The United Nations University
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Final Project 2008

APPLICATION OF A REAL-TIME qPCR METHOD FOR DETECTION OF *SALMONELLA* SPP. IN SHRIMP AND SCALLOP AND ITS PARTIAL VALIDATION

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ABSTRACT

Salmonella is one of the major causes of foodborne infection in humans, referred to as salmonellosis. It is common worldwide and widespread in fisheries products of Bangladesh because of poor post-harvest handling. The conventional microbiological detection methods of *Salmonella* require more time and need many tests for the confirmation of pathogenicity. Therefore, the requirement for more rapid and conformatory 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. In addition, its partial validation will be developed further for routine analysis of shrimp and fish. 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.

Keywords: Polymerase Chain Reaction (PCR), *Salmonella* spp. real-time PCR, shrimp.

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TERMS

A standardised PCR method
A standardised PCR method is a method that constantly gives the same results for a given sample when analysis is repeated several times and when performed by different analysts in different laboratories (Malorny et al. 2003).

Selectivity
Selectivity refers to the extent to which it can determine particular analyte in a complex mixture without interference from other components in the mixture.

Linearity
Linearity is the ability of an analytical procedure to produce results that are proportional to the concentration of analyte in samples within a given concentration range.

Accuracy
The relative accuracy is defined as the degree of correspondence between the response obtained by the reference method (traditional microbiological method) and the response obtained by the alternative method (here real-time PCR) on identical samples.

Precision
The degree to which further **measurements** or calculations show the same or similar **results**

Limit of detection
The limit of detection is the lowest quantity of a substance that can be distinguished from the absence of that substance (blank value) within a stated **confidence limit** (generally 1%).

Limit of Quantification
The Limit of Quantification (LOQ) is the limit at which we can reasonably tell the difference between two different values.

Sensitivity
The sensitivity is defined as the percentage of positive samples giving a correct positive signal. The term sensitivity has been used to describe the smallest amount of a target organism that can be reliably detected by a PCR-based technique.

Repeatability
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.

Seer fish
Seer fish refers to a subfamily of the Scombridae or Mackerel family. Seer fish include such species as: Pacific king mackerel (Scomberomorus guttatus), streaked Spanish mackerel (Scomberomorus lineolatus), spotted Spanish mackerel (Scomberomorus guttus), king mackerel (Scomberomorus commerson) and wahoo (Acanthocybium solandri).

LIST OF ABBREVIATIONS

AHCYTOEN	-	Cytolytic Enterotoxin Gene
bp	-	base pair
BPW	-	Buffered Peptone Water
CCA	-	Central Competent Authority
CFU	-	Colony Forming Units
CFU	-	Colony Forming Unit
CT	-	Threshold Cycle
DFBMD	-	Division of Food Borne Bacterial and Mycotic Diseases, United States of America.
DNA	-	Deoxyribo Nucleic Acid
EDTA	-	Ethylene Diamine Tetra Acetic Acid
ELISA	-	Enzyme-Linked Immuno Sorbent Assay
EU	-	Europen Union
GLP	-	Good Laboratory Practice
GSS	-	Global Salm-Surv
HPLC	-	High Performance Liquid Chromatography
ICDDRB	-	International Centre for Diarrhoeal Disease Research, Bangladesh
ISO	-	**International Organization for Standardization**
LD	-	Limit of Detection
LIA	-	Lysine Iron Agar
LQ	-	Limit of Quantification
NARST	-	National Association of Research in Science Teaching
NMKL	-	Nordic Committee on Food Analysis
PCA	-	Plate Count Agar
PCR	-	Polymerase Chain Reaction
RAPD	-	The Random Amplified Polymorphic DNA
RFLP	-	**Restriction Fragment Length Polymorphism**
RT-PCR	-	Real-Time Polymerase Chain Reaction
RV	-	Rappaport Vassiliadis
SPC	-	Standard Plate Count
TBE	-	Tris/ Borate/EDTA
TMA	-	**Trimethylamine**
TSA	-	Tryptic Soy Agar
TSI	-	Triple Sugar Iron
TVBN	-	Total Volatile Base Nitrogen
UNAK	-	University of Akureyri
UNIDO	-	United Nations Industrial Development Organization
UNU-FTP	-	United Nations University-Fisheries Training Programme
USA	-	United States of America
USFDA	-	United States Food and Drug Administration
WHO	-	World Health Organization
WSSV	-	White Spot Syndrome Virus
XLD	-	**Xylose Lysine Desoxycholate**

1 INTRODUCTION

1.1 Present fisheries situation of Bangladesh

Bangladesh is a South Asian country with abundant of fishery resources both in inland and in marine water. Each year Bangladesh produces about 2.4 million metric tonnes of fishery products and exports about 0.74 million metric tonnes of fishery products (DoF 2007). Most of the exportable products are shrimp from aquaculture and marine catch. Bangladesh also produces and exports different species of white fish mainly to the EU, USA, Japan and Middle East. About 4.90% of the total export earnings comes from fisheries (DoF 2007).

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 (USAID, Bangladesh 2006). 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. (Das et al. 2007). In 2008, a total of 30 consignments of Bangladeshi fishery products were refused by the USA due to *Salmonella* contamination (Import Refusal Reports for OASIS 2009).

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 modernise the laboratory.

Within a very short time, PCR could be set up for regular pathogenic analysis of fish samples. For the laboratory, this reduces labour, 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.

[1]ISO 17025 - General Requirements for the Competence of Calibration and Testing Laboratories

1.2 Food hazards

Food is essential for health and well-being. Food may also be a cause of illness when it is hazardous. Food safety has become an issue of special importance for the food industry. There are many opportunities for food to be contaminated between production and consumption. Fish can become contaminated at the farm, processing plants, during storage and transport or in the sea.

Foodborne hazards can be classified as biological, chemical or physical. Biological hazards are caused by parasites, viruses or bacteria. Chemical contaminants in foods can come from industrial and agricultural sources, from food processing or from the food itself. Toxic chemicals also come from biological sources such as moulds and algae. Foreign objects present in food could constitute a physical hazard (glass, metal etc.) to the consumer.

Foodborne illness caused by microorganisms is a large and growing public health problem. Foodborne illness is caused by infection and intoxication. Infection occurs when living bacteria are ingested into the stomach with food of sufficient numbers. These bacteria then pass into the small intestine where they multiply and produce symptoms. Intoxications happen when the bacteria grow in the food producing a toxin. When the food is eaten the toxin, rather than the microorganisms, causes illness symptoms of intoxication.

Microorganisms are everywhere and they can spoil food. Pathogenic microorganisms can be part of a food's natural microflora, or may be contaminants. Contaminants can come from the normal environment or a polluted environment, pests and pets, the food handler and equipment. Bacteria, viruses, moulds and parasites are examples of microorganisms that can cause foodborne illness. Diseases caused by microorganisms in food include pathogens such as *Salmonella* spp., Campylobacter spp., Vibrio spp., Escherichia coli etc. A common type of foodborne infection is **Salmonellosis**. This is caused by ***Salmonella*** bacteria that are frequently found in poultry, eggs and fish. Shrimps and scallops accounted for 58% of the *Salmonella* violations compared to other foodborne pathogens from the USFDA (United States Food and Drug Administration) imported fisheries products in 2001 (Jane et al. 2003) and *Salmonella* is the second most common cause of foodborne illness in the United States (U.S. Public Health Service 2009).

1.3 *Salmonella*

The genus *Salmonella* belongs to the family Enterobacteriaceae. *Salmonella* are facultative anaerobic Gram-negative rods. They are non-spore forming, usually motile with peritrichous flagella, capable of growing on ordinary media. They are pathogenic to man and animals (OIE 2005). *Salmonella* spp. are mainly transmitted by the faecal-oral route. Fish and invertebrates can also infect. *Salmonella*, when present, are usually found in low numbers in foods and often in the presence of considerably larger numbers of other members of Enterobacteriaceae. In foods which have been heated, refrigerated, frozen or dried, viable *Salmonella* bacteria may be present.

Salmonella bacteria cause foodborne illness. Every year approximately 40,000 cases of Salmonellosis are reported in the United States (DFBMD 2009). Salmonellosis is a common foodborne illness in most industrial countries (Tirado and Schmidt 2001).

Salmonellosis is common worldwide but it is widespread in the area where animal husbandry is practiced because it is common mostly in domestic animals and birds. Raw meat and poultry are therefore often contaminated with *Salmonella*. *Salmonella* contamination in fish and shellfish due to growth in polluted water has been a problem in many parts of the world. Aquaculture and firmed fish in the tropical region are the major risk area for *Salmonella* contamination due to unhygienic handling and use of poultry droppings as fertilizer in the culture water (OIE 2005). *Salmonella* serovar Enteritidis and *Salmonella* serovar Typhimurium are the two most common serovars found worldwide. Others serovars are limited to specific geographical regions (OIE 2005).

1.4 Methods of *Salmonella* detection

The conventional detection methods are based on cultures which are generally time-consuming but they are high in selectivity and sensitivity. Traditional pathogen detection methods are sensitive enough but are slow. Therefore, new methods are needed that exceed their performance.

The immunology-based methods for bacteria detection provide very powerful analytical tools for a wide range of targets. They can be used to capture and extract the targeted pathogen from the bacterial suspension by introducing antibody coated magnetic beads in it (Gu et al. 2006).

Polymerase Chain Reaction (PCR) methods provide conclusive and unambiguous results, recent advances in PCR technology, namely real-time-PCR now enable obtaining results in a few hours.

Colony counting methods, PCR and immunology-based methods are the most common tools used for pathogen detection. The most popular methods are culture and colony counting methods (Leoni and Legnani 2001) and the PCR (Bej et al. 1991).

On the other hand, recent advances in PCR technology, expressly real-time PCR (Levi et al. 2003) now enable obtaining results in a few hours. The PCR is a lot less time-consuming than other techniques. It takes from 5 to 24 hours to produce a detection result but this depends on the specific PCR variation used and this does not include any previous enrichment steps.

1.5 Method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. The method validation gives quantitativity, qualitativity and reliability of the method for the future use. Results from method validation could be use to judge the quality and consistency of analytical results. Method validation is an integral part of Good Laboratory Practice (GLP). The partially developed PCR method was a non-standard laboratory based method. The parameters of the non-standard PCR method are: selectivity, linearity, accuracy, precision, Limit of Detection (LD), Limit of Quantification (LQ), sensitivity and repeatability, which were assessed along with relevancy to ultimate requirements.

The recorded results and procedures were used for validation and a statement was made as to whether the method is fit for the intended use. The techniques used for the determination of the performance of the method included the followings:

a) Calibration using reference sample of *Salmonella* spp.
b) Comparison of results achieved with conventional method.
c) Systematic assessment of the factors influencing the results.

1.6 Polymerase Chain Reaction (PCR)

The basic protocol of PCR for rapid detection bacteria in samples is: denaturation of DNA to single strands, annealing of sequence specific primers and extension by polymerase 25-40 cycles. The PCR results in a product that represents a 50-10,000 bp specific part of a genome that can be analysed by gel electrophoresis or DNA sequencing.

RT-qPCR is a **laboratory technique** based on PCR, which is used to amplify and simultaneously quantify a targeted **DNA** molecule. It enables simultaneously, both detection and quantification of a specific DNA sequence in a sample in real-time after each amplification cycle. Two common methods of quantification involve **fluorescent dyes** that intercalate with double-stranded DNA during PCR and modified DNA **oligonucleotide** probes that **illuminate** after hybridization with complementary DNA and extension. Real-time qPCR is a combination of two steps: the amplification of the DNA using PCR and the detection and quantification of amplified DNA in real-time.

The accuracy and reliability depends on template quantity, quality and optimal assay design. Real-time PCR (qPCR) uses fluorescent dyes to combine the amplification and detection steps of the PCR reaction; the fluorescent signal is proportional to the amount of DNA produced during PCR cycles. The use of probes labelled with different reporter dyes allows the detection and quantification of multiple target genes in a single PCR reaction.

PCR should not be considering a substitute for conventional microbiology techniques, but it is rapid and requires less time to achieve competence than conventional microbiology. Conventional microbiology is often considered to be less technically demanding than PCR. However, PCR can detect bacteria that can not be grown in culture.

PCR complements and enhances the traditional microbiological methods by increasing speed, sensitivity, and specificity for detecting pathogens in fish. PCR can be performed rapidly in the field and limits the number of cultures and isolations to the few samples identified as positive by PCR to decrease the workload, labour, resources, and costs.

