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Rapid Quantification of *Salmonella* in Seafood Using Real-Time PCR Assay

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A quantitative detection method for *Salmonella* in seafood was developed using a SYBR Green-based real-time PCR assay. The assay was developed using pure *Salmonella* DNA at different dilution levels [*i.e.*, 1,000 to 2 genome equivalents (GE)]. The sensitivity of the real-time assay for *Salmonella* in seeded seafood samples was determined, and the minimum detection level was 20 CFU/g, whereas a detection level of 2 CFU/ml was obtained for pure culture in water with an efficiency of ≥85%. The real-time assay was evaluated in repeated experiments with seeded seafood samples and the regression coefficient (R^2) values were calculated. The performance of the real-time assay was further assessed with naturally contaminated seafood samples, where 4 out of 9 seafood samples tested positive for *Salmonella* and harbored cells <100 GE/g, which were not detected by direct plating on *Salmonella* Chromagar media. Thus, the method developed here will be useful for the rapid quantification of *Salmonella* in seafood, as the assay can be completed within 2–3 h. In addition, with the ability to detect a low number of *Salmonella* cells in seafood, this proposed method can be used to generate quantitative data on *Salmonella* in seafood, facilitating the implementation of control measures for *Salmonella* contamination in seafood at harvest and post-harvest levels.

Keywords: *Salmonella*, seafood, quantitative detection, real-time PCR

Salmonella infections are the second most common cause of foodborne outbreaks in humans. Approximately 1.4 million cases of salmonellosis are reported in the U.S. each year, and similar outbreaks are reported in Asian and European countries [6]. One report on the incidence of salmonellosis in the U.S. between 1988 and 1992 revealed that 7.4% of the outbreaks were associated with consumption of fish or shellfish [3]. In another long-term study (1990–1898) of *Salmonella* contamination of imported and domestic seafood in the U.S., field laboratories of the USFDA found that 11,312 imported (7.1%) and 768 domestic (1.3%) seafood samples tested positive for *Salmonella* [10]. Contamination in coastal areas and the post-harvest handling of seafood have both been found to be responsible for *Salmonella* contamination in fish and seafood [9, 13]. The influence of environmental factors and human activity on the presence of *Salmonella* serovars has also been monitored in the marine environment of the Galicia region in Spain [18]. However, since the load of *Salmonella* in most cases of food and feed contamination is usually low in number [5, 23], some culture methods may not be able to detect the presence of *Salmonella*. Thus, effective quantification of the presence of the contaminant is important in order to determine the quantitative microbial risk assessment measures. According to the Codex Alimentarius, risk analysis in food includes the identification and quantification of hazards.

Currently, nearly all quantitative data generated for *Salmonella* are obtained using traditional bacteriological methods [4, 11]. Yet, the quantitative detection of *Salmonella* in food using the conventional culture method is tedious and time-consuming, thereby limiting its usage in routine analysis. Alternatively, PCR-based methods have now been standardized and are being used to detect pathogens in food-testing laboratories [15]. Most recently, the development of a real-time PCR assay for detecting pathogens in food and environmental samples has shown potential for the quantification of pathogenic microorganisms [12, 14]. However, despite reports on the quantitative detection of *Salmonella* in meat and dairy products, a real-time PCR-based quantitative method is not yet available for the rapid enumeration of *Salmonella* in seafood. Accordingly, the aim of this study was to develop a rapid culture-independent quantitative real-time assay for *Salmonella* in seafood samples.

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Materials and Methods

Isolation and Quantification of DNA from Pure *Salmonella* Culture and Seeded Seafood Samples

Salmonella enterica subsp. *enterica* Typhimurium (MTCC 1624) culture was grown in 5 ml of a brain heart infusion broth (Difco, MI, U.S.A.) at 37°C for 18 h and the genomic DNA was isolated using the method described by Ausubel *et al.* [2] and a bacterial genomic DNA isolation kit (Sigma, India). The concentration and purity of the DNA were determined with a UV-Vis spectrophotometer (Cary 100, Varian, Australia) [22]. The pure DNA obtained from *Salmonella* Typhimurium was decimally diluted from 500 ng to 0.002 pg/10 µl in a sterile Tris-EDTA buffer (TE), pH 8, and stored at −20°C until further use.

