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Detection of *Salmonella Spp.* in *Pandalus borealis* and *Chlamys islandica* by a real time qPCR method and partial validation of qPCR in a accredited quality control (QC) laboratory of Bangladesh

Md. Golam Sarwer[1]*, Fayejun Nesa[2], A. K. Jilani Chowdhury[3]

1 Fish Inspection and Quality Control (FIQC) Laboratory, Chittagong, Bangladesh.
2 Department of Pharmacy, BGC Trust University, Bangladesh.
3 Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, Bangladesh.

E-mail : sarwerfiqc@gmail.com Contact No. : +8801712729011

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Abstract

Salmonellosis is common worldwide and widespread in fisheries products of South Asia, especially Bangladesh because of poor post-harvest handling. For that, *Salmonella* is considered as one of the major causes of foodborne infection in public health which referred to as salmonellosis. More time and need many tests for the confirmation of pathogenicity is required by the conventional microbiological detection methods of *Salmonella.* Therefore, the requirement for more rapid and confirmatory methods of *Salmonella* detection becomes apparent. The purpose of this study was to apply the real-time qPCR (Quantitative Polymerase Chain Reaction) method for detection of *Salmonella* spp. in shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*), which is more convenient and less time consuming than conventional microbiological methods. PCR and real-time qPCR were initially tested and compared with pure culture of seven *Salmonella* spp. Each detection method was tested using artificially contaminated shrimp and scallop samples. The method validation of the real-time PCR method was performed according to ISO 17025 in contrast to qualitative, quantitative and reliability criteria of validation. The sensitivity of the real-time PCR, as well as the decreased time requirements of this detection method, would suggest its usefulness in commercial laboratory practices.

Key words : Polymerase Chain Reaction (PCR), *Salmonella* spp., Real-time PCR, Shrimp.

INTRODUCTION

Bangladesh has a good geographical location which is suitable for both marine and freshwater fish production. Due to high demand for shrimps, prawn and other crustaceans in the world market some entrepreneurs stepped into a new sophisticated industrial processing to earn foreign currency utilizing available resources of shrimps, prawns and other crustaceans. Shrimps and Prawns are the main exportable items which earn a substantial amount as BDT2744.12 cr. against 50368 ton of product with demandable variation in 2007-2008[5] and in 2009-2010 the earnings comes to BDT 3408.52 cr. [5]. Production of shrimp by culture and capture fisheries increased to a great extent in the beginning of 1980's in Bangladesh. In 2007-2008, 217,877.05 hector have been brought under shrimp culture[5]. Although shrimp farming has had a significant impact on the economy of Bangladesh, it is generally agreed. Major export items from Bangladesh are raw shrimp block frozen, IQF Shrimp and prawn, consumer pack of raw frozen shrimp, dry, salted and dehydrated fish and a little quantity of value added shrimp products. 63% of frozen shrimp exported to the European countries and 34%to USA and middle-east counties from Bangladesh[1].

Due to insufficient attention toward standard of hygiene and quality of the product, reasonable numbers of seafood products have gone out of business. As a result, the export of shrimp products has suffered considerable losses in rejection from 1975 to 1978 and the country was placed under automatic detention by United State Food and Drug Authority USFDA[12]. In October 1979, Bangladesh was black listed along with other countries by USFDA for having the evidence of Salmonella, filth, flies, cockroach and other insects in frozen shrimps frog legs. It faced heavy loss in the form of rejection and of relatively low price offered by the foreign buyers for fish products from Bangladesh.

Salmonella spp. is common in fisheries products of Bangladesh because of poor handling of fish from culture and capture grounds to processing. *Salmonella* contamination mostly occurs in post-harvest handling stages rather than in pre-harvest production sources[3]. In Bangladesh, people prefer to buy and consume live or fresh fish, which is a source of easy *Salmonella* contamination through rough handling and due to lack of hygienic conditions during the process. A microbiological analysis of 12 species of raw fish carried out in 2007 from fish markets of Bangladesh found that two-thirds of the samples contained with *Salmonella* spp. [4]. In 2008, a total of 30 consignments of Bangladeshi fishery products were refused by the USA due to *Salmonella* contamination[4].

The Department of Fisheries is the Central Competent Authority (CCA) for quality and safety assurance of fish and fishery products. In Bangladesh, there are three regional microbiological laboratories in the Fish Inspection and Quality Control office under the Department of Fisheries to verify the exportable fishery products in Dhaka, Chittagong and Khulna, which are equipped with microbiological and chemical laboratory facilities. The regional laboratories have microbiological test facilities like SPC (Standard Plate Count), E. coli, Vibrio cholere, Vibrio parahaemolyticus, Staphyllococcus aureus, Listeria monocytogen, *Salmonella* spp. etc. and chemical tests like TVBN and TMA.

The UNIDO (United Nations Industrial Development Organization) is conducting a project called “Strengthening of Fish Inspection and Quality Control Service in Bangladesh”. One

part of the project is to install ELISA (Enzyme-Linked Immuno Sorbent Assay), PCR (Polymerase Chain Reaction), HPLC (High Performance Liquid Chromatography) and other necessary techniques, methods and equipments to modernize the laboratory. Within a very short time, PCR could be set up for regular pathogenic analysis of fish samples. For the laboratory, this reduces labor, resources, and finally costs. PCR gives confirmatory results more quickly than by following the complete traditional microbiological identification scheme. Another part of the UNIDO project is to increase the competence of the laboratories in such a way that they can be accredited according to the ISO 17025**1** standard to be competent to carry out tests using standard and non-standard methods and laboratory-developed methods.

So the main objective of the current investigation was to apply the real-time qPCR (Quantitative Polymerase Chain Reaction) method for detection of *Salmonella* spp. in shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*), which is more convenient and less time consuming than conventional microbiological methods in Quality Control (QC) laboratory especially microbiological unit of Chittagong.

MATERIALS AND METHODS

Study area selection for facilities

Microbiological unit of Quality Control (QC) laboratory, Chittagong was selected for the study. Various national and international laboratory experts have been working with lab personnel to sustainable increase of biological characteristics analysis of fish and fishery products in view of sustaining Bangladesh export performance in world seafood markets through integration of qualified personnel, standard methods, appropriate equipment and quality assurance system by following biosafety and biosecurity properties. Study data were collected during June 2014 to February 2015.

Salmonella strains

The *Salmonella* strains were obtained from the BCSIR Laboratory, Bangladesh. All the stains had been stored on nutrient agar (Difco) at -20C. From the nutrient agar small aliquots were transferred to PCA (Plate Count Agar) (Difco) and incubated at 37°C for 24 hours (Figure 1). From the PCA a loop-full of *Salmonella* colony was transferred to both TSA (Tryptic Soy Agar) (Difco) and TSI (Triple Sugar Iron) (Difco) agar tube. The TSA plate was incubated for 24 hours at 37°C. The loop-full of *Salmonella* strains were streaked on the TSI agar tube slope surface and stabbed on the butt and the TSA tubes were incubated at 37°C for 24 hours. From the TSI tubes a loop-full of *Salmonella* strains were grown on 10 mL nutrient broth (Difco) for 24 hours at 37°C. The *Salmonella* content of the nutrient broth was approximately 109 CFU/mL, from where 0.1 mL of the cultured nutrient broth was transferred to 10 mL 0.15% peptone water (Difco) solution which was 10-7 CFU/mL and a series of serial dilutions (10-5, 10-3, 10-2 and 10-1 CFU/mL respectively) of peptone water were made. From the peptone water dilutions (10-3, 10-2 and 10-1) 1mL of *Salmonella* containing peptone water was inoculated to TSA agar plates and was incubated at 37°C for 24 hours (Figure 1). The concentrations of cells were determined by viable counts on the TSA plates.