There are two types of real-time PCR techniques. The first one is TaqMan and SYBR Green I. TaqMan PCR is fluorescent-probe-based, here fluorescent-probe-based assays require availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily applied. In SYBR Green I PCR use of the doublestranded DNA binding dye SYBR Green I for detection of PCR products has overcome this limitation by allowing real-time PCR to be applied without the need for probes linked to fluorescent molecules (Aarts et al. 2001).

SYBR-Green real-time qRT-PCR assay provides a rapid outcome compared to others. The detection is based on the binding of the SYBR-Green dye into double stranded PCR products, which is a sequence independent process.

1.7 PCR in Bangladesh

In Bangladesh PCR has been used mainly for detection of viral diseases (EUS or Epizootic Ulcerative Syndrome) of shrimp and white fish. A PCR-equipped lab was installed in Dhaka in 2006 and another one in Cox's Bazar only for detection of white spot diseases. The first PCR tested larvae was released in April 2004 in Cox's Bazar. The common use of PCR in the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB) is for research of diarrhoeal disease in Bangladesh. A cytolytic enterotoxin gene (AHCYTOEN) in **Aeromonas hydrophila** was identified by PCR techniques by Rahman et al. (2002). The Random Amplified Polymorphic DNA (RAPD), **Restriction Fragment Length Polymorphism** (RFLP) and microsatellite markers have already been used to delineate some sort of distinct population and/or stock groups of Catla (Catla catla) (Alam and Islam 2005), Magur (Clarias batrachus) (Islam et al. 2007) and Hilsa shad (Tenualosa ilisha) in Bangladesh with different levels of genetic distances. A population bottleneck[2] has been identified in magur fish (Clarias batrachus) (Islam et al. 2007). Detection of whitespot syndrome virus (WSSV) in cultured shrimps (Penaeus monodon), P. monodon brooders, shrimp postlarvae and water samples in Bangladesh was carried out by PCR using five different pairs of primers (Hossain et al. 2004).

1.8 Objectives

This study intends to develop a pathogenic detection procedure in a most precise way using real-time PCR for the further enhancement of laboratory services of the competent authority. The project intends to achieve this by developing intensive microbiological laboratory analysis of pathogens using real-time PCR, developing a PCR method and developing a validation protocol for it. The study will seek the practice and competency in an accredited microbiological laboratory.

The objective of the present study is to establish a simple and robust real-time PCR method using SYBR Green that would be suitable for routine analysis of *Salmonella* spp. in exportable fish samples. The study has three sub-objectives:

1. Application of the real-time qPCR method for detection of *Salmonella* spp. in shrimp and scallop.
2. Partial validation of the applied PCR method.
3. To gain competence and expertise with pathogen detection methods using real-time qPCR techniques.

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[2] A population bottleneck (or genetic bottleneck) is an evolutionary event in which a significant percentage of a population or species is killed or otherwise prevented from reproducing (Wikipedia).

2 LITERATURE REVIEW

The *Salmonella* bacterium was first isolated from pigs suffering from hog cholera by an American scientist, Dr Daniel E. Salmon, in 1885 (Bremer et al. 2003). The *Salmonella* genus includes over 2435 serotypes of bacteria (Bremer et al. 2003) and *Salmonella* have been recognised as a major cause of gastrointestinal disease in both humans and animals (Darwin and Miller 1999). *Salmonella* is one of the major causes of zoonotic infection in the world. *Salmonella*e are among the most common bacterial foodborne pathogens worldwide (Eleni et al. 2006a).

Drinking contaminated water is a major source of *Salmonella* worldwide. *Salmonella* spp. have been found in raw meats, poultry, eggs, milk and dairy products, fish, shrimp, frog legs, yeast, coconut, sauces and salad, cake mixes, dried gelatin, peanut butter, cocoa and chocolate. Farmed shrimp might be contaminated with *Salmonella* due to their culture in poor quality growing waters (Bremer et al. 2003). *Salmonella* have also been found in fishmeal and in fish feed factories (Trond et al. 2003).

The most common global distributed *Salmonella* human serotype is *Salmonella* Enteritidis (61%) which is also common in Asia (33%) (Global Salm-Surv (GSS) 2006). In 2002, a total of five serotypes were reported among the 15 most common human serotypes from all six regions of the world: *Salmonella* serovar Enteritidis, *Salmonella* serovar Typhimurium, *Salmonella* serovar infantis, *Salmonella* serovar Montevideo, and *Salmonella* typhi (Eleni et al. 2006b). Domestic animals act as a reservoir for the foodborne spread of serovars such as **S.** Typhimurium which accounts for the high incidence of non-typhoid ***Salmonella*** infections worldwide. **S.** Typhimurium DT104 has developed resistance to five commonly prescribed antibiotics and is a major concern in many countries because of its rapid spread during the 1990s (Food Safety (WHO) 2008).

An estimated 500 people in the United States are infected with typhoid by *Salmonella* annually. Most cases of documented typhoid disease are related to foreign travel to developing nations such as India (30%), Pakistan (13%), Mexico (12%), Bangladesh (8%), Philippines (8%), and Haiti (5%) (Michael and Dirk 2008). The USFDA (United States Food and Drug Administration) noted an overall incidence of 7.2% in imported and 1.3% in domestic seafood during a nine year study (1990-1998) of 11,312 imported and 768 domestic seafood samples (Heinitz et al. 2000). Nearly 10% of imported and 2.8% of domestic raw seafood were positive for *Salmonella*. Distribution of *Salmonella* in seafood on a regional basis indicated the incidence to be highest in the central Pacific and Africa and lowest in Europe/Russia and North America (12% versus 1.6%). Data on a country basis indicated Vietnam to have the highest (30%) and Republic of Korea the lowest (0.7%) rates. While the most frequent serotypes in imported seafood were *Salmonella* Weltevreden (1st), *Salmonella* Senftenberg (2nd), *Salmonella* Lexington and *Salmonella* Paratyphi-B (3rd), the top 20 list included *Salmonella* Enteritidis (5th), *Salmonella* Newport (6th), *Salmonella* Thompson (7th), *Salmonella* Typhimurium (12th), and *Salmonella* Anatum (13th) commonly involved in foodborne illness in the United States (Heinitz et al. 2000).

Salmonella has been isolated from freshwater fish culture ponds in many countries. A survey in Japan showed that *Salmonella* spp. are present in 21% of eel culture ponds (Saheki et al. 1989) and the incidence of *Salmonella* in freshwater cultured catfish in the USA was estimated to be 5% (Wyatt et al. 1979). The incidence of *Salmonella* in

seafoods from India has been reported by a number of investigators. Varma et al. (1985) reported the presence of *Salmonella* in 7.46% of frozen peeled and deveined shrimp. Iyer and Shrivastava (1989) demonstrated the presence of *Salmonella* in 12% peeled and deveined[3] shrimp, 10% headless, shell-on shrimp, 14% peeled and undeveined shrimp, 17% lobsters, 14% cuttlefish, 25% catfish and 20% seer fish[4]. The presence of *Salmonella* in fish and fishery products has been reported from other Asian countries such as Sri Lanka (Fonseka 1990), Thailand (Rattagool et al. 1990), Taiwan (Chio and Chen 1981) and Indonesia (Sunarya et al. 1990). This pathogen is a public health problem associated with fish and fishery products (Kumar et al. 2003).

The number of incidence of *Salmonella* in shrimp from USFDA regulated products was 369 out of 4,724 samples from 2001-2005 and two unusual serotypes *Salmonella* Augustenborg and *Salmonella* Tteko were isolated from the imported shrimp of Bangladesh (Madson 2006). In a *Salmonella* outbreak in Bangladesh by food poisoning in March 1980, 10 of 11 members of a family who ate a food called jalar jao[5] experienced acute gastroenteritis within four-12 hours. All 10 patients were hospitalised at the International Center for Diarrheal Disease Research, Bangladesh (ICDDRB) field hospital at Matlab, Bangladesh. Jalar jao may be an important vehicle for transmitting pathogenic organisms (Yunus et al. 1981).

Bangladesh is a third world country where the use of antibiotics is not controlled neither in dose nor duration. Since 1997, treatment failures with ciprofloxacin have been reported from Bangladesh and other countries due to infection with nalidixic acid resistant *Salmonella* enterica serovar Typhi (NARST) that had decreased susceptibility to ciprofloxacin (Rahman et al. 2005). *Salmonella* typhi stains were found resistant against β-lactam antibiotic, penicillin (Parvez et al. 2004). Salmonellosis pneumonia was found common in a research survey in Dhaka Shishu Hospital, Bangladesh in 2001 by WHO (World Health Organization), out of 200 people eight had *Salmonella* (including 4 S. typhi) caused pneumonia (Factor et al. 2001).

The incidence of *Salmonella* spp. in tropical seafood was studied using standard microbiological techniques and polymerase chain reaction by Kumar et al. (2003). Simultaneous detection of Escherichia coli O157:H7, Listeria monocytogenes and *Salmonella* strains by real-time PCR was evaluated using artificially contaminated fresh produce. The real-time PCR successfully detected all three pathogens when fresh produce was washed with artificially contaminated water containing E. coli O157:H7[6] and S. enterica serovar Typhimurium down to the predicted level of 1 to 10 cells/mL and L. monocytogenes at 1000 cells/mL (Arvind 2002). Effects of sample preparation and bacterial concentration on *Salmonella* enterica detection in poultry meat using culture methods and PCR assaying of preenrichment broths was done by Kanki et al. (2008). The S. Typhimurium isolates were analyzed by PCR to detect the presence of virulence genes invA and spvC (Cheng-Hsun and Jonathan 1996). A rapid and sensitive 8 hour PCR assay has been developed for detection of *Salmonella* serovars in seafood by (Kumar et al. 2008a). A total of 110 fresh and raw seafood

[3] Deveined - to remove the vein or veins or digestive tract (dorsal vein) of a shrimp.
[4] Seer fish refers to a subfamily of the Scombridae or Mackerel family (Wikipedia).
[5] Jalar jao- is traditionally eaten food made from rice and sugar or molasses, eaten cold by rural people during hot summer months.
[6] *Escherichia coli* O157:H7: is an enterohemorrhagic strain of the bacterium *Escherichia coli* and a cause of foodborne illness.

samples were analysed for the presence of *Salmonella* using different enrichment periods prior to PCR assay. Seafood samples included in this study were fish, shrimp, mussel, crab, edible oyster, and clam, collected from local fish markets in Cochin (India). Evaluation of culture, ELISA (Enzyme-Linked Immuno Sorbent Assay) and PCR assays for the detection of *Salmonella* in seafood was done. The PCR assay exhibited 31.6% positive for *Salmonella* followed by ELISA (23.7%) and culture method (21.3%) (Kumar et al. 2008 b). Nowadays PCR assay is very popular among researchers for rapid diagnostic and screening of a large number of samples simultaneously (Kumar et al. 2008 b). A recent study evaluated the efficiency of culture, ELISA and PCR assays for the detection of *Salmonella* in naturally contaminated seafood. In the study, 215 seafood samples comprising fish, shrimp, crab, clam, mussel, oyster, squid, cuttlefish and octopus from the fish market of Cochin (India), were compared by culture, ELISA and PCR methods (Anonymous 2008). Comparison of PCR-ELISA and LightCycler real-time PCR assays for detecting *Salmonella* spp. in milk and meat samples was done by (Perellea et al. 2004).

Diagnostic real-time PCR for detection of *Salmonella* from 110 various food samples (chicken rinses, minced meat, fish, and raw milk) were investigated for *Salmonella* by Malorny et al. (2004). The diagnostic accuracy was shown to be 100% compared to the traditional culture method. The overall analysis time of the PCR method was approximately 24 hours in contrast to four to five days of analysis time for the traditional culture method. This study was planned to assess the inter-laboratory performance of this diagnostic PCR method.

111 samples of raw imported aqua-cultured shrimp have been examined for bacterial pathogens and for pathogen viruses by Mitzscherling and Kuhne (2008). The samples originated from Southeast Asia except for eight Latin American samples. Most samples (40) were taken from Bangladesh. The bacteriological quality of these samples was analysed in terms of aerobic plate count, Escherichia coli, Staphylococcus aureus, *Salmonella* spp., Listeria monocytogenes and Vibrio species. Rotavirus, norovirus and hepatitis A-virus were detected by using a nested RT-PCR. The aerobic plate count was found to be in a range between 4,8×102 to 1,3×109 CFU[7]/gm. Fourteen (14) samples (12.6 %) showed an aerobic plate count >107 cfu/g. One sample was found to be contaminated with Escherichia coli at a level of 1,9×103 cfu/g. Staphylococcus aureus was isolated from 16 samples. Listeria monocytogenes was not detected in any of the shrimp samples examined. *Salmonella* spp. was found in eight samples. *Salmonella* Weltevreden was the most frequently isolated serovar (Mitzscherling and Kuhne 2008).