Fish (*Rastrelliger kanagurta*) and shrimp (*Penaeus monodon*) confirmed to be free from *Salmonella* by conventional culture and conventional PCR methods were used for the seeding experiments. The fish and shrimp (25 g) samples were blended separately with 225 ml of buffered peptone water (Difco, MI, U.S.A.) in a homogenizer at 200 rpm for 1 min. Portions (10 ml) containing 1 g of tissue were transferred to 50-ml flasks, and seven different seeding levels of *Salmonella* (*i.e.*, 2, 2×10, 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 CFU/g), were used to seed the flasks containing fish and shrimp homogenates. DNA was then extracted from the seeded homogenate preparations (10 ml) from each dilution without enrichment. Next, the fish and shrimp seeded homogenates were centrifuged at 500 rpm at 4°C for 2 min in centrifuge tubes (Eppendorf, Germany), and the supernatants were transferred to fresh centrifuge tubes and centrifuged at 10,000 rpm at 4°C for 2 min. Thereafter, DNA was isolated from tubes containing different dilutions of seeded cells (2 to 2×10^6 CFU/g) using a genomic DNA isolation kit (Sigma, India).

Real-Time PCR Assay

The real-time thermocycler used in this study was a Chromo 4 Real-time system (MJ Research Corp., U.S.A.). A *Salmonella*-specific *inv*A (F–GTGAAATTATCGCCACGTTCGGGCAA and R–TCATCGCAC CGTCAAAGGAACC) primer was used to develop the real-time assay [20], which was carried out with 25 µl of a real-time PCR mixture consisting of 12.5 µl of 2× SYBR Green Supermix (Sigma, India) and 0.6 µl of 10 µM primers (each). Next, 10 µl of the target DNA solution was added to the reaction mixture, and a final reaction volume of 25 µl was attained with 1.3 µl of sterile milli-Q water. The SYBR Green Supermix contained dNTP (0.4 mM), *Taq* polymerase, 6 mM $MgCl_2$, 100 mM KCl, and 40 mM Tris-HCl (pH 8.4). The real-time PCR was performed with an initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 94°C for 15 s, primer annealing at 64°C for 10 s, and a primer extension at 72°C for 20 s. The melting curve (T_m) analysis of the final PCR product was carried out from 60 to 95°C at 1°C intervals. The T_m value of the PCR product was determined using MJ Research Monitor analysis software, version 3.0 (U.S.A.).

Development of Standard Curves

The standard curves were constructed from serially diluted cells (2×10^6 to 2 CFU/ml or g) of *Salmonella* in water, fish, and shrimp samples. The sample DNA was extracted, quantified, and amplified as described in the previous section. The amplification signals produced by the different dilutions of *Salmonella* in the water, fish, and shrimp samples were plotted against quantified DNA of *Salmonella* from different seafood matrices and standard curves constructed. The correlation coefficient (R^2) and efficiency of the amplification were calculated. All the seeded experiments were repeated five times in duplicate to ascertain the reliability and repeatability of the real-time assays.

Quantitative Detection of *Salmonella* in Naturally Contaminated Seafood Samples

The quantitative detection of *Salmonella* in fish, shrimp, clams, and squid collected from Cochin (India) markets was used to enumerate *Salmonella* in naturally contaminated seafood samples. A total of 28 seafood samples were analyzed for the quantitative detection of *Salmonella* in seafood. The seafood samples (25 g) were homogenized with 225 ml of buffered peptone water (Difco, MI, U.S.A.) in a stomacher blender for 1 min at 150 rpm. The preparation of the DNA for the real-time assay was immediately performed using 10 ml of the sample homogenate without enrichment, and the real-time assay performed thereafter, as discussed earlier.

The homogenized seafood samples used for the real-time PCR were simultaneously analyzed for *Salmonella* using a conventional culture method on *Salmonella* Chromagar media. One ml of the homogenized seafood samples in different dilutions (1:10 to 1:1,000) was plated onto the *Salmonella* Chromagar (Oxoid, U.K.) containing Novobiocin (5 mg/l) and Cefsulodin (12 mg/l). The plates were then incubated at 37°C for 24 h and typical colonies (mauve color) counted as *Salmonella*. Further confirmation of *Salmonella* colonies from the Chromagar plates was performed as per the steps described in the standard method of the U.S. Food and Drug Administration [1].