Preparation of pure culture of *Salmonella* spp. for DNA extraction

One mL of the cultured nutrient broth containing *Salmonella* was mixed homoginately with 10 mL of a selective enrichment medium, Rappaport Vassiliadis (RV) and incubated for 5 hours at 42°C in a circulating water bath. One mL of aliquots of a 5 hour enriched RV was used for DNA extraction for the real-time PCR assay of pure culture. Before use, the sample was stored at -20°C for upcoming PCR analysis.

Preparation of spiked shrimp and scallop samples and estimation of the contamination levels

The shrimp (Pandalus borealis) and scallop (Chlamys islandica) were used for artificial contamination, bought from the Superstore, Bangladesh. The *Salmonella* serovar Montevideo was made contaminated with both the samples. The *Salmonella* serovar Montevideo concentration in the nutrient broth was 38 x109 CFU/mL. For contamination level 1, 0.66 mL of the 10-6 CFU/mL *Salmonella* culture nutrient broth dilution and for contamination level 2, 3.3 mL of the 102 CFU/mL dilution of the nutrient browth was inoculated and mixed homoginately with the 25 g each shrimp and scallop samples. 25 g *Salmonella* contaminated shrimp and scallop samples each were mixed and homoginated with 225 mL Buffered Peptone Water (BPW) (Difco, USA) and incubated for 20 hours at 37°C. From each four pre enriched aliquots, 1 mL was homoginated with 10 mL of RV broth and incubated at 42°C for 24 hours for the continuation of traditional microbial tests and similarly other four RV broth were inoculated and incubated at 42°C for 5 hours for DNA extraction (Figure 1). The presence of *Salmonella* was verified according to NMKL-71 (1999). For the verification of *Salmonella* presence 1 mL aliquot from the selective 24 hour enriched RV broth was inoculated to XLD agar plates which and incubated at 37°C for about 48 hours and finally checked for the characteristic colonies (Figure 2).

Culture of *E.coli* for the negative control of PCR and real-time PCR

One mL of *E. coli* from LST broth was inoculated into 10 mL BPW for pre-enrichment and incubated at 37ºC for 20 hours. One mL of the pre-enrichment was homoginated with 10 mL of RV broth and was incubated at 42ºC into a circulating water bath for 5 hours for DNA extraction.

Optimisation of PCR

The optimisation is essential to maximise the specificity and efficiency of PCR, it includes optimal and suitable primer sequences, appropriate primer and template DNA concentrations and annealing temperature.

Protocol of the PCR

The Polymerase Chain Reaction of *Salmonella* DNA was done by Peltier Thermal Cycler, PTC-200 (MJ Research). The programming of the DNA engine moves through five steps: initiating the programme, naming the programme, choosing a temperature control method, entering the programmes steps and entering the end or closing step. The *Salmonella* specific primers TCGTCATTCCATTACCTACC and AAACGTTGAAAAA CTGAGGA was used to amplify a 100- base pairs fragment of the invasion (invA) gene. Primers were synthesized by Sigma Genosys Ltd.(Switzerland). All runs were included a negative control without target DNA and *Salmonella* serovar Montevideo as the positive control.

Reaction set-up

A reaction master mix was prepared by adding the following

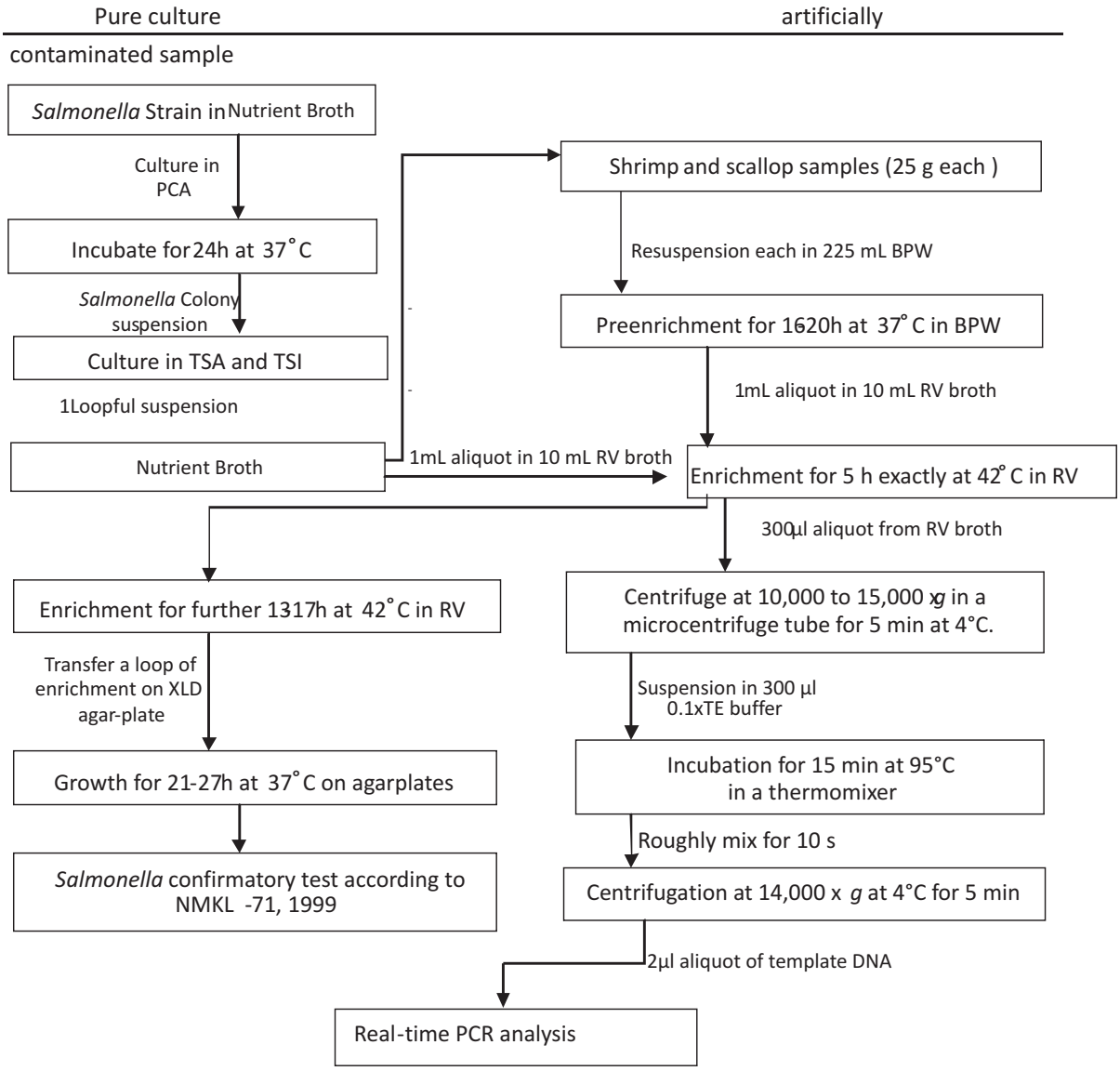

Figure 1: Flow diagram showing preparation of pure culture of *Salmonella* for spiking of shrimp and scallop samples and isolation of DNA for PCR analysis.