Development and Validation of PCR Primers for Detection of Tetracycline Efflux Genes of Gram-Negative Bacteria was done by Aminov et al. (2002). This study confirmed that the antibiotic resistance gene pool generated in animal production systems might be mobile and persistent in the environment with the potential to enter the food chain.

A validation study of two blockcycler and one capillary-based real-time PCR method for the detection of *Salmonella* in milk powder was done by Malorny et al. (2007).

[7] CFU-Colony Forming Unit.

The traditional cultural method according to EN ISO 6579:2002[8] for the detection of *Salmonella* in food was performed as the reference method. Inter-laboratory tests were done between 13 laboratories using various models of real-time PCR blockcyclers (Malorny et al. 2007).

Review on Validation of Polymerase Chain Reaction-based techniques for proxy detection of bacterial fish pathogens was done by Maura and Peter (1998). They suggested a framework for a validation protocol, problems and possible solutions for environmental applications of validation methods.

Evaluation, validation and standardisation of PCR for *Salmonella* primers within 16 laboratories were done for the quality assurance of laboratories (Malorny et al. 2003).

A validation study was carried out followed by a comparative study of a PCR method and the standard culture-based method NMKL-71 (1999)[9] for detection of *Salmonella*. Validation was performed according to the validation protocol from the Nordic validation organ for validation of alternative microbiological methods (NordVal) on 250 artificially or naturally contaminated animal feed samples. More *Salmonella*-positive samples were found with the PCR method compared to the NMKL method (Lofstrom et al. 2008).

The European Commission approved a research project on validation and standardisation in 1999 entitled "FOOD-PCR with the aim of validating and standardising the use of PCR-based methods for detection of the five major foodborne pathogens: thermophilic Campylobacter, E. coli O157, Y. enterocolitica, Listeria monocytogenes and *Salmonella* spp."(Hoorfar and Cook 2003). A consortium of 35 institutes, companies and universities from 14 EU countries and seven associated states worked on the project. The project group proposed the process of developing a standardised PCR-based method. The group also suggested a set of specific definitions of validation parameters and test controls.

A real-time PCR method for detection of *Salmonella* spp. in food samples has been developed and validated in-house. The method was evaluated with 1,293 naturally contaminated food samples and compared to the conventional cultural method (Made et al. 2004).

[8] EN ISO 6579:2002- Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of *Salmonella* spp

[9] NMKL-71, 1999: Nordic Committee on Food Analysis, *Salmonella* Detection in Foods, Method No 71, 5e ed., 1999, page 2(9).

3 MATERIALS AND METHODS

3.1 *Salmonella* strains

The *Salmonella* strains were obtained from the Promat Laboratory, Akureyri, Iceland. 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 concentration of cells were determined by viable counts on the TSA plates.

3.2 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)) (Oxoid, UK), 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.

3.3 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, Akureyri, Iceland. 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 aliquotes, 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).

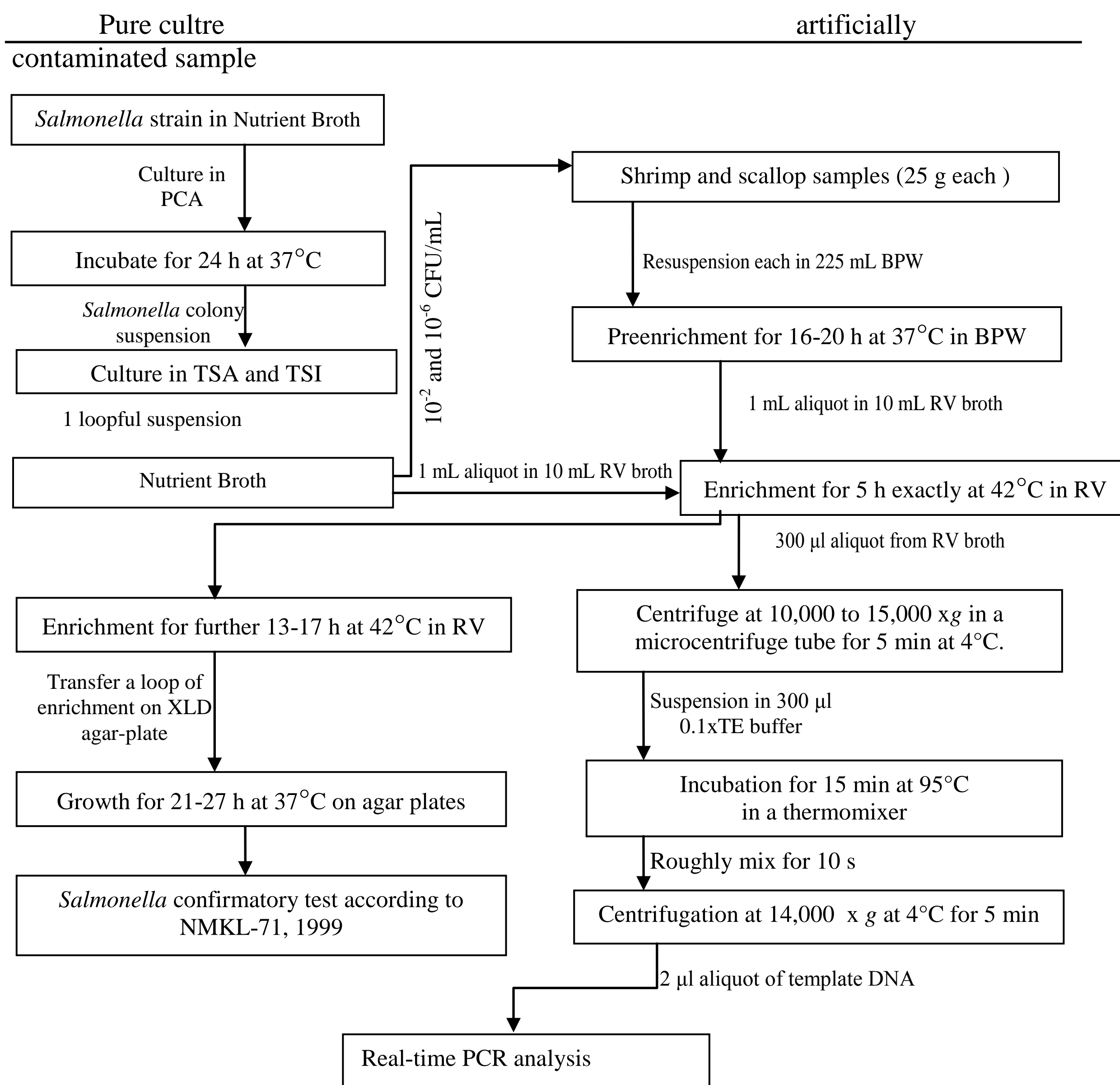


Figure 1: Flow diagram showing preparation of pure culture of *Salmonella* for spiking of shrimp and scallop samples and isolation of DNA for PCR analysis.

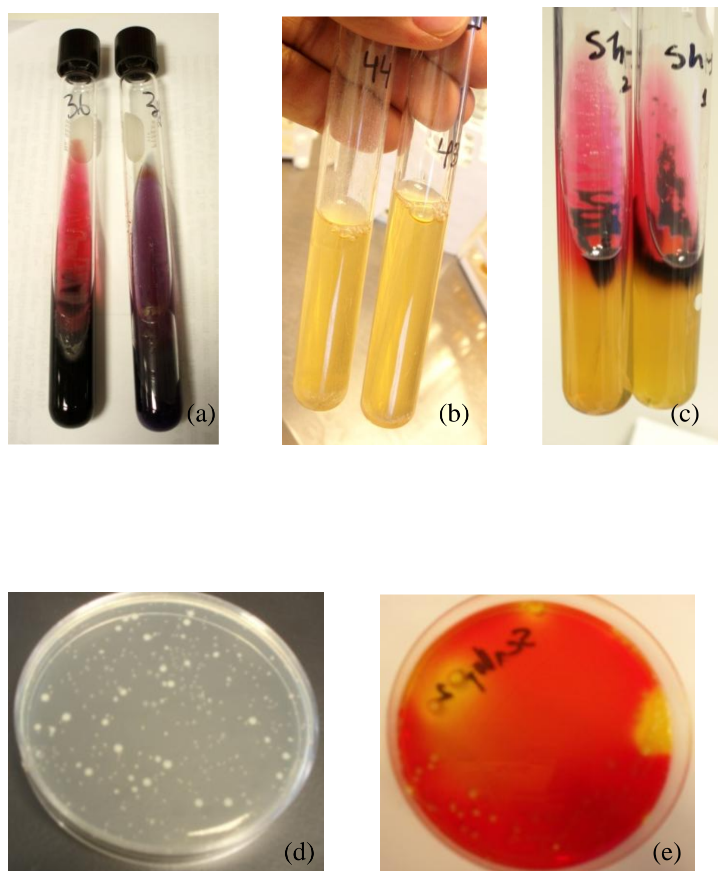


Figure 2: ***Salmonella*** **strains in (a) TSI & LIA agar tube (b) nutrient broth (c) sub-culture in TSI agar tube (d) nutrient agar plate (e) XLD agar plate**

3.4 Proficiency testing for *Salmonella*

An ampoule containing six types of freeze-dried bacteria was obtained from the National Food Administration, Microbiology Division, Upsala, Sweden. The material after reconstitution, can be used for quantitative and qualitative testing of *Salmonella* and in this project to compare the efficiency of the PCR methods with “standard” methods. The specification of the sample was:

Manufacturer:	Microbiology Division, National Food Administration, Sweden
Labelled:	2007:1
Date of manufacture:	March 16, 2007
Storage:	-18°C or lower
Durability:	December 2008

The following strains, of which the majority have been isolated directly from food, were included the ampoule:

Strain	Strain no
Micrococcus sp.	SLV-055
Klebsiella oxytoca	SLV-089
Escherichia coli	SLV-082
Staphylococcus aureus	SLV-350
Clostridium perfringens	SLV-442
Salmonella serover Dublin	SLV-242

Reconstitution of the reference samples
The ampoule was opened according to instructions. One mL of 0.1% peptone water was added and the content of the vial loosened using a sterile Pasteur pipette. The walls were carefully rinsed. The suspension was transferred to a sterile vessel containing 100 mL peptone water (a total volume of 104 mL). These 104 mL are equivalent to an undissolved sample. After careful mixing, the sample was ready for analysis.

Sample analysis
The sample was analysed for the presence of *Salmonella* using conventional microbiological procedure (NMKL-71 1999), PCR and real-time PCR methods. The sample contains approximately seven *Salmonella* Dublin per mL of reconstituted aliquote, 1 mL of the solution was inoculated to 10 mL BPW, incubated at 37ºC for 20 hours for pre-enrichment. One mL was homoginated with 10 mL of RV broth and incubated at 42ºC in a circulating water bath for 24 hours for the continuation of traditional microbial tests and similarly RV broth was inoculated and incubated at 42ºC for 5 hours for DNA extraction.

For the verification of *Salmonella* presence, 1 mL aliquot from the selective 24 hour enriched RV broth was inoculated to XLD agar plates which was incubated at 37ºC for about 48 hours and finally checked for the characteristic colonies.

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

The E. coli culture was obtained from the Promat Laboratory, Akureyri, Iceland. 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.

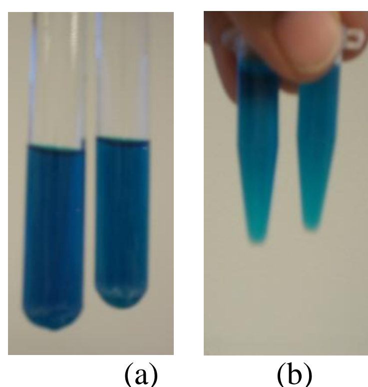
3.6 *Salmonella* detection in environmental swab samples

3.6.1 Sample collection

Forty (40) environmental samples were taken by swabbing at different locations in the neighbourhood of a fish processing facility in northern Iceland. Samples were transported to the laboratory on ice, and analysis was started within 24 hours.

