

## Quantitative Detection of *Salmonella typhimurium* Contamination in Milk, Using Real-Time PCR

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**Abstract** A rapid and quantitative real-time PCR was developed to target the invasion A (*invA*) gene of *Salmonella* spp. We developed quantitative standard curves based on plasmids containing the *invA* gene. Based on these curves, we detected *Salmonella* spp. in artificially contaminated buffered peptone water (BPW) and milk samples. We were able to determine the *invA* gene copy number per ml of food samples, with the minimum detection limit of  $4.1 \times 10^3$  copies/ml of BPW and  $3.3 \times 10^3$  copies/ml of milk. When applied directly to detect and quantify *Salmonella* spp. in BPW and milk, the present real-time PCR assay was as sensitive as the plate count method; however, copy numbers were one to two logs higher than the colony-forming units obtained by the plate count methods. In the present work, the real-time PCR assay was shown to significantly reduce the total time necessary for the detection of *Salmonella* spp. in foods and to provide an important model for other foodborne pathogens.

**Key words:** Copy number, *invA* gene, real-time PCR, *Salmonella*

Salmonellosis infection is one of the most common foodborne diseases in the world in both humans and animals [12]. The majority of the cases are due to consumption of contaminated animal products such as eggs, poultry, raw meats, raw milk, and other dairy products that have not been pasteurized or are handled inappropriately. Milk and milk products have been identified as a vehicle for transmission in approximately 5% of the salmonellosis cases, although the sources of infection in most cases remain unidentified [20]. Conventional bacteriological methods for the detection of *Salmonella* spp. in foods, such as the ISO 6579 standard method, are based on pre-enrichment in buffered peptone water (BPW) followed by selective

enrichment and plating, taking up to 5 days to complete. Therefore, a rapid and sensitive primary screening method with a proper sampling plan is required to detect *Salmonella* in the food industry. New methods based on polymerase chain reaction (PCR) have increasingly been used for rapid, sensitive, and specific detection of foodborne pathogens [4, 5, 11]. Although PCR tests are considered attractive, traditional PCR methods require amplification in a thermocycler, separation of amplification product by gel electrophoresis, followed by hybridization with a probe. These approaches have had limited success, since these modifications make the overall pathogen detection procedure labor intensive, time consuming, and difficult to automate with non-accurate quantification. Recent advances in the synthesis of fluorogenic probes and the development of instrumentation for continuous monitoring of fluorescence have facilitated the development of real-time PCR assays for specific automated detection and quantification of amplified bacterial products. The 5' nuclease assay, known as the TaqMan assay, is widely used for rapid detection of foodborne pathogens such as *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 [9, 19, 21]. The main TaqMan assay is the use of three oligonucleotides in the PCR reaction. Two of the primers (forward and reverse) allow amplification of the product to which a third dual-labeled fluorogenic oligonucleotide, the TaqMan probe, will anneal. The 5' nuclease assay uses the 5'→3' nuclease activity of *Taq* DNA polymerase, which digests an internal fluorogenic probe, resulting in an increase of reporter fluorescence signal that can be detected on a fluorescence spectrometer in a real-time manner. Therefore, the reporter dye fluorescence intensity generated by the probe's digestion is proportional to the amount of the PCR products being produced. The measurement of fluorescence throughout the reaction eliminates the need for post-PCR processing steps, such as gel electrophoresis and ethidium bromide staining of target DNA, thus easing automation of the technique and large-scale sample processing. Moreover,

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**Table 1.** Bacteria used in this study.

Species	Strain No.
<i>Salmonella typhimurium</i>	ATCC 13311
<i>Salmonella typhimurium</i>	ATCC 19585
<i>Salmonella typhi</i>	ATCC 33459
<i>Listeria monocytogenes</i>	ATCC 19111
<i>Listeria monocytogenes</i>	ATCC 19115
<i>Listeria monocytogenes</i>	ATCC 19119
<i>Vibrio parahemolyticus</i>	ATCC 27969
<i>E. coli</i> O157:H7 932	ATCC 43894
<i>Shigella sonnei</i>	ATCC 25931

there is potentially less contamination of the PCR mixture with target DNA, because the reaction tubes remain closed throughout the assay. Real-time PCR assays also allow for the precise quantification of target DNA, which is correlated with the size of the bacterial population present in the sample.