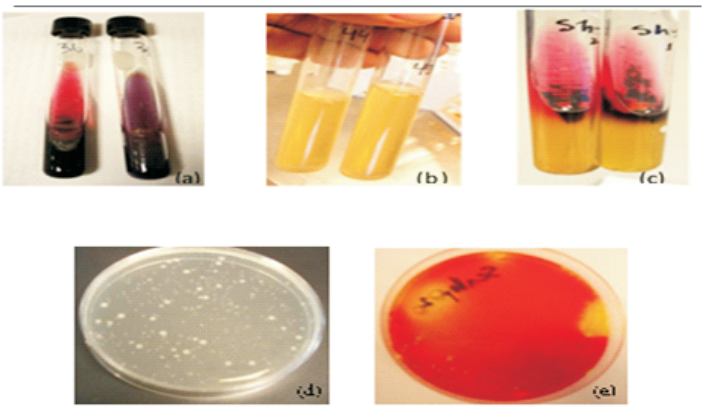

Fig. 2: *Salmonella* strains in (a) TSI & LIA agar tube (b) nutrient broth (c) sub-culture in TSI agar tube (d) nutrient agar plate (e) XLD agar plate

Table 1: Reaction mixture used for assay development of the PCR

10x PCR Buffer 2.50 µl (Provides a final concentration of 2.5 mM MgCl2)	2.50 µl
DNTP	2.00 µl
Taq Polymerage (1 U)**1**	0.30 µl
Forward Primer (3U/µl)	1.00 µl
Reverse Primers (3U/µl)	1.00 µl
Template DNA	2.00 µl
Water, nuclease-free	to 25 µl
Total Volume	25 µl

(Table 1) components (except template DNA) for each 25 µl reaction to an eppendorf tube at room temperature, all solutions were gently vortexed and briefly centrifuged after thawing.

The master mix was mixed throughly and dispensed in appropriate volumes into PCR plates. Template DNA was added to a volume of 2.00 µl to the individual PCR tubes containing the master mix. The reactions were mixed by centrifuge gently without creating bubbles. Thermal cycling conditions were: 94°C for 5 min, followed by 30 cycles of 94°C for 50 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A final extension of 72°C for 7 minutes was employed. All thermal cycling conditions were performed using a three-step cycling protocol. The PCR assay was run on 2% agarose gel electrophoresis with ethidium bromide staining. The voltage and time of the gel electrophoresis was 70 Watt for 1.5 hours. DNA moves towards positive anode due to the negative charges on its phophate backbone. The gel was stained into ethidium bromide solution for at least 15 minutes.

Preparation of reaction mixture for agarose gel electrophoresis

Two (2) µL of 6x loading dye solution (MBI, Fermentas) was mixed with 10 µl of DNA sample on a piece of aluminium foil wrap. After mixing a 10 µl solution it was inserted into the gel hole and 3 µL of 100 base pair DNA ladder (MBI, Fermentas) were inserted into a hole. For each gel run there was a negative control of template DNA.

Preparation of agarose gel

Two (2) g of Agarose powder (Sigma, Germany) were mixed and homogenated with 200 mL of 0.5x TBE; 50 mL of 10x TBE were mixed into 1 l of distilled water to prepare 0.5x TBE and 5x TBE were prepared into a stock solution of 54 g of Tris base (TRIZMA-BASE, Sigma, USA), 27.5 g of boric acid and 20 mL of 0.5 M EDTA (pH 8.0).

Preparation of ethidium bromide gel coloring bath

The ethidium bromide solution was used to bath the agarose gel. 65 µl of 10 mg/mL ethidium bromide (Plus One, Pharmacia Biotech) was added to 649.35 mL of TBE. The final solution was 1 µg/mL ethidium bromide bath, ready to use. A positive response was defined as the presence of a visible band at the expected size, while a negative response was defined as the lack of any band at the expected size. The gel was documented with a digital camera.

Spiked shrimp and scallop analysis for *Salmonella* on PCR

Two concentrations (106 CFU/mL and 103 CFU/mL) of *Salmonella* serovar Montevideo contaminated shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) were run on PCR gel-electroferosis. The *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin were as positive control and non-template DNA as negative control.

PCR of the proficiency testing sample

The *Salmonella* serovar Dublin from the proficiency testing sample contained approx. seven *Salmonella* Dublin per mL of reconstituted aliquote, were run on PCR gel- electrophoresis and SYBR Green Real-time qPCR.

Environmental swab samples

Forty environmental samples were taken by swabbing at different locations in the neighborhood of a fish processing facility in northern Bangladesh and were analyzed by the SYBR Green Real-time qPCR.

Creating a gDNA standard curve for *Salmonella* serovar Montevideo from real-time qPCR

Genomic DNA (gDNA) is commonly used as standards in quantitative PCR. The mass of gDNA corresponds to copy numbers of target nucleic acid sequences[2]. A standard curve was prepared in which a gene of interest is present at 1,000,000 copies, 100,000 copies, 10,000 copies, 1000 copies, 100 copies and 10 copies.

The size of the *Salmonella* genome is approximately 4.8 million bp (haploid). The mass of the genome was calculated by inserting the genome-size value in the following formula (Applied Biosystems 2003).

$$m = [n]\left[1.096e^{-21}\ \frac{g}{bp}\right]$$ where n = genome size (bp)

m = mass

e^{-21} = x 10-2

A standard curve was obtained for the real-time PCR reaction by analyzing 10-fold serial dilutions of a *Salmonella* serovar Montevideo. Log-linear regression analysis was performed using Microsoft Excel 2007 software. To establish the reliability of SYBR Green the real-time PCR method, there producibility of the standard curve was verified with the standard curve produced by

Power SYBR Green qPCR Master Mix. Following Table 9, components (except template DNA) for each 25 µl reaction were added to a tube at room temperature to get a final volume of 25 µl. The Master Mix was mixed throughly and dispensed in appropriate volumes into PCR plates. The reactions were mixed by centrifuge gently without creating bubbles.

Real-time qPCR with spiked shrimp and scallop

Two concentrations (106 CFU/mL and 103 CFU/mL) of *Salmonella* serovar Montevideo contaminated shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) were run on real-time PCR using Power SYBR Green qPCR Master Mix. Non-spiked shrimp, scallop and non-DNA templates were used as negative control. The *Salmonella* serovar Montevideo was a positive control.

Quantification *Salmonella* DNA from shrimp and scallop samples

Real-time PCR is often used to obtain quantitative information on the DNA concentration of the unknown samples. The concentrations are derived as a ratio relative to the DNA content in the other samples. The concentrations of the unknown samples are derived from the ratio of known concentration of standard samples.

Validation

In this work, validation was done in four steps by qualitatively and quantitatively determining *Salmonella* by PCR and RT-qPCR in pure cultures of seven *Salmonella* strains, spiked shrimp and scallop samples, a proficiency testing sample and Environmental swab samples. In all four steps results from PCR analysis were compared directly or indirectly to the "conventional" culture method.

Statistics

Repeatability of qualitative data was defined as the percentage chance of finding the same result, positive or negative, from two identical samples analysed in the same laboratory under predefined repeatability conditions. Standard curves were constructed by plotting quantity versus threshold cycle (CT) produced for the target gene. For a comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of standard curves were calculated by performing a linear regression analysis with Microsoft Excel 2007.

RESULTS

Optimisation of PCR

Primer sequence

Four pairs of *Salmonella* specific primers were initially selected. They are Shima (122 bp), invA119 (119 bp), ttr 6/4 (94 bp) and Sal/inv 139/141(284). All the four pairs were tested with the DNA of *Salmonella* serovar Montevideo strains. The invA119 (119 bp) primar pairs showed the best gel-electrophoresis band (Figure 4) signal compared to the others, so the invA119 primer pairs were used for all the next experiments.

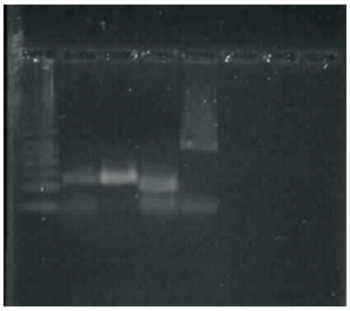

Fig. 4: PCR amplification of *Salmonella* serovar Montevideo on primer set (1) Shima 122 bp (2) invA 119 bp (3) ttr6/4 94 bp (4) Sal/inv 139/141 284 bp. InvA 119 bp (arrow mark) shows the best band.