3.6.2 Sample preparation for DNA extraction

The swab samples were homogenate with 10 mL BPW and were incubated at 37±1°C for 20 hours. Transfer 1 mL of each pre-enrichment bottle to 10 mL of Rappaport - Vassiliadis soya peptone broth, previously warmed to 42°C and incubated at 42°C for 48 hours in a circulating water bath for the continuation of traditional microbial tests and similarly the other RV broth was inoculated and was incubated at 42°C for 5 hours for DNA extraction (Figure 3).



(a) (b)

Figure 3: RV broth ready for DNA extraction in (a) 25 mL test-tube, (b) 1.5 mL tube

3.7 DNA extraction

DNA extraction is the the removal of Deoxyribonucleic Acid (DNA) from the cells of bacteria. It is the early step of PCR analysis to break open (lyse) the cells of bacteria containing the DNA of interest.

3.7.1 Method:1

For the DNA extraction, 300µl each of 5 hour incubated RV enrichment broth were used as cell suspension.

<u>Cell Lysis</u>
Three hundred (300) µL cell suspension (containing 0.5- 1.5 billion cells) was added to a 1.5 mL microfuge tube on ice and was centrifuged at 13,000 g for 5 seconds to pellet cells and the supernatant was removed with a pipet. 300 µL Cell Lysis Solution was added into the tube and was pipet up and down to suspend cells and then incubated at 80ºC for 5 minutes to lyse cells. Now, in this condition cells are stable in Cell Lysis Solution for at least two years at room temperature.

<u>Protein precipitation</u>
The sample was cooled down from 80°C to room temperature by placing it on ice for 1 minute and 100 µL protein precipitation solution was added to the cell lysate. The solution was vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate. The solution was then centrifused at 13,000 g for 3 minutes. The precipitated proteins formed a tight pellet. For some samples, the pellet was not tight enough, then the vortex was done vigorously at high

speed for 20 seconds and was incubated on ice for 5 minutes and was centrifuge again at 13,000 g for 3 minutes.

DNA precipitation
The DNA containing supernatant was poured (leaving behind the precipitated protein pellet) into a clean 1.5 mL microfuge tube containing 300 µL 100% isopropanol (2-propanol). The sample was mixed by inverting gently 50 times and was centrifuged at 13,000 g for 1 minute to make the DNA visible as a small white pellet. The supernatant was discarded and the tube was drained on clean absorbent paper, 300 µL 70% ethanol was added into the tube and was inverted several times to wash the DNA. Then the tube was centrifuged at 13,000 g for 1 minute and ethanol was poured carefully and was inverted on clean absorbent paper to air dry for 5-10 minutes.

DNA hydration
Fifty (50) µL DNA Hydration Solution was added to the tube and DNA was rehydrated by incubating for 1 hour at 65°C. The tube containing DNA was kept at room temperature overnight before PCR analysis.

3.7.2 Method: 2

For the DNA extraction 300 µL each of 5 hours incubated RV enrichment broth were used, the enriched RV culture was centrifuged at 10,000 to 15,000 g in a microcentrifuge tube for 5 minutes at 4°C. The supernatant was carefully discarded and the cell pellet was suspended in 300 µL 0.1xTE buffer (1 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]). The suspension of the microcentrifuge tube was incubated for 15 minutes at 95°C in a water bath, afterwards roughly mixed for 10 seconds. After centrifugation at 14,000 g at 4°C for 5 minutes, the supernatant containing DNA was carefully transferred to a new microcentrifuge tube and stored on ice until use in the real-time PCR. A 2 µL aliquot was used as the template DNA for the PCR.

3.8 Calculation of the amount of DNA

The quantification of DNA was measured using a fluorometer (Invitrogen). 200 µl working solution was made for each sample by mixing and homogenating 199 µL of Quant- iT buffer with 1 µl of Quant- iT reagent. 10 µL of each two standards were homogenated with 190 µL of working solutions. 198 µL of the working solution was mixed homogenated with 2 µL of extracted DNA sample in a new tube; the final volume was 200 µL. The two standard solutions were placed into a fluorometer, and then the sample solutions were placed into the fluorometer to measure the quantity of DNA.

3.9 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.

Primer sequence
PCR primers are oligonucleotides which are typically 15-30 bases long. When choosing two PCR primers (Table 1), it is important that they not contain bases complementary to eachother. Complementarity at the 3' ends was avoided to minimise

the primer-dimer. A 40% - 60% G+C content is recommended for each primer, avoiding internal secondary structure and long stretches of any one base. For the experiment, 45% and 40% G+C content of left and right primers were used.

Primers were used in real-time RT-PCR as described in Table 1 and were purchased from Sigma Genosys Ltd. (Switzerland).

Table 1: Overview of primers and corresponding characteristics

Primer	Targeted sequence	Amplicon size (bp)	Primer concen (µM)	Forward primer, Reverse primer sequence (5′-3′)	Reference
Shima	himA	122	0.2	CGTGCTCTGGAAAACGGTGAG CGTGCTGTAATAGGAATATCTTCA	(Chen et al. 1999)
InvA	invA	119	0.2	TCGTCATTCCATTACCTACC AAACGTTGAAAAACTGAGGA	(Mi Nam et al. 2005)
ttr6/ttr4	Ttr RSBCA locus	94	0.2	CTCACCAGGAGATTACAACATGG AGCTCAGACCAAAAGTGACCATC	(Malorny et al. 2004)
InvA141/139	InvA	284	0.2	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	(Malorny et al. 2003)

The four pairs of *Salmonella* specific primers were initially selected which are Shima (122 bp), invA119 (119 bp), ttr6/4 (94 bp) and Sal/inv 139/141 (284), all the four pairs were tested with the DNA of *Salmonella* serovar Montevideo strains.

Primer concentration

The amount of primers available during the PCR reaction influences the ultimate results. The concentration of primer in the amplification reaction should be between 0.1 and 1.0 µM. Low primer concentration generally ensures cleaner product and lower background. The primer concentration into the reaction mixture is also important for better implification. For the optimisation of PCR, different concentrations of primers were used (Table 3) in the reaction mixture to find out the suitable primer set for good PCR amplification. All the primer sets were run on the same reaction mixture (Table 2) with 2 µl *Salmonella* serover Montevideo as template DNA.

Table 2: The reaction mixture used for primer concentration for optimisation of the PCR

10x PCR buffer (Provides a final concentration of 2.5 mM MgCl2)	2.50 µl
DNTP	2.00 µl
Taq polymerase (1 U)	0.30 µl
Template DNA	2.00 µl
Water, nuclease-free	to 25 µl
Total volume	25 µl

Table 3: Primer concentrations used for optimisation

Primer concentration (µM)	
Forward	Reverse
0.3	0.3
0.1	0.3
0.1	0.1
0.2	0.2
0.3	0.5

Annealing temperature

Annealing temperature is one of the most important parameters that need adjustment in the PCR reaction. The PCR reaction temperature is lowered to 50-65°C seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 5ºC below the melting temperature (Tm) of the primers used. In the experiment for the optimisation of PCR, the PCR reaction mixture (Table 2) was used in three different annealing temperatures (55°C, 58°C and 60°C) to find out the better efficiency of the PCR reactions.

3.10 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 programme's steps and entering the end or closing step.

The *Salmonella* specific primers TCGTCATTCCATTACCTACC and AAACGTTGAAAAACTGAGGA 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 (Table 4) components (except template DNA) for each 25 µl reaction to a eppendorf tube at room temperature, all solutions were gently vortexed and briefly centrifuged after thawing.

Table 4: Reaction mixture used for assay development of the PCR

10x PCR Buffer (Provides a final concentration of 2.5 mM MgCl2)	2.50 µl
DNTP	2.00 µl
Taq Polymerage (1 U)[10]	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

[10] Taq DNA Polymerase (Prokaria, Iceland)

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.

3.11 *Salmonella* strains in PCR

The selected *Salmonella* strains were tested with the 16S rDNA primer (5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-TACGGCTACCTTGTTACGACTT-3). The 16S rDNA sequence is a gene encoding small sub-unit ribosomal RNA, it acts as a common primer to nonselectively amplified any bacterial DNA in the sample.

Six *Salmonella* strains were run on PCR using the reaction mixture (Table 4) with 1 µl each primer set, which are *Salmonella* Tennesse, *Salmonella* serovar Montevideo, *Salmonella* serovar Schwarzengrund, *Salmonella* serovar Agona, *Salmonella* serovar Bredeney, and *Salmonella* serovar Infantis. The same volume (2 µl) of the template DNA were used for each *Salmonella* spp.

3.12 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.

3.13 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.

3.14 Environmental swab samples

Forty environmental samples were taken by swabbing at different locations in the neighbourhood of a **fish processing** facility in northern Iceland and were analysed by the SYBR Green Real-time qPCR.

3.15 SYBR Green real-time qPCR

3.15.1 Concept of SYBR Green real-time qPCR

SYBR-Green real-time qRT-PCR assay provides a rapid outcome. The detection is based on the binding of the SYBR-Green dye into double stranded PCR products, which is a sequence independent process.

The sensitivity of detection with SYBR-Green may therefore be compromised by the formation of primer-dimers, lack of specificity of the primers, primer concentration and the formation of secondary structures in the PCR product. All of these factors could lead to the creation of unexpected double-stranded DNA products, which would incorporate SYBR-Green and register a fluorescent signal.

3.15.2 Protocol of the SYBR real-time qPCR

Real-time PCR and data analysis was performed in the MiniOpticon™ real-time detection system with Supports Opticon Monitor™ version 3.1 (Bio-Rad Laboratories) using Maxim® SYBR Green qPCR Master Mix (2X) (Fermentas).

The *Salmonella* specific primers invA (TCGTCATTCCATTACCTACC and AAACGTTGAAAAACTGAGGA) were used to amplify a 100 base pairs fragment of the invasion (invA) gene. Primers were synthesised by Sigma Genosys Ltd. (Switzerland). All runs included a negative control without target DNA and *Salmonella* serovar Montevideo as the positive control.

<u>Reaction set-up</u>
A master mix was prepared by adding the following (Table 5) components (except template DNA) for each 25 µl reaction to a tube at room temperature. All solutions were gently vortexed and briefly centrifuged after thawing.

Table 5: Reaction mixture used for assay development of the SYBR real-time qPCR

Maxima TM SYBR Green qPCR Master Mix (2X) (Provides a final concentration of 2.5 mM MgCl2)	25 µl
Forward Primer (3U/µl)	0.3 µM
Reverse Primers (3U/µl)	0.3 µM
Template DNA	2.0 µM
Water, nuclease-free	to 50 µl
Total Volume	50 µl

The master mix was mixed throughly and dispensed in appropriate volumes into PCR plates. Template DNAwas added to a volume of 2µl/reaction to the individual PCR tubes containing the master mix. The reactions were mixed by centrifuge gently without creating bubbles.

Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A final extension of 72°C for 5 minutes was employed. All thermal cycling conditions were performed using a three-step cycling protocol. Data acquisition was performed during the extension step. Reproducibility of Maxim® SYBR Green qPCR Master Mix real-time PCR was assessed by running samples independently on different days.

The designed new PCR assay was verified to the product specificity by gel electrophoresis with ethidium bromide stained 2% agarose gels (Sigma, Germany) as melting temperatures of a specific product and primer-dimers may overlap depending on the sequence composition. The gel was documented with a digital camera.

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.

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

3.16.1 Standard curve from Maxima TM SYBR Green qPCR Master Mix (2X)

Genomic DNA (gDNA) is commonly used as standards in quantitative PCR. The mass of gDNA corresponds to copy numbers of target nucleic acid sequences (Applied Biosystems 2003). 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[11]). The mass of the genome was calculated by inserting the genome-size value in the following formula (Applied Biosystems 2003).

[11] The haploid number is the number of chromosomes in a gamete of an individual (Wikipedia)

$m = [n]\left[1.096e^{-21}\ \frac{g}{bp}\right]$ where n = genome size (bp)
m = mass
e-21 =x 10-2

The formula above was derived as follows:

$$m = [n][\frac{1\text{mole}}{6.023e23\text{ molecules (bp)}}]\left[\frac{660\ g}{\text{mole}}\right] = [n]\left[\frac{1.096e^{-21}\ g}{bp}\right]$$

where:

n = DNA size (bp)
m = mass
Avogadros number = 6.023e23 molecules / 1 mole
Average Molecular Weight (MW) of a double-stranded DNA molecule = 660 g/mole

The mass of the *Salmonella* genome (haploid) is calculated as follows:

$$m = [4.8e^{6}\ bp]\left[\frac{1.096e^{-21}\ g}{bp}\right] = 5.26e^{-15}\ g = 5.26e^{-3}\ pg = 0.00526\ pg$$

The mass of the genome was divided by the copy number of the gene of interest per haploid genome. The invA gene is a target that exists as a single copy gene per haploid genome.

$$\frac{0.00526\ pg}{genome} \div \frac{1\ copy\ RNase\ P}{genome} = \frac{0.00526\ pg}{1\ copy\ RNase\ P}$$

Therefore, 0.00526 pg of *Salmonella* gDNA contains one copy of the invA gene.