Results

Real-Time Assay for Pure *Salmonella* Culture DNA

A real-time assay was developed for pure DNA isolated from *Salmonella* Typhimurium, and subsequent assays were based on a decimal dilution of pure DNA used as known standards to determine the DNA concentration in the test samples. The minimum detection sensitivity was 0.005 pg of pure DNA in a PCR reaction. The linear range of detection spanned from 7 log cycles of pure DNA ranging

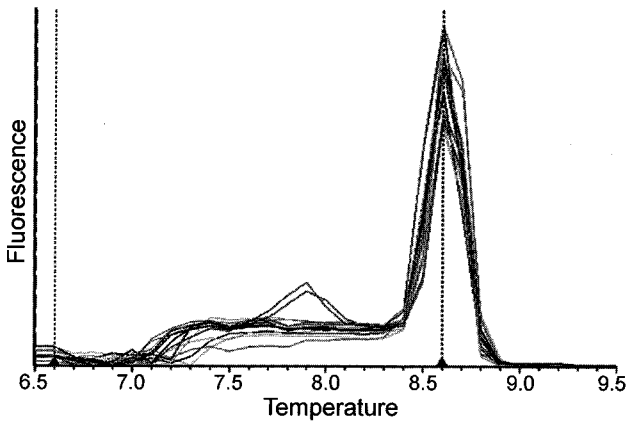

Fig. 1. Melting curve (T_m) analysis of *Salmonella inv*A gene amplicons obtained from inoculating around with the cell range 2×10^6 to 2 CFU/g in seafood.

from 5,000 pg to 0.005 pg (1,000 to 1 GE). The standard plot showed that the regression coefficient was linear (R^2 0.993) over a 7 log dilution range. One cell of *Salmonella* Typhimurium DNA corresponds to 0.005 pg, and the minimum detection level of the newly developed real-time PCR was also 0.005 pg. The maximum threshold Ct value was 39.4±0.48, and there was no subsequent amplification when the amount of DNA was further reduced to 0.002 pg. Thus, the results indicated that the level of detection was up to 0.005 pg when using a real-time PCR for different concentrations of *Salmonella* genomic DNA. The T_m value for the amplified product of the *inv*A gene was observed uniformly at 86±1°C for all the real-time PCR assays (Fig. 1).

Development of Standard Curves and Quantification of *Salmonella* in Seeded Fish and Shrimp Samples

When carrying out the real-time assay using the DNA extracted from seeded seafood (2 to 2×10^6 CFU/g), the results showed a linear plot of Ct values against a cell range from 20 to 2×10^6 CFU/g (Fig. 2 and 3). Thus, the standard curves showed a linear relation between the Ct values and the concentration of sample DNA, derived from 20 to 10^6 CFU/g of seafood tissue with regression coefficient (R^2) values ranging from 0.913 to 0.999. A linear plot of Ct values against the log concentration (2 to 2×10^6 CFU/ml) in water revealed that the cell detection was consistently linear up to 2 CFU/ml (Fig. 4). The maximum threshold Ct value for the seafood samples was determined at 38.07 ±0.12. A linear plot of the Ct values and DNA from different dilutions of the seeded fish and shrimp samples showed regression coefficient (R^2) values for the fish and shrimp homogenates seeded with 2×10, 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 CFU/g in repetitive experiments (Table 1).

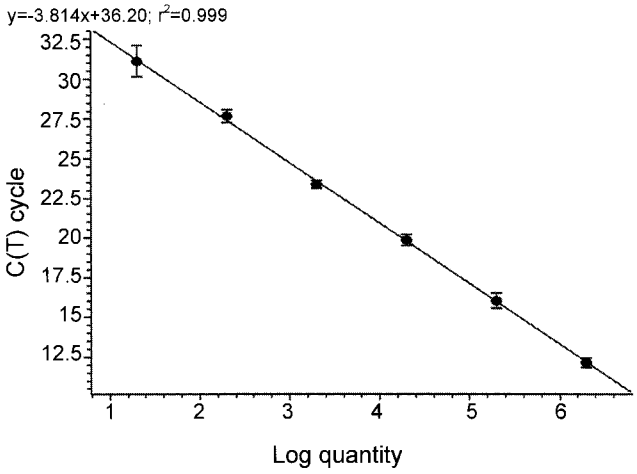

Fig. 3. Detection of *Salmonella* in seeded shrimp homogenates using real-time PCR.
Ct values are plotted against log CFU/g and the plot shows the regression coefficient (R^2) value. Data are reported as means and standard deviation of 3 repeat experiments.

Quantification of *Salmonella* Load in Naturally Contaminated Seafood Samples

The quantitative data showing *Salmonella* cell load in naturally contaminated seafood are presented in Table 2. The lowest *Salmonella* cell load was detected in a fish (*Sardinella longiceps*) sample (35 GE/g of fish sample), whereas the highest *Salmonella* load (1.8×10^4 GE/g) was detected in a shrimp sample. The results also showed that 4 out of the 10 seafood samples tested positive for *Salmonella* cells <100 GE/g, yet all the samples tested negative for *Salmonella* when using a direct plating method on *Salmonella* Chromagar media.