In this study, we developed a real-time PCR assay with the TaqMan probe system to target *Salmonella*, specifically the *invA* gene sequence, and to quantify *Salmonella typhimurium* in artificially contaminated milk.

## MATERIALS AND METHODS

### Bacterial Strains, Culture Conditions, and Sample Collection

All bacteria used in this study are listed in Table 1. Cultures of *Salmonella* were routinely started from freezer stocks for growth on Luria-Bertani (LB) agar medium. Following overnight incubation at 37°C, a single colony was selected and incubated into 50 ml of LB in a 125-ml Erlenmeyer flask. Pasteurized whole milk was purchased from local stores. Milk sample and BPW were artificially contaminated with a bacterial culture, having an optical density of 0.66 (at 600 nm) corresponding to 10<sup>8</sup> CFU/ml, by adding 1 ml of 10-fold dilutions into milk samples and 9 ml of BPW, respectively. Total colony numbers were determined by estimating viable cell counts on BPW agar plates in triplicate. The traditional enrichment method for the detection of *Salmonella* in artificially contaminated samples was based on the method of Malorny *et al.* [21]. Briefly, artificially contaminated milk sample and BPW (25 ml) were mixed

in 225 ml of BPW and pre-enriched for 8 h at 37°C without shaking.

### Preparation of DNA Samples

Template DNA was isolated by modifying the boiling method of Lee *et al.*. Briefly, a 1 ml aliquot of each sample was centrifuged at 14,000 ×g at 4°C for 5 min. The supernatant was carefully removed, and the cell pellet was washed in 1 ml TE buffer and then mixed in 200 µl of TE buffer [10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0)]. The tube was incubated for 15 min at 95°C in a water bath and immediately chilled on ice for 5 min. After centrifugation at 14,000 ×g at 4°C for 5 min, the supernatant containing DNA was carefully transferred to a new microcentrifuge tube and adjusted to a final volume of 150 µl. A 1 µl aliquot was used as template DNA for the PCR. Plasmid DNA used as a standard curve was routinely purified using a Qiagen plasmid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All DNA preparations were stored at -20°C until use.

### Primers for PCR

The DNA primers used in this study were amplified from the invasion A (*invA*) gene of *Salmonella typhimurium* (GenBank accession no. NC 003197). The PCR was performed using a thermocycler (Astec PC 808, Japan). The reaction mixture (25 µl) contained 1× PCR buffer (Takara, Tokyo, Japan), 0.2 mM each deoxynucleoside triphosphates (containing each deoxynucleoside triphosphate at 2 mM concentration), 10 pmol each primer, 0.2 U Taq polymerase, 1 µl template DNA, and sterile distilled water to make up 25 µl. Amplification was carried out using the following cycles: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 90 s, annealing at 64°C for 80 s, and extension 72°C for 2 min, and final extension at 72°C for 7 min, followed by cooling to 4°C. The PCR was verified by mixing 2 µl of PCR product with 2 µl of loading dye and electrophoresis on a 2% (w/v) agarose gel for 30 min at 50 V.

### Primers and Probe for *invA* to Quantify *Salmonella typhimurium*

Primers and probe used for the detection and quantification of *Salmonella typhimurium* are shown in Table 2. The

**Table 2.** Oligonucleotide primers and probe used in the PCR.

Oligonucleotide	Sequence (5'-3')	Nucleotide position in <i>invA</i>	Reference
SAL F	ATGGCGGCGAATTACGAGCA	1,751–1,770	This study (for PCR)
SAL R	ACAGACGTAAGGAGGACAAG	1,924–1,943	This study (for PCR)
SAL4F	CGCAAAGGGATCCGTCAGA	1,804–1,822	This study (for RT-PCR)
SAL4R	GGAGGCTTCCGGGTCAAG	1,846–1,863	This study (for RT-PCR)
SAL4M1	FAM <sup>a</sup> CCTCTGGCAGTACCTC	1,823–1,839	This study (for RT-PCR)

<sup>a</sup>FAM, 6-carboxyfluorescein (the reporter dye).