The mass of gDNA was calculated which contains the copy numbers of interest, that is 1,000,000 to 10 copies (Table 6).

Therefore, copy number of interest × mass of haploid genome = mass of gDNA needed.

The mass of gDNA needed was divided by the volume to be pipetted into each reaction.

Table 6: Six dilutions series in 2 µl template DNA of *Salmonella* serovar Montevideo

Copy Number		Mass of gDNA needed (pg)		Final concentration (pg/µl) of gDNA.
1000000		5260		2630
100000		526		263
10000	X0.00526 pg	52,6	/2 µl	26,3
1000		5,26		2,63
100		0,526		0,263
10		0,0526		0,0263

A serial dilution of the gDNA was prepared using the following formula:

$C_1V_1 = C_2V_2$

The stock concentration of *Salmonella* gDNA was determined by spectrophotometric analysis to be 4.21 µg/µl or 4210 pg/ µl. Therefore, in this example, C1= 4210 pg/µl. Each dilution prepared has a final volume (V2) of 100 µl.

So, dilution no. 1:

$$\left[4210\frac{pg}{\mu l}\right][V_1] = \left[2630\frac{pg}{\mu l}\right][100\mu l]$$

V1= 62.47 µl

Volume of diluent = 100 µl – 62.47 µl = 37.53 µl

To achieve the final volume of 100 µl, add 62.47 µl of stock gDNA to 37.53 µl of diluent. The diluent can be sterile 1X TE (1mM Tris, 0.1mM EDTA, pH8.0) or sterile, nuclease-free H2O. For the diluton of *Salmonella* serovar Montevideo nuclease-free H2O were used. Dilutions 2 to 5 were calculated using the same types of calculations (C1V1 = C2V2) as presented above for dilution no. 1. The following table (Table 7) presents the calculated volumes of gDNA and diluent for all dilutions.

Table 7: Copy of invA gene in dilutions of template DNA of *Salmonella* serovar Montevideo.

Dilution No	Source of gDNA for Dilution	Initial concentration (pg/µl)	Volume of gDNA(µl)	Volume of diluent (µl)	Final Volume (µl)	Final concentration of dilution (pg/µl)	Resulting copy # invA gene/2µl
1	Stock	4210	62,47	37,53	100	2630	1000000
2	Dilution 1	2630	10	90	100	263	100000
3	Dilution 2	263	10	90	100	26,3	10000
4	Dilution 3	26,3	10	90	100	2,63	1000
5	Dilution 4	2,63	10	90	100	0,263	100
6	Dilution 5	0,263	10	90	100	0,0263	10

All the 6 dilutions were run using the following protocol (Table 8).

Table 8: Temperature Protocol for real-time PCR

Steps	Temperature	Duration
1	940C	5 minutes
2	940C	15 Seconds
3	600C	30 Seconds
4	720C	30 Seconds
5	Go to Step 2	40 Times
6	720C	7 minutes
7	End	

Standard curve

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.

Standard curve from Power SYBR Green qPCR master mix

To establish the reliability of SYBR Green the real-time PCR method, the reproducibility 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. Template DNA was added to a volume of 2.5 µl/reaction to the individual PCR tubes containing the Master Mix. The reactions were mixed by centrifuge gently without creating bubbles.

Table 9: Reaction mixture used for assay development of the SYBR real-time qPCR

Power SYBR Green qPCR Master Mix (Provides a final concentration of 2.5 mM MgCl2)	12.5 µl
Forward Primer	0.1 µM (1 µl)
Reverse Primers	0.1 µM (1 µl)
Template DNA	2.5 µl
Water, nuclease-free	to 25 µl
Total Volume	25 µl

Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A final extension of 72°C for 5 minutes was employed. Data acquisition was performed during the extension step using StepOne (Applied Biosystems) real-time PCR system.

3.17 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.

3.18 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. The ratio of templates from the CT values can be calculated because the CT values are related to the initial template concentration.

Each PCR cycle doubles the amount of PCR product. It is possible to compare copy number ratios by comparing their CT values. The relationship between copy number

and CT value is inversely related, the higher the CT, the lower the copy number of starting template and since the cycling reaction causes the template to double, the relationship is also logarithmic:
The copy number is proportional to:

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

= Ratio of standard DNA template to sample DNA template

From the above equation, the number of known invA genes of *Salmonella* and the unknown number of invA genes were calculated.

3.19 Validation

"Validation is the conformation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled." (ISO/IEC 17025:2005(E)).

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 (National Food Administration, Uppsala, Sweden), and
Environmental swab samples.

In all four steps results from PCR analysis were compared directly or indirectly to the "conventional" culture method.

4 STATISTICS

A PCR reaction is positive if the amplification curve indicates a clear exponential increase of the PCR product and crosses the threshold line before cycle 40.

For level L0, the specificity was defined as the percentage of negative samples giving a correct negative signal. For levels L1 and L2, the sensitivity was defined as the percentage of positive samples giving a correct positive signal. The relative accuracy is defined as the degree of correspondence between the response obtained by the reference method (traditional microbiological method) and the response obtained by the alternative method (here real-time PCR) on identical samples.

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.

5 RESULTS

5.1 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-electroferosis band (Figure 4) signal compared to the others, so the invA119 primer pairs were used for all the next experiments.

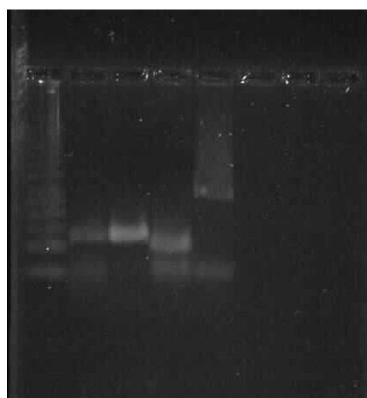


Figure 4: PCR amplification of *Salmonella* serovar Montevideo on primer set (1) Shima 122 bp (2) invA 119 bp (3) ttr6/4 94 bp (4) Sal/inv 139/141 284 bp. InvA 119 bp (arrow mark) shows the best band.

Primer concentration

From the different concentration of primers (Table 3) 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.

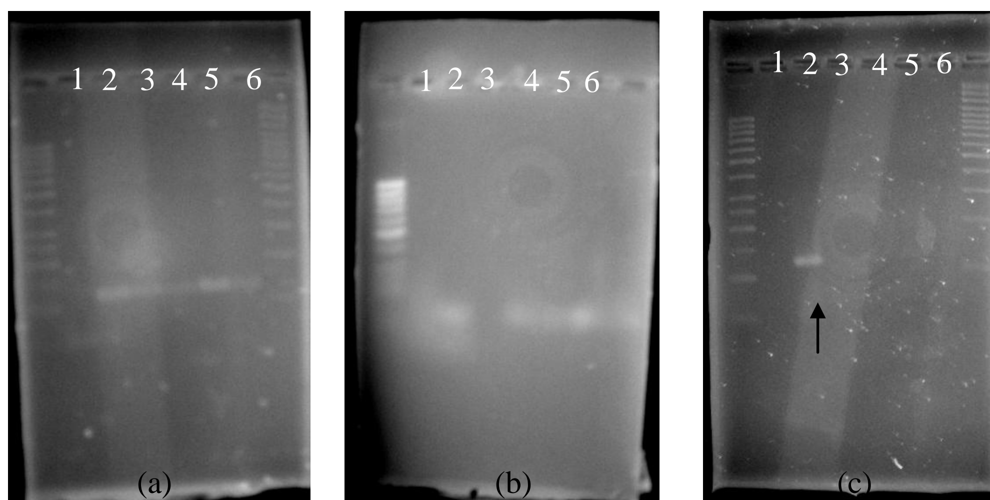


Figure 5: PCR amplification of *Salmonella* spp. on 2% agarose gel by 119-base pair invasion (invA) gene primer set in three different annealing temperatures (a) 55C (b) 58°C (c) 60°C; and six different concentrations of primer sets: 1. 0.3 µM Forward (F) and Reverse (R) primer each with no template 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-electroferosis (arrow sign in c) without any primer-dimer.

5.2 *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 *Salmonella* (Table 10). The lowest concentration was found in the *Salmonella* serovar Agona and the highest concentration in the *Salmonella* serovar Montevideo.

Table 10: Concentration of six *Salmonella* spp. used for assay development was collected from Promat; Akureyri, Iceland; all strains were sampled from Fish of Iceland.

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

The DNA concentrations of six *Salmonella* spp. measured by fluorometer (Invitrogen) shown the following (Table 11) concentrations. The highest DNA concentration was found in *Salmonella* serovar Tennesse and the lowest concentration in *Salmonella* serovar Schwarzengrund.

Table 11: 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

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.

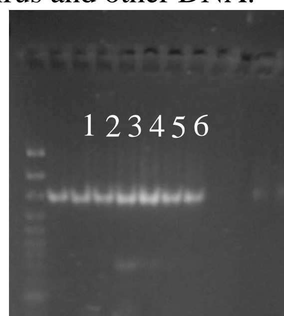


Figure 6: PCR amplification of six different *Salmonella* spp. on 1.5% agarose gel by 16S rDNA primer set. 1. 100 bp DNA ladder. 2. *Salmonella* serovar Montevideo. 3. *Salmonella* serovar Tennesse. 4. *Salmonella* serovar Montevideo. 5. *Salmonella* Schwarzengrund. 6. *Salmonella* Agona.

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.

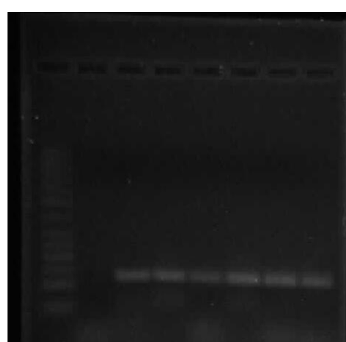


Figure 7: PCR amplification of six *Salmonella* spp. on 2.5% agarose gel by invA 119 bp Primer set. 1. 50 bp DNA ladder. 2. No Template DNA 3. *Salmonella* serovar Tennesse. 4. *Salmonella* serovar Montevideo. 5. *Salmonella* serovar Schwarzengrund. 6. *Salmonella* serovar Agona. 7. *Salmonella* serovar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

5.3 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 12). The scallop had highest concentration of *Salmonella* of 21.1 µg/mL.

Table 12: Quantity of *Salmonella* serovar Montevideo DNA in Pandalus borealis and Chlamys islandica.

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

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.

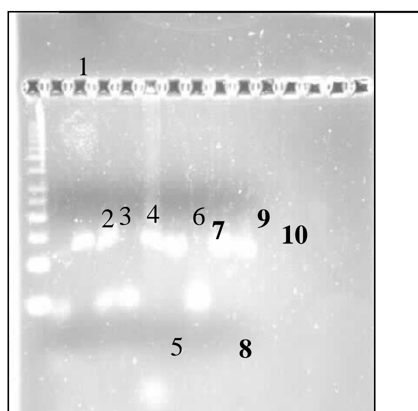


Figure 8: PCR amplification of *Salmonella* serovar Montevideo contaminated with shrimp (Pandalus borealis) and scallop (Chlamys islandica) on 2.5% agarose. 1. 50 bp DNA ladder 2. Negative control (non-template DNA) 3. *Salmonella* serovar Montevideo as positive control 4. *Salmonella* serover Dubllin 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)

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.

5.4 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.