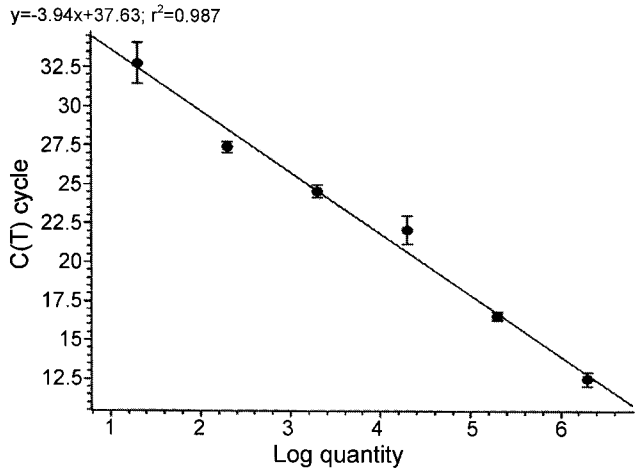

Fig. 2. Detection of *Salmonella* in seeded fish homogenates using real-time PCR.
Ct values are plotted against log CFU/g and the plot shows the regression coefficient (R^2) value. Data are reported as means and standard deviation of 3 repeat experiments.

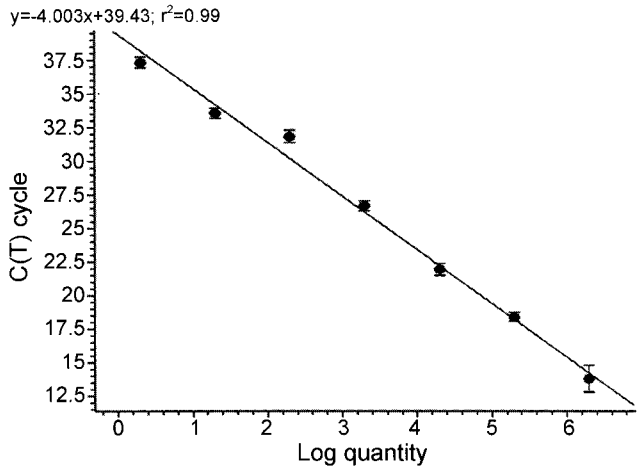

Fig. 4. Real-time PCR assay dilution for *Salmonella* pure culture without enrichment.
Ct values are plotted against log CFU/ml and the plot shows the regression coefficient (R^2) value. Data are reported as means and standard deviation of 3 repeat experiments.

Table 1. Regression coefficient (R^2) analysis of a real-time PCR assay for *Salmonella* in water, fish, and shrimp samples.

Exp. No.	Water		Fish		Shrimp	
	Slope (y)	R^2	Slope (y)	R^2	Slope (y)	R^2
1	-4.003x+39.43	0.990	-3.94x+37.63	0.987	-3.81x+36.20	0.999
2	-3.641x+43.26	0.979	-3.602x+37.72	0.994	-2.69x+35.59	0.963
3	-2.56x+36.72	0.984	-3.525x+36.49	0.913	-3.27x+31.98	0.988
4	-3.427x+27.20	0.997	-3.351x+38.07	0.946	-4.29x+28.05	0.976
5	-4.29x+28.05	0.996	-2.7x+37.12	0.966	-3.42x+42.70	0.992

Values calculated from seven 10-fold dilutions in duplicate.

The development of rapid quantitative methods is highly desirable for the enumeration of *Salmonella* cells in naturally contaminated seafood samples. The real-time PCR assay developed here for the quantification of *Salmonella* in seafood was found to be very useful for rapid enumeration, and was able to detect *Salmonella* (*i.e.*, 20 CFU/g) in seeded fish and shrimp samples, whereas in a pure culture (without seafood), the detection limit was 2 CFU/ml. The present results were also in agreement with an earlier study that reported on the detection of *Salmonella* (5 CFU/reaction) using a real-time PCR assay [16]. The quantitative detection of *Vibrio vulnificaus* in clam meat was previously reported at 100 CFU/g [24]. Similarly, the minimum detection limit for *Listeria monocytogenes* in water and skimmed milk was reported at 6 to 60 CFU/ml [19]. Interestingly, the level of real-time detection was not consistent for <100 *Salmonella* cells in a sample in which the DNA extraction was carried out using a phenol chloroform method (data not provided). The probable reason was that the extraction of the genomic DNA involved a multistep extraction process with enzymatic digestion, followed by phenol:chloroform separation, leading to the loss of a minute quantity of genomic DNA.