PCR primers and fluorogenic probe for the TaqMan assay were designed using Primer Express 2.0 software (Applied Biosystems, Forester City, CA, U.S.A.) in order to specifically amplify the *Salmonella typhimurium* invasion A (*invA*) gene. The oligonucleotides and all reagents for the PCR reactions used in this study were synthesized and purchased from Applied Biosystems. The probe was labeled with fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5' end, and Minor Groove Binder (MGB) probe with non-fluorescent quencher (NFQ) at the 3' end of the probe. The TaqMan MGB probe was recently developed to be more specific than conventional probes. Fluorescence quenching is more efficient, giving increased sensitivity, and the higher melting temperature allows the design of significantly shorter probes that are also more specific, especially if there is a mismatch in the MGB region of the duplex [16, 22]. The amplification reactions were performed in a total volume of 25  $\mu$ l. Thermal cycling was performed using a two-step PCR protocol: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The template (1  $\mu$ l) was transferred to 25  $\mu$ l of PCR reaction mix consisting of TaqMan Universal PCR Master Mix (12.5  $\mu$ l), 1.25  $\mu$ l of primer and probe mixture (900 nM and 250 nM final concentration, respectively) and 10.25  $\mu$ l of water in a 96-well microwell plate. The intensities of the fluorescent dyes in each reaction were read automatically during PCR cycling in an ABI PRISM 7000 sequence detector (Applied Biosystems). The real-time amplification data were analyzed using ABI PRISM 7000 SDS software (Applied Biosystems) installed on the sequence detector.

**Construction of a Plasmid DNA for the Standard Curve**  
Primers SAL-F and SAL-R amplified a 193-bp fragment in the *invA* gene of *Salmonella typhimurium* DNA. This fragment was amplified, purified, and ligated into the plasmid pGEM-T easy, using pGEM-T easy Vector System (Promega, Madison, WI, U.S.A.). Plasmid DNA containing the *invA* fragment was purified using the Takara spin Mini prep kit (Takara, Otsu, Japan) by following the manufacturer's instructions. The DNA concentration of the purified plasmid was estimated by absorbance at 260 nm and expressed in copy number of plasmid per 1  $\mu$ l. The copy number of plasmid DNA was calculated as follows [23]. Weight in Daltons ( $g/mol$ )=(bp size of plasmid size) (330 Da $\times$ 2 nucleotides/bp). Hence, ( $g/mol$ )/Avogadro's number= $g/molecule$  =copy number. Knowing the copy number and concentration of plasmid DNA, the precise number of molecules added to subsequent real-time PCR runs can be calculated, thus providing a standard for specific DNA quantification. Ten-fold serial dilutions ( $10^5$  to 10 copy per 1  $\mu$ l) of the plasmid in water were prepared for the evaluation of the assay sensitivity and used as a standard curve in real time PCR. The Ct values were set above the highest 6-carboxyfluorescein endpoint fluorescence signals of the negative samples. The

samples were considered negative if the Ct values exceeded 40 cycles. The Ct values from unknown samples were plotted on the standard curve, and the number of *invA* gene copies per ml was calculated.

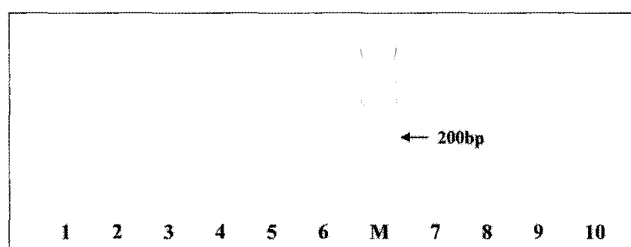
#### Calculation of the Copy Number of the *invA* Gene in Milk by Real-Time Quantitative PCR

The copy number of the *invA* gene per ml milk was calculated by multiplying the copy number per PCR with a factor of 150, since DNA was isolated from 1 ml; the DNA was suspended in a final volume of 150  $\mu$ l, from which 1  $\mu$ l was used for real-time PCR.