5.5 Environmental swab samples

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

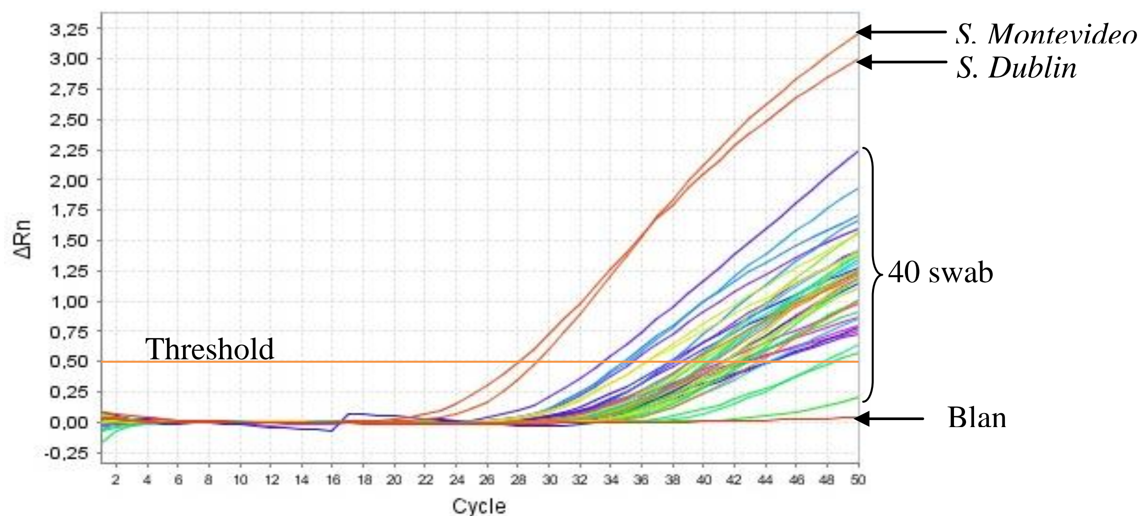


Figure 9: Fluorescence quantity of 40 swab samples, *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin as positive contol and non-template DNA as negative control.

From the 40 swab samples, most of the swabs showed positive on the real-time PCR run, the fluresscence 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).

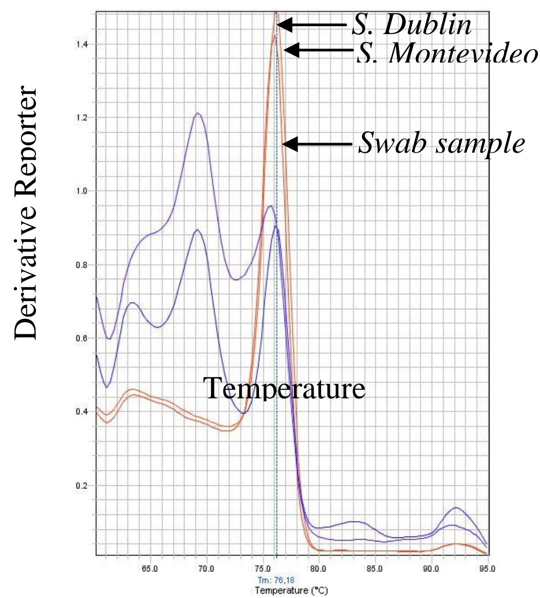


Figure 10: Melting curve analysis of two swab samples, *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin as positive contol and non-template DNA as negative control.

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).

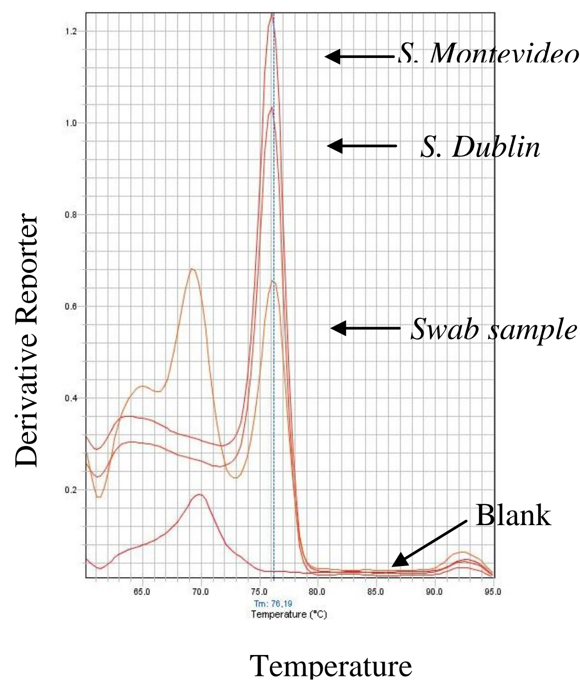


Figure 11: Melting curve analysis of two swab samples, *Salmonella* serovar Montevideo and *Salmonella* serovar Dublin as positive contol and non-template DNA as negative control.

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

5.6.1 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) (Appendix-1).

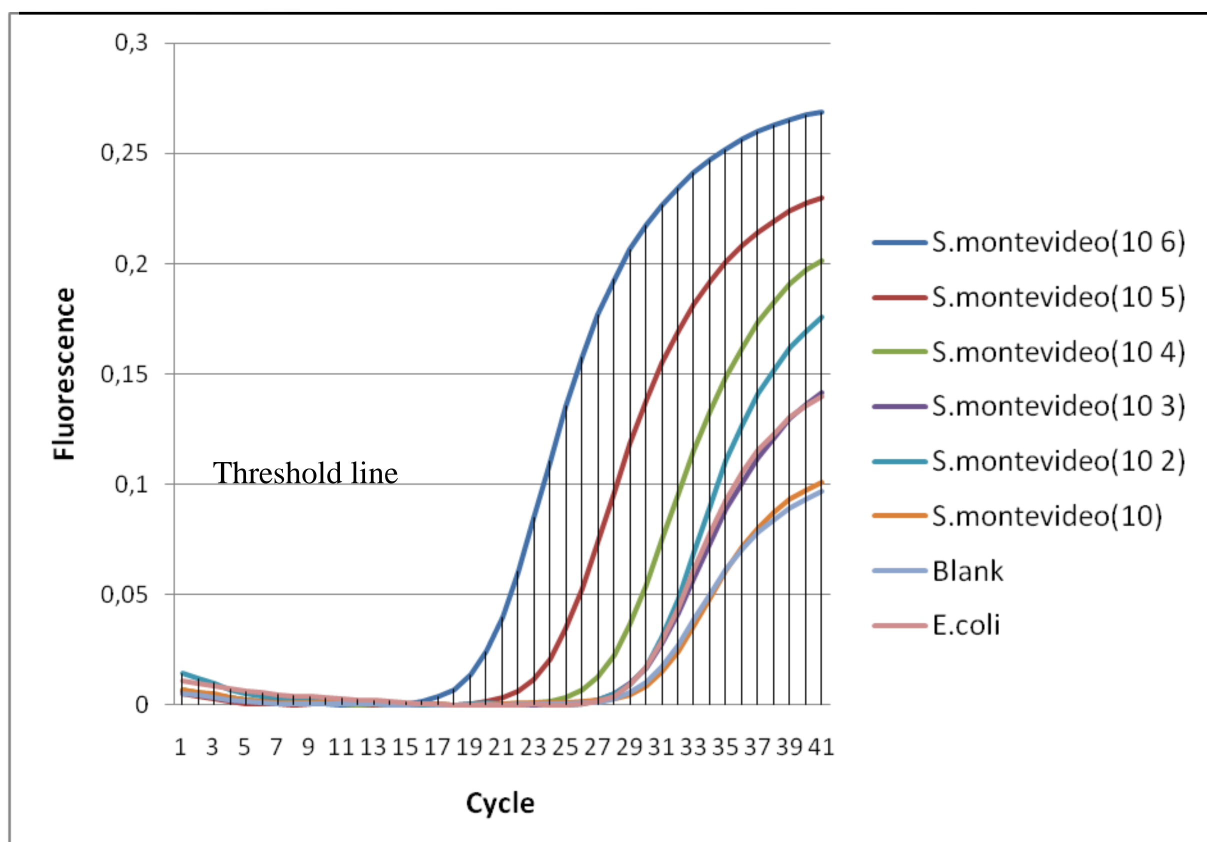


Figure 12: Fluorescence quantity shows 40 cycles in real-time PCR of six dilution series (106, 105, 104, 103, 102, 101) of *Salmonella* serovar Montevideo in Maxima TM SYBR Green qPCR Master Mix (2X), E.coli and non-DNA template were used as negative control.

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).

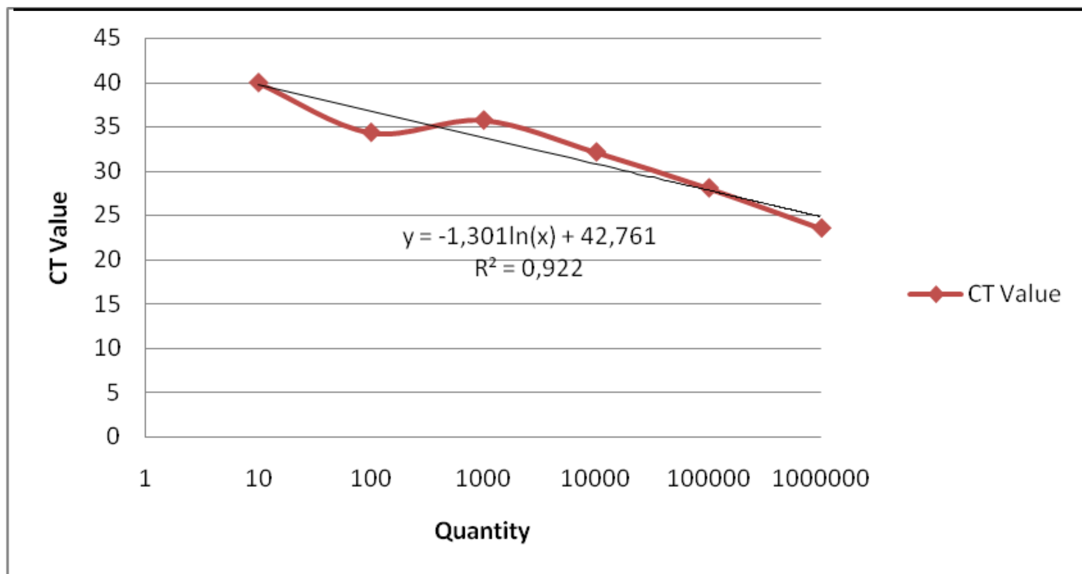


Figure 13: Standard curve of the 10-fold serial dilutions (106 to 101) of *Salmonella* serover Montevideo in the real-time qPCR in Maxima TM SYBR Green qPCR Master Mix (2X).

5.6.2 Standard curve from Power SYBR Green Qpcr Master Mix

A standard curve was obtained using the power SYBR Green 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 (Appendix-2).

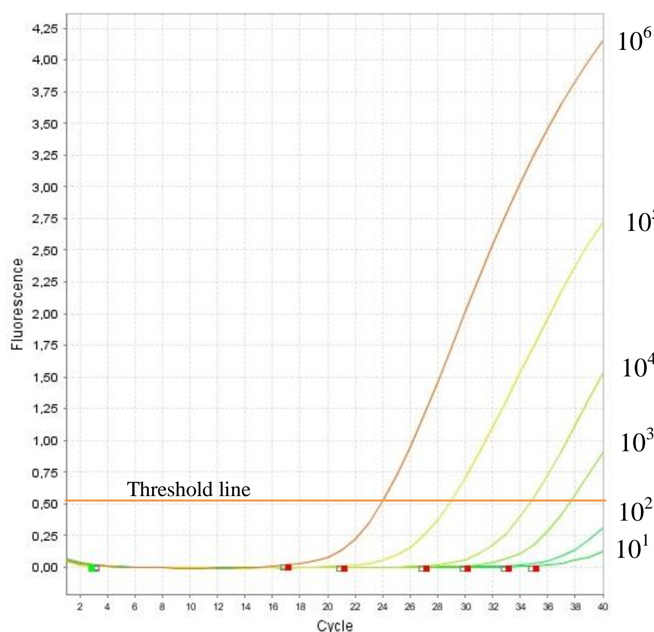


Figure 14: Fluorescence quantity shows 40 cycles in real-time PCR of six dilution series (106, 105, 104, 103, 102, 101) of *Salmonella* serovar Montevideo invA gene in Power SYBR Green qPCR Master Mix.

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.