Primer concentration

From the different concentration of primers, the better efficiency is shown in 0.2 µM primer concentration (Figure 5c) which was selected for the next intended use of the project.

Annealing temperature

After comparing three different annealing temperatures (55°C, 58°C and 60°C), the 60°C annealing temperature showed better efficiency of the PCR reactions (Figure 5c). For the next PCR reactions, the 60°C annealing temperature was used.

Salmonella strains

All the six *Salmonella* spp. were tested in a microbiology laboratory using the traditional microbiological method and showed *Salmonella* positive in TSI and LIA agar (Figure 2), concentrations were found from the six different strains of

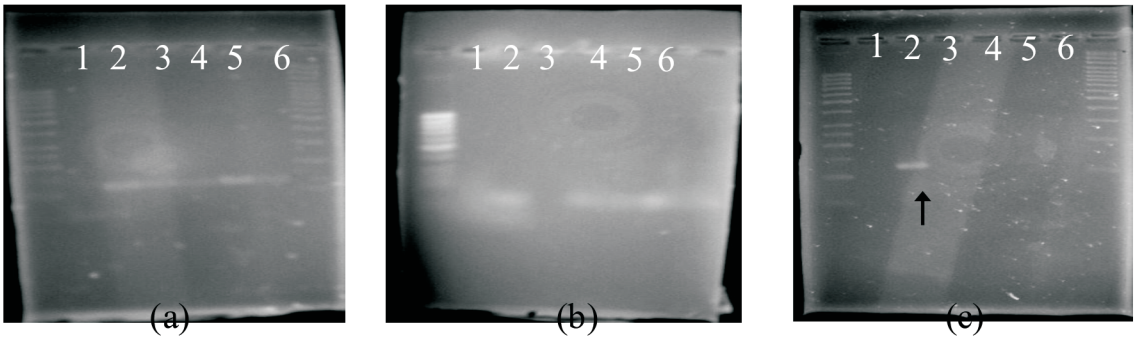

Fig. 5: PCR amplification of *Salmonella* spp. on 2% agarose gel by 119-base pair invasion (invA) gene primer set in three different annealing temperatures (a) 55°C (b) 58°C (c) 60°C; and six different concentrations of primer sets: 1. 0.3 µM Forward (F) and Reverse (R) primer each with no template DNA. 2. 0.3 µM F and R primer. 3. 0.1 µM F and 0.3 µM R primer. 4. 0.1 µM F and R primer. 5. 0.2 µM Fand R primer. 6. 0.3 µM F and 0.1µM R primer. 0.3 µM F and R primer and 60°C annealing temperature shows the best efficiency on gel-electrophoresis (arrow sign in c) without any primer-dimer.

Table 2: Concentration of six *Salmonella* spp. used for assay development was collected from Chittagong, Bangladesh; all strains were sampled from Fish of Bangladesh.

Salmonella spp.	CFU/mL
1. *Salmonella* serovar Tennesse	9 x109
2. *Salmonella* serovar Montevideo	38 x109
3. *Salmonella* serovar Schwarzengrund	4 x109
4. *Salmonella* serovar Agona	3 x109
5. *Salmonella* serovar Bredeney	7 x109
6. *Salmonella* serovar Infantis	4 x109

Salmonella (Table 2). The lowest concentration was found in the *Salmonella* serovar Agona and the highest concentration in the *Salmonella* serovar Montevideo.

The six selected *Salmonella* strains were tested with the universal 16S rDNA primer (Figure 7). It proved that the strains were bacteria, because the 16S rDNA primer separates bacteria DNA from virus and other DNA.

All the six *Salmonella* spp. showed positive bands on 2.5% agarose gel electroferosis (Figure 7) after PCR reactions with *Salmonella* Specific invA(119 bp) primer. The invA gene primer absorbed the *Salmonella* DNA from the strains, so it proved that PCR reaction easily detected the *Salmonella* spp.

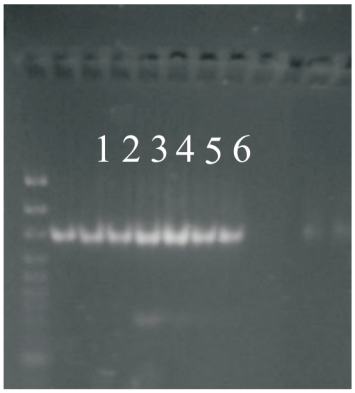

Fig. 6: PCR amplification of six different *Salmonella* spp. on 1.5% agarose gel by 16S rDNA primer set. 1. 100 bp DNA ladder. 2. *Salmonella* serovar Montevideo. 3. *Salmonella* serovar Tennesse. 4. *Salmonella* serovar Montevideo. 5. *Salmonella* Schwarzengrund. 6. *Salmonella* Agona.

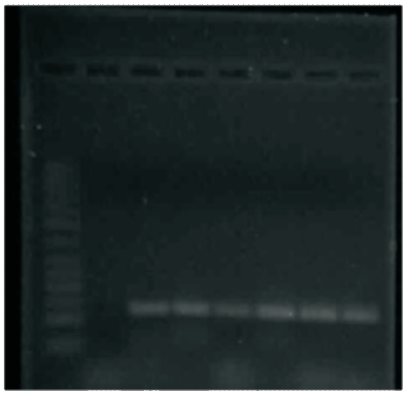

Fig. 7: PCR amplification of six *Salmonella* spp. on 2.5% agarose gel by invA 119 bp Primer set. 1. 50 bp DNA ladder. 2. No Template DNA 3. *Salmonella* serovar Tennesse. 4. *Salmonella* serovar Montevideo. 5. *Salmonella* serovar Schwarzengrund. 6. *Salmonella* serovar Agona. 7. *Salmonella* serovar Bredeney. 8. *Salmonella* serovar Infantis.

Table 3: Quantity of DNA in six *Salmonella* strains.

***Salmonella* spp.**	**Concentration (µg/mL or ηg/µl)**
1. *Salmonella* serovar Tennesse	8.62
2. *Salmonella* serovar Montevideo	4.21
3. *Salmonella* serovar Schwarzengrund	3.48
4. *Salmonella* serovar Agona	3.68
5. *Salmonella* serovar Bredeney	4.56
6. *Salmonella* serovar Infantis	4.42

Table 4: Quantity of Salmonella serovar Montevideo DNA in *Pandalus borealis* and *Chlamys islandica.*

Species	Concentration (µg/mL)
Pandalus borealis	4.98
Chlamys islandica	21.1

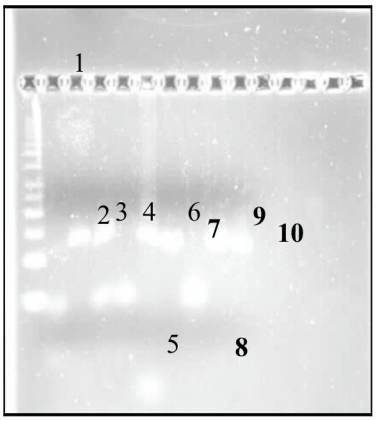

Fig. 8: PCR amplification of *Salmonella* serovar Montevideo contaminated with shrimp (*Pandalus borealis*) and scallop (Chlamys islandica) on 2.5% agarose. 1. 50 bp DNA ladder 2. Negative control (non-template DNA) 3. *Salmonella* serovar Montevideo as positive control 4. *Salmonella serover* Dubllin as positive control 5. Non-spiked *Pandalus borealis* 6. *Pandalus borealis* (106 CFU/mL) 7. *Pandalus borealis* (103 CFU/mL) 8. Non-spiked Chlamys islandica 9. *Chlamys islandica* (106 CFU/mL) 10. *Chlamys islandica* (103 CFU/mL)

Spiked shrimp and scallop samples

Two concentrations (106 CFU/mL and 103 CFU/mL) of *Salmonella* serovar Montevideo contaminated shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) were tested using traditional microbiological methods and showed positive in TSI and LIA agar, the concentration was measured by fluorometer (Invitrogen) (Table 4). The scallop had highest concentration of *Salmonella* of 21.1 µg/mL.