## RESULTS

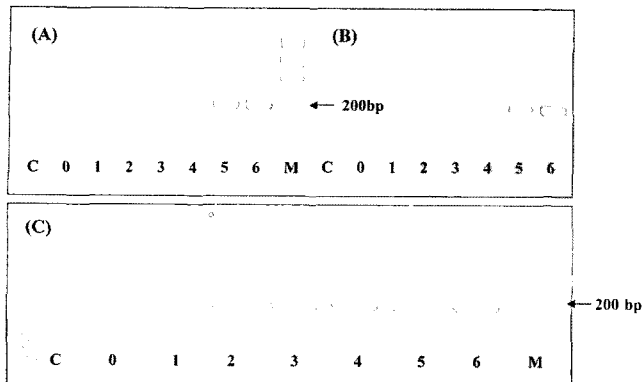
#### Detection Limits of *Salmonella* Strains by Conventional PCR

The primers SAL F/R were derived from the conserved region of the *invA* gene, and were able to prime the synthesis of a 193-bp fragment, when *Salmonella* DNA was used as a template. The oligonucleotides used were designed on the basis of the nucleotide sequence of the *invA* gene, which was used as the target sequence for amplification. This gene, which is essential for the invasion of epithelial cells by *Salmonella* and is a critical component in the pathogenesis of the disease produced by these organisms [7, 8], has been shown to be present and functional in most *Salmonella* serotypes [9]. As shown in Fig. 1, the specific PCR products of 193 bp were obtained for all the different *Salmonella* strains (lanes 1 to 3) tested, whereas no amplification products were obtained with several non-*Salmonella* strains (lanes 4 to 9). These results clearly indicate that the primers SAL F/R have a high affinity for the correct target sequence and are specific for *Salmonella* spp. Next, BPW and milk samples were artificially contaminated with serial 10-fold dilutions of



**Fig. 1.** Specificity of the PCR assay.

(Lane 1, *Salmonella typhimurium* ATCC 13311; lane 2, *Salmonella typhimurium* ATCC 19585; lane 3, *Salmonella typhi* ATCC 33459; lane 4, *Listeria monocytogenes* ATCC 19111; lane 5, *Listeria monocytogenes* ATCC 19115; lane 6, *Listeria monocytogenes* ATCC 19119; lane 7, *Vibrio parahaemolyticus* ATCC 27969; lane 8, *E. coli* O157:H7 932 ATCC 43894; lane 9, *Shigella sonnei* ATCC 25931; lane 10, negative control (without template); M, 100 bp DNA ladder.



**Fig. 2.** Sensitivity of the PCR assay with primers SAL F/R for *Salmonella*.

(A) BPW, (B) whole milk, (C) after enrichment for 8 h incubation. C: control (no template); 1–6:  $10^6$ – $10^0$  (CFU/ml); M: 100 bp DNA ladder.

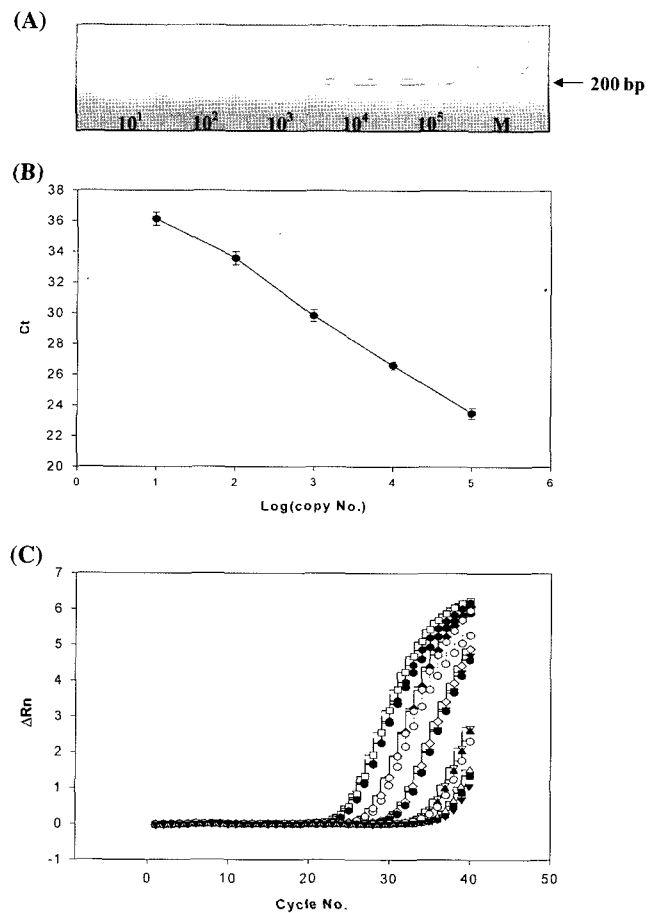
*Salmonella typhimurium* suspensions: The contamination ranged from  $10^6$  to  $10^0$  CFU/ml of BPW and milk, and the sensitivity limits of sample detection were  $10^2$  CFU/ml BPW and  $10^3$  CFU/ml milk. When the pre-enrichment step (8 h at  $37^\circ\text{C}$ ) using BPW broth was performed prior to the PCR assay, as low as 1–9 CFU/ml of the milk sample could be detected (Fig. 2). Therefore, the pre-enrichment step appears to be useful for enhancing the signal and also for increasing the number of live bacteria, thereby avoiding false positives.

