3 min, the supernatant was discarded. Then, 1 mL of sterile distilled water was added, followed by vigorous vortexing and centrifuging, and the pellet was resuspended in 200 μL PrepMan Ultra Reagent. The sample was boiled for 10 min and cooled at room temperature for 2 min. After centrifugation at 16,000 × g for 3 min, the supernatant was collected in a new tube for use as the DNA template. The DNeasy Tissue Kit protocol for gram-negative bacteria was performed in accordance with the manufacturer's instructions. The DNA template was eluted in sterile distilled water.

Real-time PCR was performed on samples from the three DNA extraction methods and the Ct values for the three methods were compared for each of the enrichment broths. Four trials of the same experiment were performed with quadruplicate samples.

Food sample preparation

Steamed pork was purchased from a grocery in Seoul, Korea. The bulk sample (500 g) was inoculated with low levels of SE inocula (7, 14, and 24 CFU/ 500 g), mixed well, and divided into 20 samples (25 g each). Each portion was aseptically placed into a sterilized stomacher bag to detect SE by conventional methods and real-time PCR. A positive control was prepared by spiking 25 g of the sample homogenate with approximately 10^8 CFU/mL of SE. For negative controls, uninoculated food (25 g) and sterilized PBS (25 mL) were also prepared. After overnight stabilization at 4°C, all 22 samples, including the control samples, were mixed with 225 mL BPW followed by homogenization in a laboratory Stomacher blender for 30 sec and incubation at 37°C for 24 h.

SE detection in food samples using culture method and real-time PCR

One hundred microliters of enriched BPW were added to 10 mL of RV and MKTTn and incubated at 42°C for 24 h for selective enrichment.

For the culture method, a loopful of RV broth was streaked onto xylose lysine deoxycholate agar (Difco, USA), and the plates were incubated overnight at 37°C. Presumptively positive samples, black colonies on the XLD plate, were confirmed as SE by reactions on triple sugar iron (Difco, USA) slants and with "O" and "H" antisera (Difco, USA).

For real-time PCR, DNA templates were extracted from 1 mL samples of pre-enriched BPW, RV, and MKTTn in centrifuge tubes, using both PrepMan Ultra Reagent and PrepMan Ultra Reagent with an additional washing step as previously described.

Statistical analysis

The Ct values of all experiments were analyzed statistically with InStat software (Version 3.05; GraphPad, USA). The Ct value of each trial and *p* values for comparisons between the control group and the experiment groups were analyzed in each experiment.

Results

Inhibitory effect of culture media on PCR

PCR inhibitory effect of enrichment media on detection of SE was assessed by directly adding different amounts of media into the PCR mixture. When the media were directly added into the PCR mixtures, the PCR reactions were inhibited, as indicated by higher Ct values as the volume of RV and MKTTn added to the mix was increased (Table 2). The inhibitory effect of BPW on the PCR reaction was

Table 2. Inhibitory effect of different volumes of *Salmonella* enrichment media added into real-time PCR mixture on detection of *Salmonella* Enteritidis (n = 4)

Volume (μL)	Ct ± SD				
	Trial	PBS (control)	BPW	RV	MKTTn
5	Trial 1†	17.8 ± 0.0	18.23 ± 0.1	34.5 ± 0.5*	ND
	Trial 2‡	18.3 ± 0.0	18.7 ± 0.0	31.5 ± 2.5*	ND
3	Trial 1	17.8 ± 0.1	18.2 ± 0.0	18.7 ± 0.1	28.2 ± 0.4*
	Trial 2	18.3 ± 0.1	18.5 ± 0.0	18.4 ± 0.17	29.0 ± 3.6*
2	Trial 1	17.9 ± 0.0	18.1 ± 0.0	17.0 ± 0.0	20.1 ± 1.8*
	Trial 2	18.5 ± 0.0	18.5 ± 0.0	17.4 ± 0.1	21.0 ± 0.1
1	Trial 1	17.9 ± 0.0	18.1 ± 0.0	17.1 ± 0.1	18.0 ± 0.1
	Trial 2	18.5 ± 0.0	18.5 ± 0.0	17.4 ± 0.0	18.3 ± 0.0