The template DNA from *Salmonella* serovar Montevideo contaminated shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) were analysed with PCR gel- electrophoresis, showed strong band (Figure 9). The *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin were as positive control and non-template DNA as negative control.

The PCR easily detected 103 CFU/mL of *Salmonella* Montevideo from shrimp and scallop, the non-spiked shrimp and scallop showed no band (Figure 9(5) and (8)) with the invA 119 bp primer, possibly other bacteria may present in the non-spiked shrimp and scallop.

Reference sample

The reference *Salmonella* serover Dublin which was collected from the Microbiology Division, National Food Administration, Sweden, contains approximately seven *Salmonella* serover Dublin per mL of reconstituted aliquote, were run on PCR gel-electrophoresis shown positive band (Figure 9(4)). Therefore, PCR was able to detect reference *Salmonella* serover Dublin. It was the important part quantitative validation of the PCR method.

Environmental swab samples

Among the 40 collected swab samples from different locations in the neighbourhood of a fish processing facility in northern Bangladesh, one sample that showed a positive response was taken from personal rest area but this need to be confirmed.

From the 40 swab samples, most of the swabs showed positive on the real-time PCR run, the flurescence quantity (ΔRn) crossed the threshold lines from the 26 cycles which means they are possibly positive. Among all the possible *Salmonella* positive sample,s only one sample (sample 28) proved the conformation of the presence of *Salmonella* in melting curve analysis (Figure 11). It showed the same melting temperature as like *Salmonella* Montevideo and *Salmonella* Dublin (Figure 11).

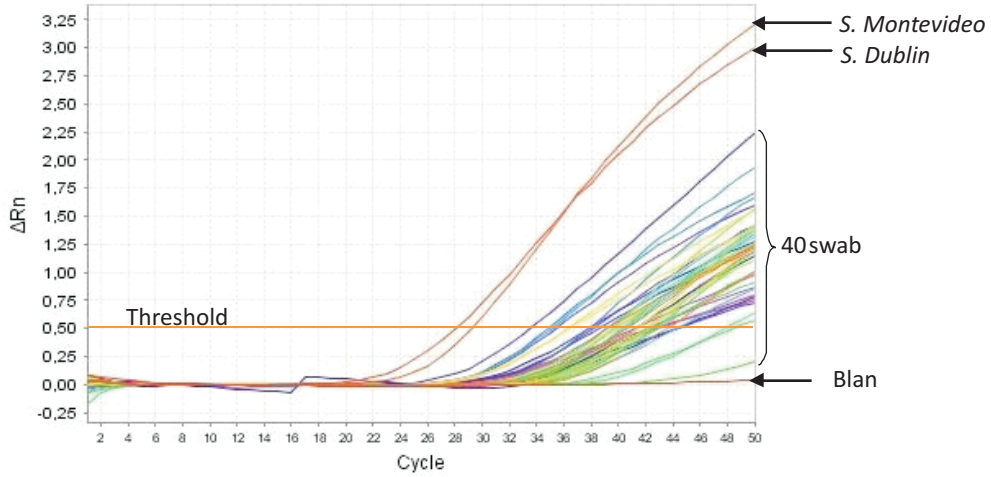

Figure 9: Fluorescence quantity of 40 swab samples, *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin as positive control and non template DNA as negative control.

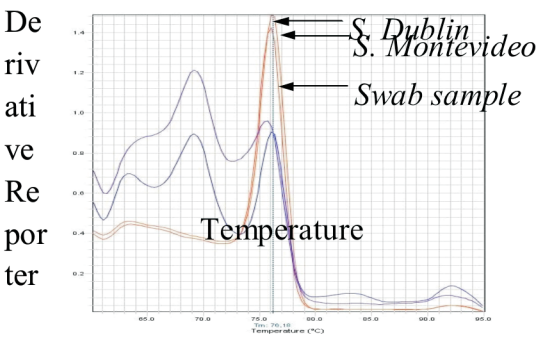

Fig. 10: Melting curve analysis of two swab samples, *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin as positive control and non-template DNA as negative control.

For the conformation of the presence of *Salmonella* in the swab sample 28, the melting curve analysis was repeated (Figure 12). It showed the same melting temperature as positive control (*Salmonella* serovar Montevideo and *Salmonella* serovar Dublin).

Creating a gDNA standard curve for *Salmonella* serovar Montevideo from real-time qPCR

a. Standard curve from Maxima TM SYBR Green Qpcr Master Mix (2X)

Six serial dilutions (106 to 101) of *Salmonella* serovar Montevideo were analysed in real-time qPCR, show a relation of Threshold Cycle (CT) value with the fluorescence signal (Figure 12).

A standard curve was obtained for the real-time PCR reaction by analysing 10-fold serial dilutions of *Salmonella* serovar Montevideo. Log-linear regression analysis was performed using Microsoft Excel 2007 software. There was a good correlation (R2=0.922) between the CT values and the copy numbers of invA gene of *Salmonella* serovar Montevideo (Figure 14).

b. Standard curve from Power SYBR Green Qpcr Master Mix

A standard curve was obtained using the power SYBR Green

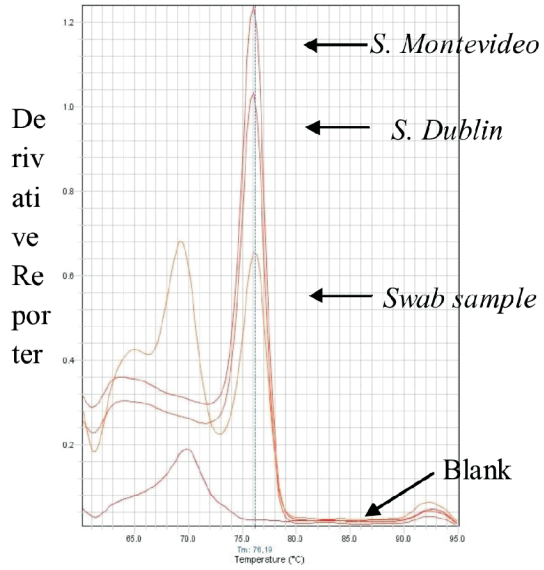

Fig. 11: Melting curve analysis of two swab samples, *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin as positive contol and non-template DNA as negative control.

Qpcr Master Mix in the Applied Biosystems StepOne Real-Time PCR. Ten-fold six serial dilutions (106 to 101) were used to produce an amplification plot (Figure 16) of fluorescence quantity vs. CT value.

A standard curve was obtained by analysing 10-fold serial dilutions of a *Salmonella* serovar Montevideo. Each sample was used in triplicate to get an average CT value. Log-linear regression analysis was performed using Microsoft Excel 2007 software.

SYBR Green qPCR Master Mix.