#### Detection Range of the Real-Time PCR

A standard curve was obtained using 10-fold serial dilutions of positive plasmid controls for *Salmonella* spp. The PCR products (193 bp) of the plasmid DNA were confirmed on a gel (Fig. 3). It is noted that the band of low concentrations ( $10^1$  copies/ $\mu\text{l}$ ) was not clearly visible on the gel, but showed positive Ct values generated by the real-time PCR assay. To generate a standard curve, the threshold cycle (Ct) of these standard dilutions was plotted against the number of plasmid copies used as an input. The standard curve showed a linear correlation coefficient of 0.9974 that was highly significant, and the slope value of the standard curve was  $-3.228$  (Fig. 3). The slope of the standard curve was used for the calculation of the PCR efficiency (E) by the following equation:  $E=10^{1/s}-1$  [15]. The amplification plot is  $-3.228$ , corresponding to a 99.5% efficiency of the PCR assay. All samples used in this study were amplified and detected with Ct values in the range of 21–36, corresponding to  $10^5$  to  $10^1$  plasmid copies, respectively.

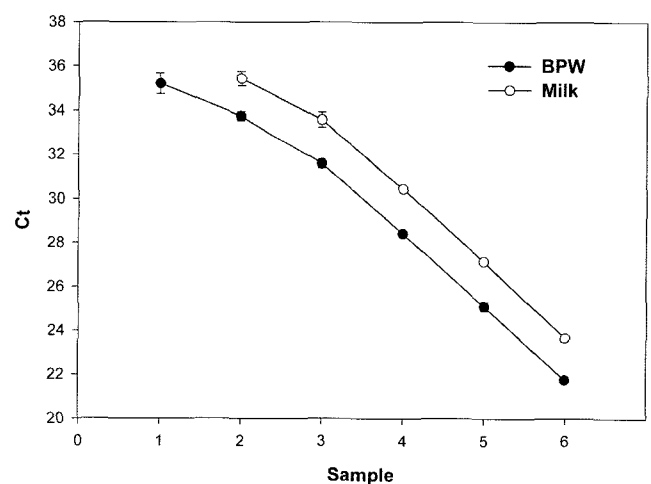
#### Evaluation of Detection Limits by Real-Time PCR Using Artificially Contaminated Milk

In order to evaluate the TaqMan assay developed in this study for *Salmonella* spp., the copy numbers of this organism



**Fig. 3.** Standard curve for a 10-fold serial dilution of the control plasmid.

(A) Agarose gel electrophoresis of amplification products of dilution series. M: 100 bp DNA ladder. (B) Standard curve showing correlation between Ct and initial copy number of *invA* ( $\log C_0$ ). Slope:  $-3.228$ ; intercept: 39.611;  $R^2$ : 0.9974. (C) The fluorescence emission ( $\Delta Rn$ ) is plotted against the number of PCR cycle.