Table 2. Quantification of *Salmonella* in naturally contaminated seafood samples.

Sl.	Seafood	Real-time assay (genome equivalent/g)	*Salmonella* Chromagar method (CFU/g)
1.	Fish (*Rastrelliger kanagurta*)	0	0
2.	Fish (*Sardinella longiceps*)	2.8×10^2	1×10
3.	Shrimp (*Penaeus monodon*)	1.8×10^4	1×10^2
4.	Fish (*Rastrelliger kanagurta*)	0	0
5.	Fish (*Sardinella longiceps*)	0	0
6.	Fish (*Rastrelliger kanagurta*)	0	0
7.	Fish (*Sardinella longiceps*)	35	0
8.	Shrimp (*Penaeus monodon*)	0	0
9.	Shrimp (*Metapenaeus dobsoni*)	0	0
10.	Shrimp (*Penaeus monodon*)	80	0
11.	Squid (*Loligo* spp.)	0	0
12.	Clam (*Villorita cyprinoides*)	49	0
13.	Mussel (*Perna indica*)	0	0
14.	Clam (*Villorita cyprinoides*)	9×10	0
15.	Squid (*Loligo* spp.)	1.5×10^3	8×10
16.	Clam (*Villorita cyprinoides*)	0	0
17.	Clam (*Villorita cyprinoides*)	0	0
18.	Squid (*Loligo* spp.)	0	0
19.	Mussel (*Perna indica*)	0	0
20.	Shrimp (*Metapenaeus dobsoni*)	0	0
21.	Fish (*Sardinella longiceps*)	2×10^4	4×10^2
22.	Shrimp (*Penaeus monodon*)	0	0
23.	Clams (*Villorita cyprinoides*)	0	0
24.	Fish (*Sardinella longiceps*)	5×10^3	6×10^2
25.	Squid (*Loligo* spp.)	0	0
26.	Shrimp (*Penaeus monodon*)	0	0
27.	Fish (*Rastrelliger kanagurta*)	0	0
28.	Fish (*Sardinella longiceps*)	0	0

The present study also focused on the quantification of *Salmonella* in seafood from a naturally contaminated environment. When using culture methods, the requirement of different enrichment steps multiplies *Salmonella* levels many fold, making it impossible to determine the actual *Salmonella* load in a sample. Although the MPN method can provide the quantitative aspect of *Salmonella* in food or seafood, this method is not widely used for routine analyses as it is very cumbersome, lengthy, and difficult to analyze more than a few samples, since 10 to 15 tubes are required to analyze one sample. In contrast, the real-time PCR assay developed here, without the involvement of an enrichment period, can provide information on the *Salmonella* load in naturally contaminated samples. Although the number of samples tested was not very high, preliminary quantitative information was provided on the *Salmonella* load in the seafood. As the presence of a low number of *Salmonella* cells in food and environmental samples has already been reported [5, 8], this highlights the need for the generation of quantitative data on *Salmonella* in food and environmental samples and a real-time method would seem to be the most efficient and suitable quantitative method for the enumeration of *Salmonella* in food and feed samples [17]. The quantitative information on *Salmonella* in the naturally contaminated fish and shrimp samples showed a varying *Salmonella* contamination load in the seafood. The present study also highlighted that the real-

time assay was able to detect as little as 35 GE/g of *Salmonella* in the seafood samples, and 4 out of the 9 positive seafood samples exhibited a *Salmonella* load <100 GE/g. The level of detection using the real-time assay was also supported by the *Salmonella* Chromagar method, although the latter method was unable to detect any *Salmonella* when the load was low in the seafood samples. The detection of *Listeria monocytogenes* at the level of 4×10^3 CFU/g in fresh cheese using a real-time PCR was already described [21], and some other studies have compared a real-time PCR assay with conventional culture methods for qualitative detection of *Salmonella* in naturally contaminated food samples [7]. However, the real-time assay developed in this study will be useful for generating quantitative data on *Salmonella* in seafood and also helpful for detecting low levels of *Salmonella* in naturally contaminated seafood. The present study also highlights the need for a robust quantitative real-time method using suitable internal amplification control (IAC) for *Salmonella*, so that the method can be evaluated at multiple centers to harmonize the quantitative real-time PCR protocol for *Salmonella* in seafood.

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