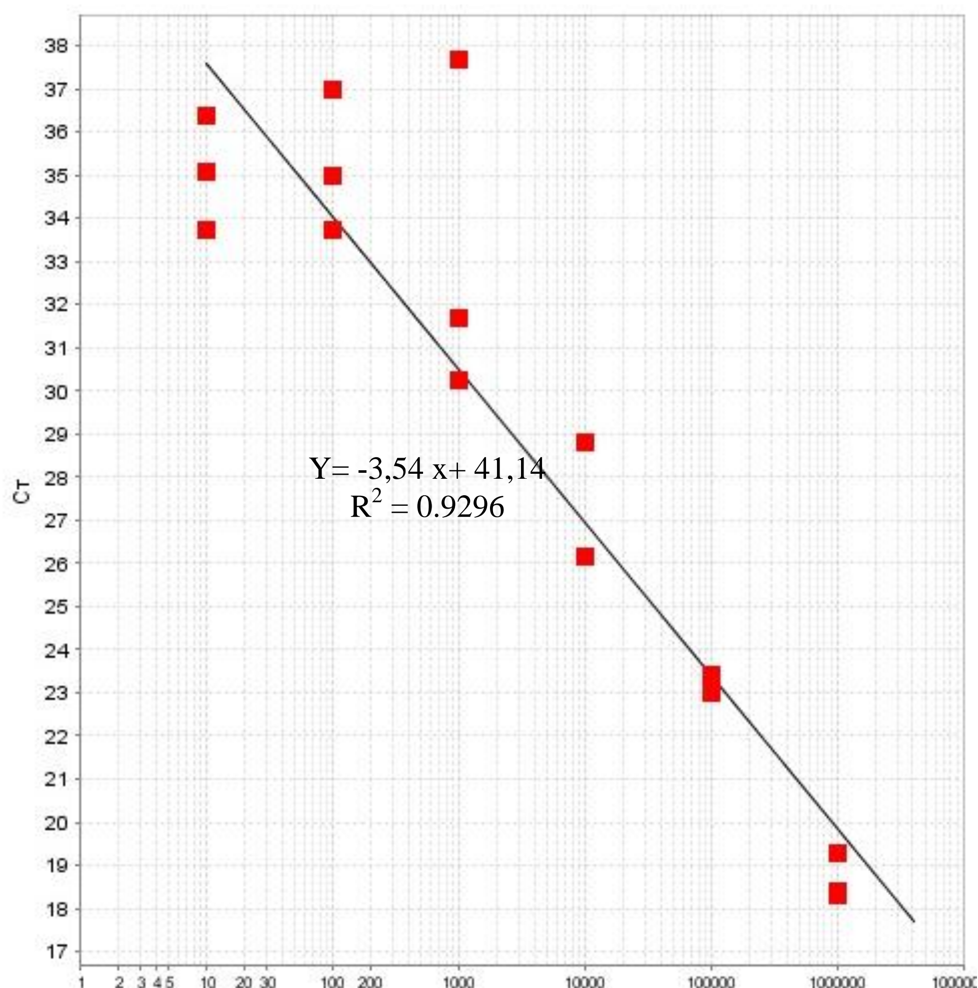


Figure 15: Standard curve of the 10-fold serial dilutions (106 to 101) of *Salmonella* serovar Montevideo (triplicate samples) in the real-time qPCR in Power SYBR Green qPCR Master Mix.

Both the dilution curves (Figures 13 and 15) showed that the real-time qPCR easily detected 103 invA gene, the flurescence 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 *Salmonella* serovar Montevideo (Figure 16). Experimental error may cause unequal difference of CT values in dilution series.

5.7 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).

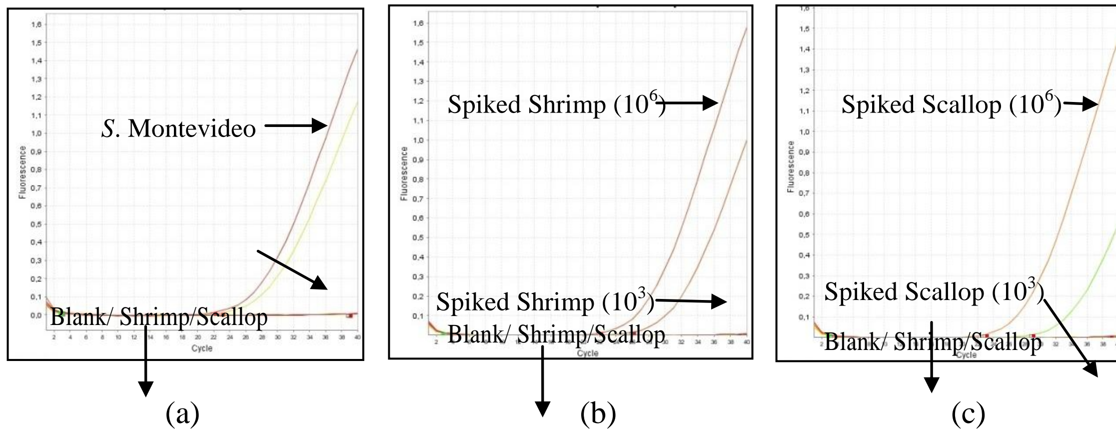


Figure 16: Fluorescence quantity (Y-axis) shows in 40 cycles (X-axis) in real-time PCR of (a) duplex *Salmonella* serovar Montevideo strains (b) Two concentrations (106 CFU/mL and 103 CFU/mL) *Salmonella* serovar Montevideo contaminated with shrimp (Pandalus borealis) (c) Two concentrations (106 CFU/mL 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).

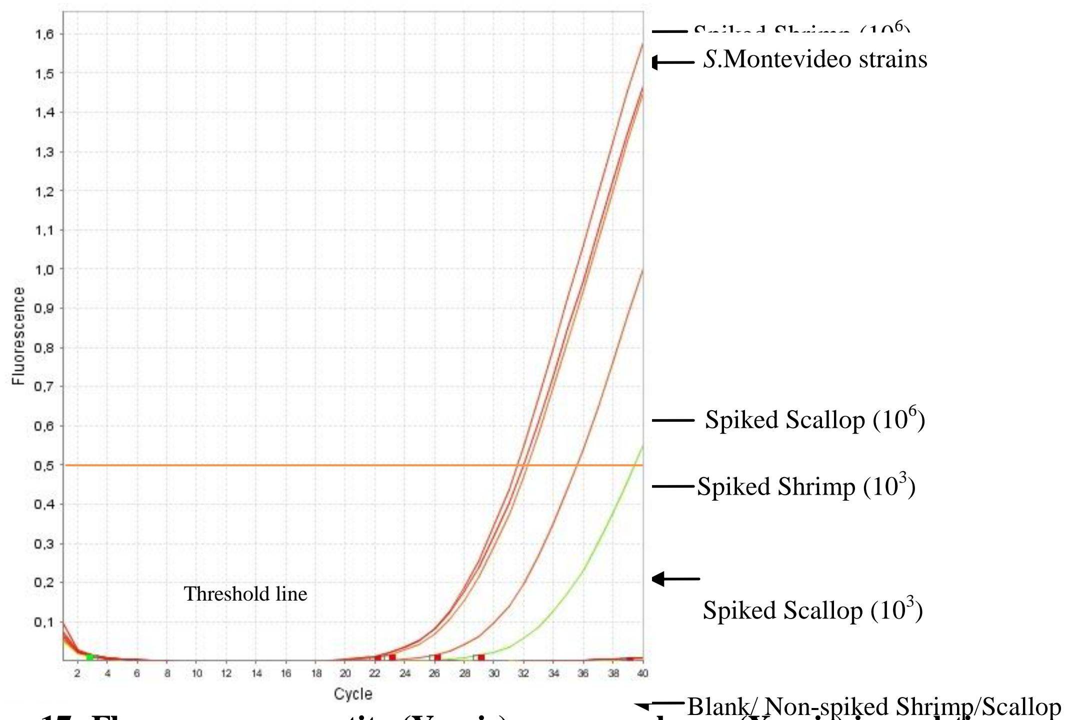


Figure 17: Fluorescence quantity (Y-axis) versus cycle no. (X-axis) in real-time PCR shows two concentrations (106 CFU/mL and 103 CFU/mL) of *Salmonella* serovar Montevideo contaminated with shrimp (Pandalus borealis) and scallop (Chlamys islandica). Non-spiked shrimp, scallop and non-DNA templates were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

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).

5.8 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.

$= 2^{-[CT(\text{Standard DNA}) - CT(\text{Sample DNA})]}$ = Ratio of Standard DNA Template to Sample DNA Template

The copy number of invA gene of *Salmonella* spp. (1000,000 invA gene) (Table 7) 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*.

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

= 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 15).

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)

Table 13: 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

5.9 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).

5.10 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.

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.

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).

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).

6 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 (Malorny et al. 2004), the second amplified the himA gene (Chen et al. 1999), while the third and the fourth pairs were situated in the invA gene (Mi Nam et al. 2005, Malorny et al, 2003, Hein et al. 2006). 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 (Heid et al. 1996). 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 experianced 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 (Table 14), 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 (Table 15).

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 are not 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.

In conclusion, the evaluation of the PCR-based method applied in this study identified the need for a nested real-time PCR, instead of the less sensitive conventional single PCR, to enable detection of pathogenic *Salmonella* in contaminated shrimp and scallop.

7 CONCLUSIONS AND RECOMMENDATIONS

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.

7.1 Future works

To continue the present work several approaches and topics can be studied more.

Direct detection of *Salmonella* from the environmental sample, prior to any microbiological enrichment steps.

The first obvious attempt is to introduce PCR methods to quantify the common bacterial community of the fish samples in real-time PCR.

An important assignment for further work would be to find out the detection limit for the common fish spoilage bacteria in different fish species; spoilage of fish and fishery products are very common in tropical countries like Bangladesh.

Delineate the uncertainty measurement regarding validation for specific spoilage bacteria and develop specific guidelines for accreditation of the developed method.

DEDICATION

I would like to dedicate this work to my wife, Bosra Khanam,
and my mother, Peara Begum, for their support and influence in my life and work.

ACKNOWLEDGEMENTS

This work was carried out at the Icelandic Institute of Natural History Laboratory, University of Akureyri (UNAK), Akureyri, Iceland, under the United Nations University-Fisheries Training Programme (UNU-FTP), Iceland.

I thank God for the strength he has provided me during this research work. I would like to express my sincere gratitude to all who have contributed to this work, especially:

Prof. Hjorleifur Einarsson, my main supervisor, for providing me with the possibility to start and fulfill this research at the University of Akureyri, and for engagement and guiding me through the process of scientific writing, continuous inspiration, selfless assistance and advice throughout this research and for being such a good and caring supervisor.

Kristinn P. Magnusson, my supervisor, for continuous and tireless support regarding PCR techniques and for introducing me into this exciting area of microbiology and giving me the opportunity to initiate my research education and work as well.

UNU-FTP, Iceland and the Department of Fisheries, Bangladesh for giving me the opportunity to come and study in Iceland and develop my knowledge and gain experience in the latest technology of fisheries science.

Mr. Thor Asgeirsson, Mr. Konrad Thorisson, Mr. Gudni Eiriksson, Ms. Sigridur Ingvarsdottir and Bjarni Eiriksson for their uninterrupted support, direction and cooperation during the entire training programme.

Jon Johannesson, Eydis Elva Thorarinsdottir and Kristbjorg Goa Sigurdardottir for **technical support during the project periods.**

Bosra Khanam, sweet daughter Rostina Artha and my mother Peara Begum, for their cordial inspiration, love, prayers, encouragement and continuous support to complete this research effort.

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Appendix 1: Real-Time PCR Fluorescence quantity against cycles in six dilution series (106,105,104,103,102,101) of *Salmonella* serovar Montevideo in Maxima TM SYBR Green qPCR Master Mix (2X), E.coli and non-DNA template were used as negative control.