Both the dilution curves (Figures 13 and 15) showed that the real-time qPCR easily detected 103 invA gene, the florescence was visible after 24 cycles. The two standard curves (Figures14 and 16) revealed that the real-time PCR can quantify *Salmonella* DNA with a particular difference in CT values in each 10-fold dilution. There was a good correlation (R2=0.922 and 0.9296) between the CT values and the copy numbers of invA gene of

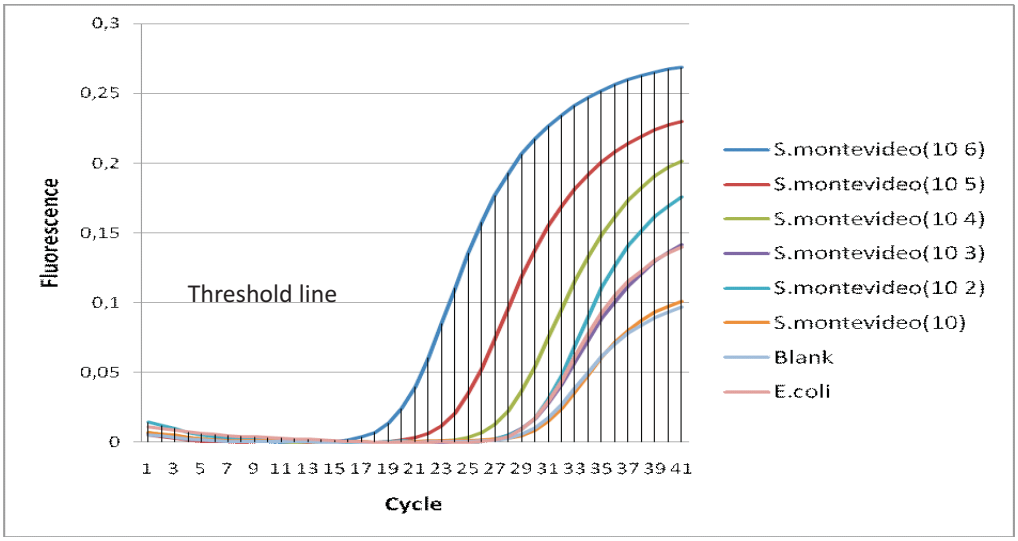

Figure 12: Fluorescence quantity shows 40 cycles in real-time PCR of six dilution

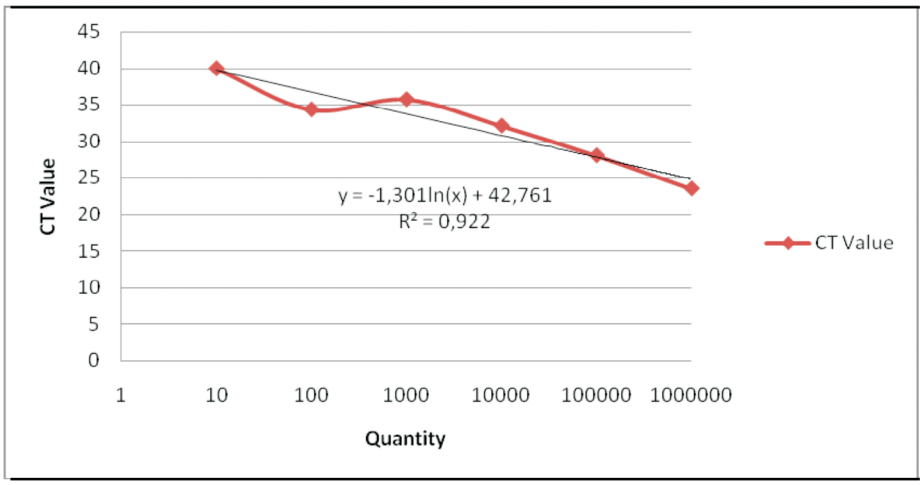

Fig. 13: Standard curve of the 10-fold serial dilutions (106 to 101) of *Salmonella* serover Montevideo in the real-time qPCR in Maxima TM SYBR Green qPCR Master Mix (2X).

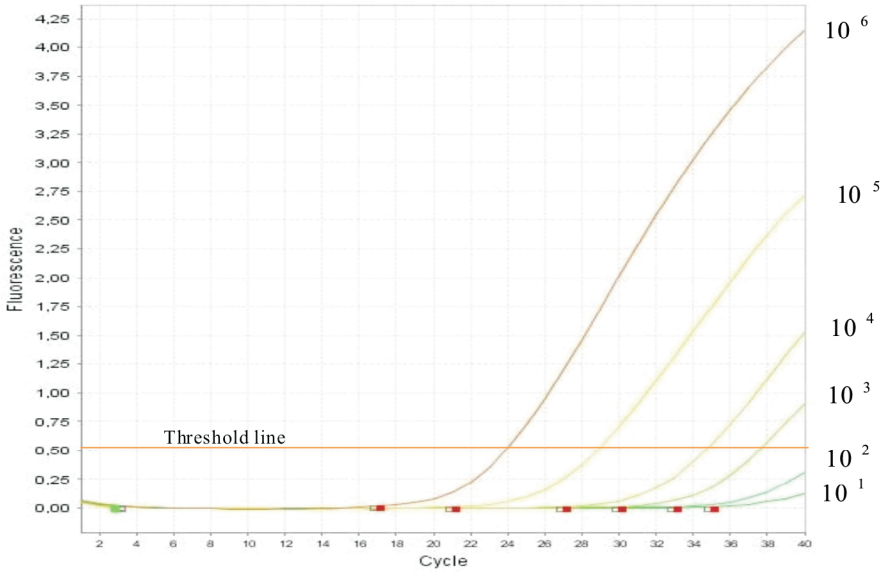

Fig. 14: Fluorescence quantity shows 40 cycles in real-time PCR of six dilution series (106, 105, 104, 103, 102, and 101) of *Salmonella* serovar Montevideo invA gene in Power SYBR Green qPCR Master Mix.

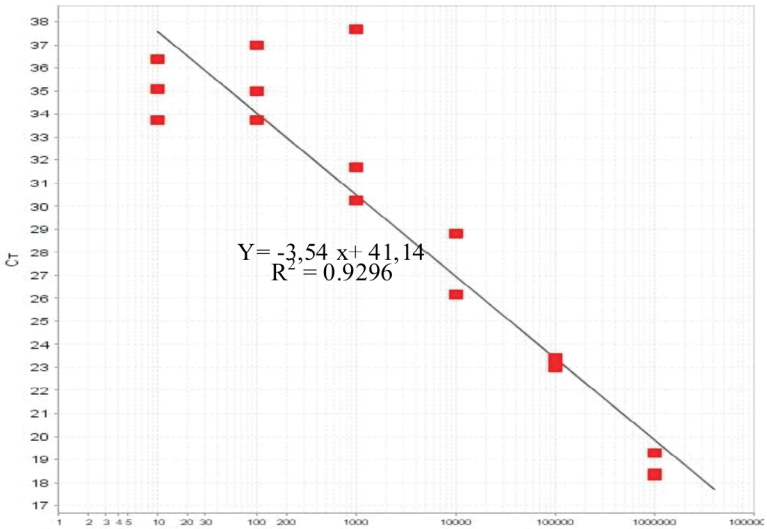

Fig. 15: Standard curve of the 10-fold serial dilutions (106 to 101) of Salmonella serovar Montevideo (triplicate samples) in the real-time qPCR in Power

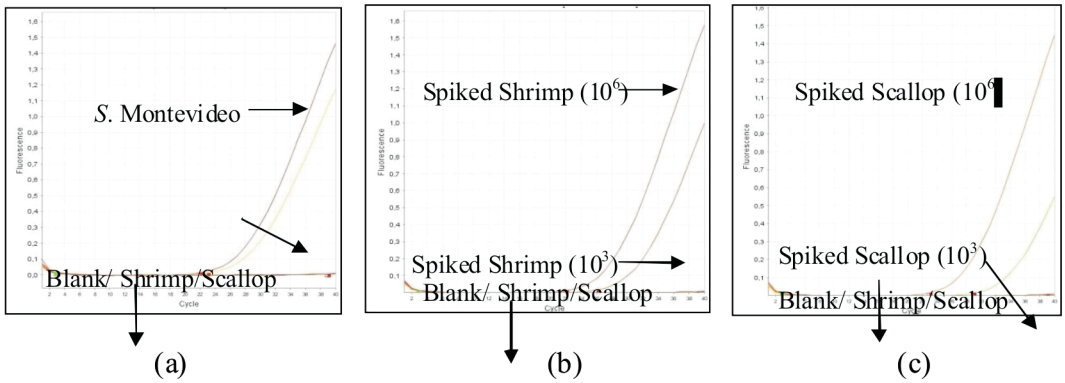

Fig. 16: Fluorescence quantity (Y-axis) shows in 40 cycles (X-axis) in real time PCR of (a) duplex *Salmonella* serovar Montevideo strains (b) Two concentrations (106 CFU/mL and 103 CFU/mL) *Salmonella* serovar Montevideo contaminated with shrimp (Pandalus borealis) (c) Two concentrations (106 CFU/mL and 103 CFU/mL) *Salmonella* serovar Montevideo contaminated with scallop (Chlamys islandica). Non-spiked shrimp, scallop and non-DNA templates were used as negative control (blank).