PBS: phosphate-buffered saline, BPW: buffered peptone water, RV: Rappaport-Vassiliadis, MKTTn: Muller-Kauffmann tetrathionate with novobiocin, Ct: threshold cycle, ND: not detected. *The Ct values are statistically different from those for PBS in the same trial ($p < 0.05$). †The number of SE cells used in Trial 1 was 1.3×10^9 CFU/mL. ‡The number of SE cells used in Trial 2 was 1.2×10^9 CFU/mL.

similar to that of the PBS control (Table 2). No PCR inhibition was observed even when 5 μL of BPW was added into the PCR mixture. The Ct values increased from 17.1 to 34.5 in Trial 1 and from 17.4 to 31.5 in Trial 2 when the volume of RV added into the PCR mixture was raised from 1 μL to 5 μL (Table 2). Differences between these values and those of the PBS control were statistically significant ($p < 0.05$). When 2 μL or higher of MKTTn was added, statistically significant differences in Ct values compared with PBS were observed, and SE was not detectable in the reaction mixture when 5 μL of MKTTn was added (Table 2).

Effect of *Salmonella* enrichment media on the sensitivity of real-time PCR

The effect of *Salmonella* enrichment media on the sensitivity of real-time PCR was determined by comparing the Ct values of equal numbers of SE cells in the different media and using the same DNA extraction method, the PrepMan Ultra Reagent protocol (Table 3).

Although there were no statistical differences between the mean Ct values of BPW and those of PBS in all trials, the mean Ct values of RV were statistically different from those of PBS in 2 out of 3 trials, and the net differences in mean Ct values between PBS and RV were 3.6 in Trial 1 and 1.8 in Trial 3 (Table 3). The mean Ct values of MKTTn were statistically different from those of PBS in all 3 trials. The net differences in mean Ct values between PBS and MKTTn were 5.8, 4.3, and 12.8 in each trial (Table 3).

Comparison of DNA extraction methods for eliminating PCR inhibitory effect of enrichment media

The current PrepMan Ultra Reagent method was modified by adding an extra washing step, with the expectation of further eliminating potential PCR inhibitors that may be present in the *Salmonella* enrichment media. The DNeasy Tissue Kit, which includes several washing steps, was also used as an alternative DNA extraction method. The mean Ct values obtained from four trials using overnight cultures of SE grown in BPW, RV, and MKTTn broth along with the unmodified DNA extraction method were 16.9, 19.1, and 30.4, respectively, with varying final concentrations of SE (Fig. 1). The net decreases in mean Ct values from the PrepMan Ultra Reagent were 1.2 (BPW), 2.4 (RV), and 14.1 (MKTTn) when the PrepMan Ultra Reagent w/additional washing step method was used, and -0.6 (BPW), 1.2 (RV), and 10.2 (MKTTn) when the DNeasy Tissue Kit was used indicating that the inhibitory effect was significantly ($p < 0.05$) removed by using modified extraction method or DNeasy Tissue Kit in MKTTn (Fig. 1).

Comparison of different enrichment broths and DNA extraction schemes

Steamed pork samples artificially contaminated with low numbers of SE cells were subjected to pre-enrichment in BPW and selective enrichment in RV or MKTTn and then analyzed by a culture method and real-time PCR with two different DNA extraction methods. The culture method, described above, identified 26 out of 60 samples as positive