**Fig. 4.** Values of Ct for detecting various populations of *Salmonella* from BPW and milk.

**Table 3.** Detection and quantification of real-time PCR with artificially contaminated sample from a standard curve.

Contamination level of <i>S. typhimurium</i> (CFU/ml)	BPW		Milk
	Copy No. of <i>invA</i> gene/ml	Copy No. of <i>invA</i> gene/ml	Plate count method CFU/ml
C (uninoculated)	ND	ND	ND
10 <sup>0</sup>	ND	ND	ND
10 <sup>1</sup>	4.1×10 <sup>3</sup>	ND	ND
10 <sup>2</sup>	1.0×10 <sup>4</sup>	3.3×10 <sup>3</sup>	4×10 <sup>2</sup>
10 <sup>3</sup>	4.7×10 <sup>4</sup>	1.2×10 <sup>4</sup>	3.4×10 <sup>3</sup>
10 <sup>4</sup>	4.4×10 <sup>5</sup>	1.0×10 <sup>5</sup>	4.2×10 <sup>4</sup>
10 <sup>5</sup>	4.6×10 <sup>6</sup>	1.1×10 <sup>6</sup>	9.0×10 <sup>4</sup>
10 <sup>6</sup>	4.8×10 <sup>7</sup>	1.3×10 <sup>7</sup>	2.3×10 <sup>6</sup>

ND: not detected.

in artificially contaminated milk samples were counted by the TaqMan assay (Fig. 4, Table 3). The sensitivity of the real-time PCR was approximately 10<sup>1</sup> CFU/ml in BPW and 10<sup>2</sup> CFU/ml in milk, having sensitivity higher than the conventional PCR. The minimum detection limit was 4.1×10<sup>3</sup> copies/ml (Ct: 35.2) in BPW and 3.3×10<sup>3</sup> copies/ml (Ct: 35.4) in milk. The calculated copy number of the *invA* gene was two logs higher than the CFUs derived by the plate count method.

## DISCUSSION

Conventional means for the identification and quantification of foodborne pathogenic microorganisms are generally laborious and time consuming. Hence, there is an urgent need to develop assays based on faster technologies, such as real-time PCR, in order to speed up the detection and quantification of microorganisms. Detection sensitivities of TaqMan assays have previously been reported to vary by approximately 50 CFU per reaction for the *Listeria monocytogenes* assay of Bassler *et al.* [1]. The *Salmonella* assay evaluated by Chen *et al.* [2] and Kimura *et al.* [14] had an analytical sensitivity of 2 CFU/reaction in pure cultures, but none of these assays was quantitative. Generally, 10<sup>7</sup>–10<sup>9</sup> cells are necessary for salmonellosis, but outbreaks by significantly lower numbers of cells have been noted. Detection of *Salmonella* in food and the determination of level of contamination are important for food safety concerns [3, 13]. Therefore, this study was applied to the direct detection and quantification of *Salmonella* spp. in milk samples that had been intentionally contaminated. The sensitivity of the real-time PCR was approximately 10<sup>1</sup> CFU/ml in BPW and 10<sup>2</sup> CFU/ml in milk, thus having sensitivity higher than the conventional PCR. Application of the assay to examine artificially contaminated milk samples resulted in copy numbers that were one log higher than the plate count method, in agreement with Mayer *et al.* [18] and Hein *et al.* [10], and the result can possibly be explained by several possibilities. The number of CFUs obtained by the

plate count method depends on the distribution of the microorganisms in the sample, and the microorganisms have to be alive and culturable. In contrast, PCR detects the DNA of microorganisms whether they are alive or not. Furthermore, the quantification by PCR depends on the physiological state of the bacteria and on the efficiency of lysis and extraction procedures. Therefore, the observed discrepancy between copy numbers of the *invA* gene and CFUs is most likely due to the fact that the stationary phase cultures contain a significant number of dead and lysed cells. The number of copies in BPW was generally higher than in milk. It is highly conceivable that milk contains substances inhibitory to the PCR reaction. In the present study, we developed an accurate and less labor-intensive real-time PCR assay with the TaqMan probe system, capable of detecting *Salmonella*, specifically the *invA* gene. This assay is expected to significantly reduce the total time spent for the detection of *Salmonella* spp. in foods and is an important model for other foodborne pathogens.

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