Salmonella serovar Montevideo (Figure 16). Experimental error may cause unequal difference of CT values in dilution series.

Real-time PCR with spiked shrimp and scallop

Two concentrations (106 CFU/mL and 103 CFU/mL) of *Salmonella* serovar Montevideo contaminated shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) were run on real-time PCR using Power SYBR Green qPCR Master Mix. Non-spiked shrimp, scallop and non-DNA template were used as negative control. The *Salmonella* serovar Montevideo was as positive control.

The *Salmonella* serovar Montevideo strains show positive thresholds (CT value 28.58 and 30.01), where the non-spiked shrimp, scallop and non-DNA templates show no thresholds (Figure 16a).

The *Salmonella* serovar Montevideo contaminated shrimp (*Pandalus borealis*) with a concentration of 106 invaA gene and 103 invA gene show a significant (standard deviation 0.65) difference (2.44) of CT values, 29.31 and 31.75 for 106 invaA and 103 invA gene respectively. *Salmonella* serovar Montevideo contaminated scallop (*Chlamys islandica*) with the same *Salmonella* specific invA gene concentrations show a significance (standard deviation 3.60) CT value difference (2.87) (Figures 16b, 16c and 17).

Quantitation of *Salmonella* DNA in shrimp and scallop

From the following equation, the number of known invA gene of *Salmonella*, the unknown number of invA gene from the shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) were calculated.

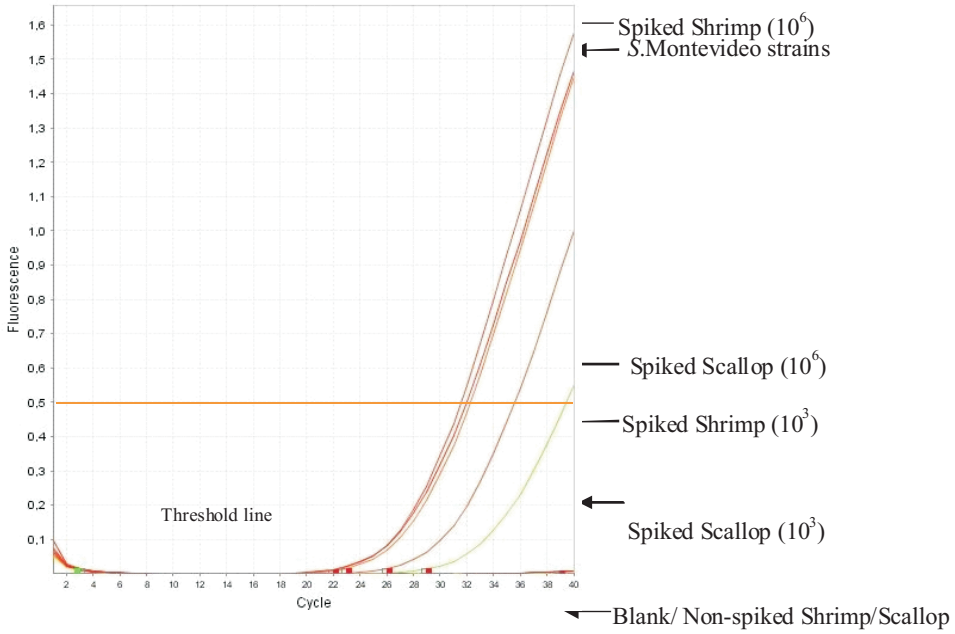

Fig. 17: Fluorescence quantity (Y-axis) versus cycle no. (X-axis) in real-time PCR shows two concentrations (106 CFU/mL and 103 CFU/mL) of *Salmonella* serovar Montevideo contaminated with shrimp (Pandalus borealis) and scallop (Chlamys islandica). Non-spiked shrimp, scallop and non-DNA templates were used as negative control (blank), *Salmonella* serovar Montevideo strains were used as positive control.

Table 5: Quantity of DNA (invA gene) in spiked shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*), calculated from the CT value of control/standard DNA (*Salmonella* serover Montevodeo).

Standard/Samples	1st CT Value	2nd CT Value	CT Mean	No of invA gene
S. Montevideo	28.58	30.01	29.29	1000000.00
Spiked Shrimp (106 CFU/mL)	28.39	30.22	29.31	986193.30
Spiked Shrimp (103 CFU/mL)	32,21	31.29	31.75	181818.18
Spiked Scallop (106 CFU/mL)	28,95	31.48	30.22	526315.79
Spiked Scallop (103 CFU/mL)	30,54	35.64	33.09	71787.51

= Ratio of Standard DNA Template to Sample DNA Template

The copy number of invA gene of *Salmonella* spp. (1000,000 invA gene) were used as control/standard DNA. The mean CT values were calculated from the two CT values of *Salmonella* serover Montevodeo, spiked shrimp (*Pandalus borealis*) (106 CFU/mL and 103 CFU/mL) and scallop (*Chlamys islandica*) (106 CFU/mL and 103 CFU/mL) which were used to calculate the quantity of invA gene of *Salmonella*.

$= 2^{-[CT\,(Standard\,DNA) - CT(Sample\,DNA)]}$

= 2-[29.29- 29.31]

= 20.02

= 1.014

Thus, the *Salmonella* serover Montevodeo (control/standard) DNA had 1.014 times more starting DNA than the *Salmonella* serover Montevodeo contaminated/spiked shrimp sample (106 CFU/mL). Therefore, px1.014 = 1000,000 p = 986193.30, where p is the number invA gene in spiked shrimp sample (106 CFU/mL). From the above example, the quantitations of invA gene were calculated from different concentrations of spiked shrimp and scallop (Table 5). Quantity of DNA (invA gene) in spiked shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*), calculated from the CT values of control/standard DNA (*Salmonella* serover Montevodeo)

Development of real-time qPCR methods for detection of *Salmonella* spp.

A real-time PCR method was developed for detection of pathogenic *Salmonella* spp. from shrimp and scallop. The method was optimised using four sets of primer pairs to get the appropriate primer pair and its optimised concentration, three anneling temperatutes were tested to the assay to get the best result. In the evaluation of the real-time PCR method on *Salmonella* spp. *Salmonella* was detected by both PCR and a traditional culture method (NMKL-71 1999). Six *Salmonella* strains were analysed with the real-time PCR method and were verified with PCR and traditional culture method. A reference sample of *Salmonella* Dublin from the Microbiology Division, National Food Administration, Sweden, was tested by the real-time PCR method, PCR and traditional method as well to establish the reliability of the developed method.