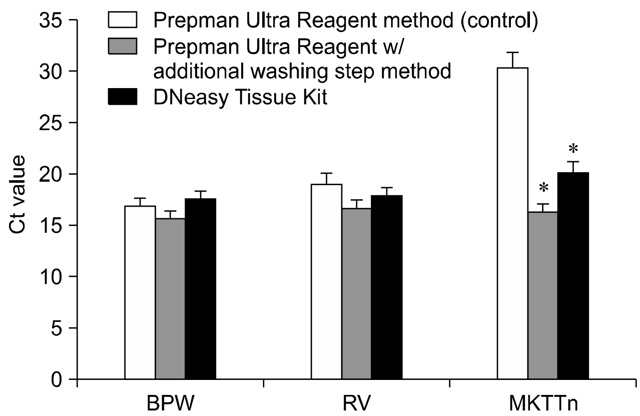

Fig. 1. Detection of *Salmonella enterica* subsp. *enterica* serotype Enteritidis (SE) in buffered peptone water (BPW), Rappaport-Vassiliadis (RV), and Muller-Kauffmann tetrathionate with novobiocin (MKTTn) overnight cultures by PrepMan Ultra Reagent w/additional washing step method and the DNeasy Tissue Kit, as compared with the PrepMan Ultra Reagent method (n = 4). Mean numbers of SE cells in each medium, enumerated from four trials, were 1.5×10^9 in BPW, 9.1×10^8 in RV, and 1.3×10^9 in MKTTn (all values are CFU/mL). The threshold cycle (Ct) values are mean ± SD values of sixteen samples, from quadruplicates in four trials. *The Ct values are statistically different from those of the PrepMan Ultra Reagent method ($p < 0.05$).

Table 3. Effect of enrichment media presence during DNA extraction on real-time PCR detection of *Salmonella* Enteritidis (n = 4)

Volume (μL)	Ct ± SD			
	PBS (control)	BPW	RV	MKTTn
Trial 1 (2.2×10^9)†	18.1 ± 0.4	18.5 ± 0.8	21.7 ± 2.7*	23.9 ± 0.3*
Trial 2 (1.8×10^9)†	18.4 ± 0.2	18.5 ± 0.1	19.8 ± 0.7	22.7 ± 0.4*
Trial 3 (1.5×10^9)†	18.7 ± 0.0	19.1 ± 0.3	20.5 ± 1.2*	31.5 ± 1.7*

*The Ct values are statistically different from those of PBS in the same trials ($p < 0.05$). †The number of SE cells used in each trial (CFU/mL).

Table 4. Comparison of conventional method versus real-time PCR combined with two DNA extraction methods for detecting SE inoculated in steamed pork

	No. of SE-positive samples / total No. of tested samples*											
	7 CFU/500 g			17 CFU/500 g			24 CFU/500 g			Total		
	Culture	Real-time PCR		Culture	Real-time PCR		Culture	Real-time PCR		Culture	Real-time PCR	
		P†	M‡		P	M		P	M		P	M
BPW	ND§	6/20	6/20	ND	6/20	7/20	ND	15/20	16/20	ND	27/60	29/60
BPW + RV	6/20	5/20	6/20	5/20	4/20	5/20	15/20	14/20	15/20	26/60	23/60	26/60
BPW + MKTTn	ND	5/20	6/20	ND	2/20	4/20	ND	10/20	15/20	ND	17/60	25/60

*There was no statistical significance between culture method and real-time PCR and between the two DNA extraction methods. †P: PrepMan Ultra Reagent was used as the DNA extraction method. ‡M: Modified PrepMan Ultra Reagent method, with an additional washing step, was used as the DNA extraction method. §ND: not done.

(Table 4). Real-time PCR using samples cultured in BPW detected an equivalent number to or more positive samples than the culture method, regardless of the DNA extraction method (Table 4). However, the numbers of positive samples detected by real-time PCR using samples grown in RV and MKTTn were lower than those detected by the culture method when PrepMan Ultra Reagent method was used as DNA extraction without statistically significant difference ($p > 0.05$) (Table 4). When the DNA extraction method was PrepMan Ultra Reagent with additional washing step method, the sensitivity of the real-time PCR was improved as the positive samples were increased from 27, 23, and 17 to 29, 26, and 25 out of 60 samples from BPW, RV, and MKTTn, respectively. However, there was no statistically significant difference ($p > 0.05$) between the two DNA extraction methods (Table 4).