Cycle	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10^{1}	Blank	*E.coli*
1	0,0059	0,0052	0,0067	0,0065	0,0143	0,0069	0,0053	0,0108
2	0,0051	0,0042	0,0058	0,0053	0,0121	0,006	0,0045	0,0099
3	0,0046	0,0031	0,0048	0,0041	0,0098	0,0051	0,0037	0,0088
4	0,0036	0,0018	0,0035	0,0026	0,007	0,0037	0,0026	0,0073
5	0,0023	0,0008	0,0023	0,0018	0,0051	0,0025	0,0018	0,0063
6	0,0019	0,0005	0,0016	0,0019	0,0038	0,0018	0,001	0,0056
7	0,0014	0,0003	0,0011	0,0019	0,0032	0,0014	0,0007	0,0049
8	0,0007	0,0002	0,0005	0,0018	0,0026	0,0009	0,0006	0,0041
9	0,0004	0,0003	0,0004	0,0019	0,0026	0,0011	0,0007	0,0039
10	0,0004	0,0005	0,0006	0,0017	0,0023	0,0011	0,0004	0,0032
11	0,0001	0,0005	0,0004	0,0012	0,0025	0,0013	0,0005	0,0031
12	0	0,0004	0,0002	0,0008	0,0017	0,0013	0,0004	0,0026
13	0	0,0002	0,0004	0,0004	0,0014	0,0014	0,0003	0,0022
14	0,0002	0	0,0001	0,0004	0,0006	0,0012	0,0001	0,0017
15	0,0008	0,0001	0,0001	0,0001	0,0005	0,0008	0	0,0013
16	0,0018	0	0,0002	0	0,0002	0,0005	0,0003	0,0008
17	0,0038	0,0001	0,0004	0,0001	0,0002	0,0003	0,0002	0,0005
18	0,0072	0,0002	0	0,0002	0	0	0	0,0001
19	0,0137	0,0008	0	0,0001	0,0006	0	0	0,0001
20	0,0242	0,0017	0,0002	0,0006	0,0009	0,0005	0,0004	0,0002
21	0,0399	0,0032	0,0002	0,0004	0,0007	0,0006	0,0002	0
22	0,0606	0,0064	0,0002	0,0003	0,0006	0,0009	0,0004	0,0002
23	0,0848	0,0118	0,0009	0,0002	0,0005	0,0011	0,0003	0,0003
24	0,1104	0,0211	0,0018	0,0004	0,0003	0,0013	0,0006	0
25	0,1352	0,0347	0,0033	0,0008	0,0001	0,0013	0,0007	0,0001
26	0,1576	0,0529	0,0067	0,0012	0,0006	0,0016	0,0009	0,0008
27	0,1769	0,074	0,0128	0,0026	0,0022	0,0022	0,0014	0,0019
28	0,1928	0,0966	0,0227	0,0054	0,0052	0,0031	0,0029	0,0043
29	0,2063	0,1186	0,0368	0,0099	0,0095	0,0049	0,0056	0,0093
30	0,2173	0,1381	0,0545	0,0166	0,0177	0,0086	0,0102	0,0172
31	0,2264	0,1552	0,0745	0,0278	0,031	0,015	0,0176	0,0291
32	0,2342	0,1696	0,0954	0,042	0,0485	0,0243	0,0276	0,0442
33	0,2411	0,1819	0,1153	0,0577	0,0692	0,036	0,0392	0,0614
34	0,2468	0,1923	0,1333	0,0738	0,0909	0,0488	0,0508	0,078
35	0,2517	0,2011	0,1486	0,0887	0,1111	0,061	0,0618	0,093
36	0,2562	0,2083	0,1619	0,1011	0,1273	0,0717	0,0708	0,1053
37	0,2596	0,2146	0,1732	0,112	0,141	0,0802	0,0784	0,1151
38	0,2625	0,2198	0,1826	0,1215	0,1519	0,0874	0,0842	0,123
39	0,265	0,2243	0,1908	0,1299	0,162	0,0933	0,0898	0,1302
40	0,2672	0,2276	0,1969	0,1368	0,1697	0,0977	0,0938	0,1358

Appendix 2: Real-Time PCR Fluorescence quantity against cycles in six dilution series (106,105,104,103,102,101) of *Salmonella* serovar Montevideo in Power SYBR Green qPCR Master Mix.

Cycle	10^6	10^5	10^4	10^3	10^2	10^1
1	0,0610649	0,0530109	0,0448034	0,052306	0,0461532	0,0700495
2	0,0293653	0,0212505	0,0189768	0,0253049	0,0176894	0,0354823
3	0,0141046	0,0107585	0,0136976	0,0146319	0,0109499	0,0201285
4	0,0052711	0,0055823	0,0071271	0,0071823	0,0075173	0,0121943
5	0,0021653	0,001933	0,0034636	0,0016374	0,0022211	0,0090114
6	-0,0016067	-0,00137	-0,0003139	0,0011723	0,0002852	0,0026175
7	-0,004998	0,000415	-0,001292	0,0006613	0,0012648	4,417E-06
8	-0,0059068	-0,0033019	-0,0012264	-0,0014561	0,0002688	-0,0005973
9	-0,0063252	-0,0049325	-0,0035779	-0,000834	0,0004973	-0,0042145
10	-0,006307	-0,0025328	-0,0042624	-0,0016813	-0,0008189	-0,0045939
11	-0,0063196	-0,0043373	-0,004111	-0,0015137	-0,0009551	-0,0057979
12	-0,005663	-0,0036709	-0,0033267	-0,0046123	-0,0020216	-0,0061715
13	-0,0033659	-0,0024881	-0,0034164	-0,002476	-0,0018613	-0,0062
14	-0,0014135	-0,0065386	-0,0036042	-0,0064992	-0,0016396	-0,0067251
15	0,001035	-0,0037932	-0,002432	-0,0056553	-0,0030976	-0,0058852
16	0,0054604	-0,0025676	-0,0026949	-0,0022609	-0,00263	-0,0040926
17	0,0138693	-0,0016919	-0,0025115	-0,0047901	-0,00232	-0,0062642
18	0,0247188	-0,0007247	-0,0041103	-0,0035417	-0,0041221	-0,0051667
19	0,047181	0,0032801	-0,0024931	-0,0036155	-0,0039665	-0,0041892
20	0,0806517	0,0052992	-0,0038345	-0,000572	-0,0031784	-0,0043539
21	0,137262	0,0106814	-0,001375	-0,0031093	-0,0028672	-0,002782
22	0,2275852	0,0173338	-0,0010385	-0,0043404	-0,0032344	-0,0023946
23	0,3552933	0,0324313	-0,0001033	-0,0017687	-0,0055425	-0,0023683
24	0,5237199	0,0564213	0,0008926	-5,865E-05	-0,0025603	-0,0022943
25	0,7197439	0,0974303	0,0030372	0,0002002	-0,0025316	-0,0014464
26	0,9509297	0,1584882	0,0069165	-0,0013726	-0,0017294	-0,0008594
27	1,1995049	0,251645	0,0105892	0,0019792	-0,0014567	-0,0005708
28	1,4616561	0,3737583	0,0216805	0,0041352	-0,001945	-0,000102
29	1,7334248	0,5297651	0,0363441	0,0074246	0,0001751	0,0022302
30	2,0144503	0,7058012	0,0611654	0,0111336	0,0007578	0,0052787
31	2,2776551	0,895952	0,1048543	0,0226323	0,0010206	0,0058752
32	2,5414813	1,1019956	0,171186	0,0378667	0,0022429	0,0076316
33	2,7859499	1,3115611	0,2668865	0,0654488	0,0035068	0,0120979
34	3,0243189	1,5360278	0,3956217	0,1078205	0,006579	0,0190199
35	3,2448554	1,7450404	0,5483267	0,1784603	0,0111918	0,0318448
36	3,4563856	1,9592212	0,7232394	0,2790731	0,020087	0,0503458
37	3,6496506	2,1739883	0,9130121	0,4111775	0,0317595	0,0854264
38	3,8280973	2,3690059	1,1119354	0,5628659	0,0535392	0,1388526
39	3,995795	2,5570455	1,3234588	0,7326682	0,0817663	0,2130098
40	4,1546717	2,7234657	1,5373226	0,9116558	0,1259494	0,311906

Appendix 3: Real-Time PCR Fluorescence quantity against cycles in two dilution series (106,103) of spiked *Salmonella* serovar Montevideo into shrimp (Pandalus borealis) and scallop (Chlamys islandica) in Power SYBR Green qPCR Master Mix. Non-spiked shrimp, scallop and non-DNA template were used as negative control.

Blank	S.Montevideo	Shrimp	Shrimp (spikedS. Montevideo,10 6)	Shrimp (spikedS. Montevideo,10 3)	Scallop	Shrimp (spikedS. Montevideo,10 6)	Shrimp (spikedS. Montevideo,10 3)
0,0732281	0,094487526	0,0605988	0,0653747	0,0560046	0,0532078	0,0739759	0,0674333
0,0232387	0,026775304	0,0228782	0,022352	0,0216538	0,017162	0,0244318	0,0242759
0,0107142	0,014089493	0,0139006	0,0122544	0,0136236	0,0109222	0,0139539	0,0142929
0,0040327	0,008216254	0,007742	0,0075313	0,0085222	0,0050631	0,0060549	0,007895
0,0046966	0,003193738	0,0051873	0,0029034	0,0043288	0,0035129	0,0037104	0,0021341
0,0028694	-0,001280818	0,0020617	0,0004637	0,0014772	0,0012547	0,001302	-0,0004531
-0,0003458	-0,002593676	-9,22E-05	-0,0036424	-0,0011411	0,0014698	-0,000555	-0,0024733
0,0009611	-0,004302159	-0,0010122	-0,00274	-0,0009348	-0,0014135	-0,0026032	-0,0014923
-0,0024327	-0,003672651	-0,0017838	-0,0045817	-0,003349	-0,0007392	-0,0032348	-0,0038993
-0,0031062	-0,005734649	-0,0025809	-0,0051949	-0,0036573	-0,002087	-0,0037123	-0,005193
-0,0019486	-0,004609692	-0,0026696	-0,0049015	-0,0041255	-0,0014828	-0,0045835	-0,0037375
-0,0041482	-0,006041729	-0,0033473	-0,0034076	-0,0045785	-0,0015074	-0,0049417	-0,0055399
-0,0019908	-0,003677129	-0,003217	-0,0037782	-0,0051221	-0,0025056	-0,0063362	-0,0054644
-0,000368	-0,00470977	-0,0016748	-0,0039887	-0,0046318	-0,0016424	-0,0060572	-0,0029334
-0,0008618	-0,004269119	-0,0035723	-0,0047387	-0,0045484	-0,0027212	-0,0057216	-0,0040472
-0,0001778	-0,003057049	-0,0022244	-0,0023853	-0,0045992	-0,0016482	-0,004998	-0,0027795
-0,0022367	-0,002088896	-0,0027608	-0,0031508	-0,0044572	-0,0023083	-0,0043518	-0,0040847
-0,0029035	-0,00118765	-0,002904	-0,0012165	-0,004554	-0,0019394	-0,0037747	-0,001293
-0,0016633	-0,000210438	-0,003198	0,0003237	-0,00438	-0,002016	-0,0008145	-0,0015598
-0,0019317	0,003006717	-0,0024238	0,0020908	-0,0039895	-0,0020791	0,0003209	-0,0002585
-0,0023647	0,006237522	-0,0026963	0,0065806	-0,0023435	-0,0019162	0,003076	0,0005751
-0,0016207	0,012691701	-0,0020071	0,0115786	-0,0007493	-0,0013701	0,0075519	0,003755
-0,0013074	0,02305061	-0,0020001	0,0204916	0,0015927	-0,002536	0,0157142	0,00579
-0,0013121	0,035072532	-0,0020546	0,033963	0,0047991	-0,0020986	0,0265939	0,0107669
-0,0020504	0,052436464	-0,0014468	0,0528741	0,0083343	-0,0022661	0,0432944	0,0194033
-0,0016424	0,083057597	-0,0018221	0,0834946	0,0144834	-0,0016683	0,0688131	0,031419
2,12E-05	0,122826733	-0,0014929	0,1264724	0,0246906	-0,0001413	0,1048084	0,0512348
-0,0015271	0,17587465	-0,0003219	0,185684	0,0406775	-0,0005542	0,1540703	0,0799742
-0,001723	0,239405632	-0,0016746	0,255549	0,0635171	-0,0005271	0,214541	0,1208103
-0,00098	0,31593132	-7,192E-05	0,3416428	0,096839	-0,0004971	0,2874775	0,1748378
-0,0010425	0,403262049	-0,0015923	0,4380608	0,1392493	-0,0021322	0,3722045	0,2446348
-0,0016978	0,501391053	0,0007709	0,5495825	0,1978203	0,0008564	0,4708922	0,3281478
-0,0010009	0,610335827	0,0009356	0,6694843	0,2678084	-7,741E-05	0,5799059	0,4247472
3,104E-05	0,724112034	0,0020474	0,7952984	0,347782	0,0008751	0,6966179	0,5301562
0,0015316	0,849540234	0,0020336	0,9275076	0,4399456	0,000746	0,8168602	0,6477287
0,0017607	0,971563637	0,0016813	1,0623664	0,5402889	0,0019315	0,9463507	0,7733109
0,0042191	1,098344803	0,003767	1,194304	0,6482072	0,0031323	1,073725	0,9008753
0,0051364	1,223675132	0,0052045	1,3233287	0,7626955	0,0040969	1,1985626	1,0270877
0,0064098	1,347298265	0,0053097	1,4553856	0,8799905	0,0060136	1,3251443	1,1567812