Two standard curves were drawn using the same six dilutions (106 to 101 CFU/mL) of *Salmonella* serovar Montevideo to verify the linearity of the method. The detection limit was determined by testing triplicates of the same six conentrations of serial dilutions. By this method, the detection limit was determined to 1000 *Salmonella* serovar Montevideo (invA gene) per PCR reaction volume of 25 µl. The quantitations of DNA from spiked shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) were calculated from the ratio of CT values of known concentrations of control/standard sample (*Salmonella* serovar Montevideo).

Validation of real-time qPCR for detection of *Salmonella* spp.

The results from PCR analysis show that the PCR based methods were able to detect and quantify *Salmonella* in all positive samples.

a. Qualitative criteria of validation

The specificity was checked out by detecting the target *Salmonella* specific DNA sequence (invA gene) from purecultures of *Salmonella*, from spiked shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) samples and from inter-laboratory test samples. The specificity was checked with the conventional microbiology method and PCR gel-electroferosis. Selectivity was demonstrated with the negative and positive process control of *Salmonella* DNA from the *Salmonella* strains. The demonstration was done repetitively (three times) using the same *Salmonella* specific primers, show the absence of interference peaks of other unexpected pathogens.

The proficiency test sample was tested in real-time PCR, "conventional method" and PCR method; all three methods showed positive results which provided the reliability properties of the validation method.

b. Quantitative criteria of validation

Sensitivity studies were performed with *Salmonella* pure cultures in broth and in shrimp samples to determine the lower detection limit of the real-time PCR assay. The real-time PCR assay was able to detect 103 CFU/mL of *Salmonella* serovar Montevideo strains 103 invA gene from the contaminated sampled. The RT-qPCR was able to detect seven *Salmonella* cells

per mLin the proficincy test sample. Standard curves were constructed using mean Threshold Cycle (CT) and various concentrations of *Salmonella* (ranging from 101 to 106 invA gene) and resulted in a linear relationship between CT and log input DNA. There was a good correlation (R2= 0.922 and 0.9296) between the CT values and the copy numbers of invA gene of *Salmonella* serovar Montevideo.

The two different contamination levels (10-3 CFU/mL and 10-6 CFU/mL) of *Salmonella* concentration into the shrimp and scallop samples were analysed in both conventional and real-time PCR methods to provide the linearity of the results. There was a significant difference of CT values of two different concentrations of shrimp (2.44) and scallop (2.87).

c. Reliability criteria of validation

The RT-qPCR method was evaluated by repeating the same run three times using the same primers of *Salmonella*. The two standard curves were constructed by Maxima TM SYBR Green Master Mix (2X) and Power SYBR Green qPCRMaster Mix, using the six serial concentrations of *Salmonella* serovar Montevideo showed a good correlation (R2= 0.922 and 0.9296).

Discussion

The aim of this study was to find a fast and a reliable method to detect *Salmonella* in fish samples mainly in shrimp and scallop. For this purpose, a real-time qPCR method was chosen. PCR can be an accurate and rapid way to amplify species-specific DNA from a given sample. The RT-qPCR method had not been running in the laboratory previously and thus it had to be "developed" and adapted from various sources, e.g. literature and previous experience. The adaption procedure for the PCR method started by obtaining six *Salmonella* strains and preparing purecultures for further work. These strains were used as positive controls for the testing of the *Salmonella* specific assays chosen for this study.

The second step was to select *Salmonella* specific primers. Four different *Salmonella* specific primer pairs were tested. The first pair targeted the ttrRSBCA locus[9], the second amplified the himA gene[4], while the third and the fourth pairs were situated in the invA gene[7,8,10]. All of the primer pairs gave positive signals in PCR using the six *Salmonella* strains, as expected. Of the four primer pairs invA 119F/R primer pair gave the best results and was best suited for the SYBR Green Real-time PCR assay. The *Salmonella* serovar Montevideo strain was used as a quantitating control as serial dilutions (101 to 106) to verify the linearity and a standard curve.

The third step was to optimise several reaction conditions, i.e. annealing temperature, appropriate primer and its concentration. Although the type of kits or machines is not crucial to get the method to work, it is worth mentioning the variety tools used with good results. To carry out real-time PCR, three kits were tested: IQ SYBR Green Supermix (Bio-Rad), Maxima SYBR Green aPCR Master Mix (Fermentas) and Power SYBR Green qPCR Master Mix (Applied Biosystem). Two thermal cyclers were used in this study MiniOpticon (BioRad) and StepOne (Applied Biosystem). A standard method to quantify DNA or copy number of genes is the Critical cycle (Ct) method[6]. For best results we used the serial dilution of *Salmonella* DNA in real-time PCR which gave a good correlation between CT values and the copy numbers of the invA gene.

The two different concentrations (103 and 106 CFU/ml) of *Salmonella* serovar Montevideo were spiked into shrimp (*Pandalus borealis*) and scallop (*Chlamys islandica*) and analysed to verify the efficiency of the developed real-time method, which was acceptable (standard deviation 0.65 for shrimp and 3.60 for scallop). The invA gene from the *Salmonella* Montevideo contaminated scrimp and scallop were quantified from the difference of CT values, the CT values are related to the initial template concentration. The two different concentrations (103 and 106CFU/ml) of *Salmonella* serovar Montevideo were spiked into the shrimp and scallop, the resultant quantity of invA gene showed a significant difference of mean CT values.

The important part of the real-time PCR method development was to analyse reference samples containing seven *Salmonella* serovar Dublin per ml of the reconstituted aliquote; the results showed a proven band on PCR gel-electrpferosis and the real-time PCR as well. This shows that the applied PCR method is highly sensitive. A few problems were experienced with the real-time PCR method. The main problem was with primer-dimers, a problem that introduces false positive signals, even in blank samples. To overcome this, it is important to opitmise the primer concentration of each primer. Variability in results mainly caused by pipetting errors, which took some time to overcome.

The developed real-time qPCR method is a non-standard laboratory based method, was partially validated according to ISO 17025 to imply in routine diagnostic of *Salmonella* spp. In a microbiological laboratory. The project intension was to validate the method in contrast to quality, quantity and reliability with the vertical link each to pure culture, spiked shrimp and scallop samples, reference samples, natural swab samples and the traditional microbiological methods. The qualitative validation was incorporated with the target *Salmonella* spp. which was detected by real-time PCR, PCR and the conventional method. Repeatation needed to comply with the reliability of the method.

A standard curve was constructed to get the lower limit of detection of *Salmonella* serover Montevideo. The 10-fold serial dilution did not show the equal CT value difference, because of unequal distribution of DNA between the dilutions which is an important consideration for identifying the low limit of detection. Two concentrations of *Salmonella* serover Montevideo were spiked in shrimp and scallop to quantify the flow of different concentrations, but the invA gene ratios were not exactly proportional to the concentration level.

The applied real-time method was validated partialy due to constraints in experience, DNA contamination and time limits for repeating the method. Therefore, it is essential to work in a broader spectrum for the development of real-time PCR method and for the development of a full validation protocol. Traditional microbiological methods based on growth which are always not good enough to assess the pathogen in food. A combination of molecular techniques and microbiological analyses should be applied to obtain the most representative picture of the microorganisms present in a fish shellfish sample. There are many factors that need to be considered, when determining the applicability of a rapid detection method for *Salmonella* contamination in a fish-processing industry. The most important of these would be sensitivity and specificity of the assay designed for real-time PCR reactions.

ACKNOWLEDGEMENT

The facilities provided to us in the Fish Inspection and Quality Control (FIQC) Laboratory, Chittagong, Bangladesh to carry out this study gratefully acknowledged.

CONCLUSIONS

From the results obtained in this study, it is concluded that the PCR method applied was able to detect and quantify *Salmonella* in pure, mixed and spiked samples. Sample processing time was 24 hours, in practical terms this means 2 days compared to 48 to 120 hours for conventional methods. The method detected *Salmonella* in environmental samples where conventional methods have failed so far. The method showed big potential and should be developed further.

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