Discussion

The PCR inhibitory effect has been an area of concern in the accurate detection of pathogens in food using PCR-based assays [13]. However, few studies have reported on the effect of enrichment media on the PCR reaction [14]. We evaluated the PCR inhibitory effects of three common enrichment media (BPW, RV, and MKTTn) used for detecting *Salmonella* spp. by comparing the Ct values obtained from real-time PCR assays. Analysis of the data statistically and quantitatively could significantly reduce the need for subjective estimation. When RV or MKTTn was directly added into PCR mixtures, Ct values were dramatically increased compared with those of PCR mixtures containing BPW or PBS, indicating a marked inhibition of the PCR reaction. The SE was not detectable with high concentrations of MKTTn in the PCR mixture. In addition, we evaluated the effects of *Salmonella* enrichment media on the sensitivity of real-time PCR by comparison of Ct values with the same number of SE cells in the media and using the same DNA extraction method, the PrepMan Ultra Reagent protocol. Detection of SE by the real-time PCR assay was significantly diminished when DNA templates were extracted from SE contained in the selective enrichment media, RV and MKTTn, compared with the same number of SE cells contained in PBS and BPW. This could be due, in part, to the potential PCR inhibitors such as malachite green oxalate in RV and bile salt in MKTT [1,4,10,17,18]. However, the differences in mean Ct values between the enrichment media and PBS, indicating the degree of inhibitory effect of the media, were not consistent across trials. It appears that the degree of PCR inhibition of each trial is considerably affected by the amount of residue from the enrichment media after the single washing step involved in the PrepMan Ultra Reagent method. This hypothesis was supported by the observation that SE detection was significantly improved after an additional washing step.

Catarame *et al*. [3] reported that detection of *Salmonella* by real-time PCR after RV enrichment was successful. Myint *et al*. [11] showed that a culture method and a PCR assay with RV and Tetrathionate-Hajna (TT-H) broth resulted in equal sensitivity of *Salmonella* detection. However, the successful detection of *Salmonella* in RV and TT-H could be attributed to the DNA extraction method, in this case, the DNeasy Tissue Kit protocol. Heller *et al*. [5] compared four commercial kits for isolation of DNA from food samples by real-time PCR: PrepMan Ultra, Bugs'n Beads, NucleoSpin food kit, and the Wizard magnetic DNA purification system for food. They reported that PrepMan Ultra was the simplest method to perform and that its results were not different significantly from the results of other kits.

However, in this study, the PrepMan Ultra Reagent method was not sufficient to remove potential PCR inhibitors from *Salmonella* enrichment media, and detection was significantly improved when DNA was extracted using PrepMan Ultra Reagent with an additional washing step or using the DNeasy Tissue Kit. The sensitivity of the real-time PCR method can be enhanced by the proper combination of centrifugation and washing steps or using silica membrane columns for DNA extraction, thus eliminating the PCR inhibitory effects of the *Salmonella* enrichment media [8,16]. Interestingly, in this study, the detection of SE was improved more by the simple addition of a washing step than by use of the complex and labor-intensive DNeasy Tissue Kit. This may be due to the washing step being enough to remove water-soluble PCR inhibitors from RV and MKTTn without significant loss of bacterial cells, while substantial cell loss may occur when the DNeasy Tissue Kit is used, due to its multiple washing, filtering, and centrifugation steps.

We also evaluated an optimum enrichment media and DNA extraction method to detect *Salmonella* spp. in food (steamed pork), using a real-time PCR assay. With the modified DNA extraction method, the real-time PCR coupled with single 24 h enrichment with BPW performed better than double 48 h enrichment with BPW plus RV or MKTTn and detected an equivalent number of positive samples or more as compared with results from the conventional culture method.

Further study is necessary to identify the components of enrichment broths responsible for these inhibitory effects. Prudent selection of an enrichment medium, combined with an optimum DNA extraction method, will result in improved detection of foodborne pathogens in various food products by real-time PCR.

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