RAPID DETECTION OF *SALMONELLA* CONTAMINATION IN FISH AND FISHERY PRODUCTS USING MOLECULAR TECHNIQUES

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by

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May 2004

Dedicated to

my beloved family members

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Dated: 26 May 2004

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I hearby declare that the thesis entitled "**RAPID DETECTION OF *SALMONELLA* CONTAMINATION IN FISH AND FISHERY PRODUCTS USING MOLECULAR TECHNIQUES**" is an authentic record of the work done by mé and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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Brundabana Sahu

(Brundabana Sahu)

सारांश

सालमोनेला को मानव के गृषित आहार के सूक्ष्म जीवाणुओं में एक मुख्य रोगाणु के रुप में जाना जाता है । खाद्योत्पन्न सालमोनेलोसिस की खतरनाक दर से वृद्धि दुनिया भर के उपभोक्ताओं, औद्योगिक ईकाइयों व नियन्त्रक शाखाओं के लिये बढ़ती हुई चिन्ता का विषय है । सालमोनेला की पहचान के परम्परागत तरीके जो आज-कल लोकप्रिय हैं वे श्रमपूर्ण व अधिक समय लेने वाले हैं । इसके अलावा ये तरीके मत्स्य परिसंस्करण उद्योग में एच.ए.सी.सी.पी. पर आधारित गुणवत्ता आश्वासन तन्त्र लागू करने हेतु क्रिटिकल नियन्त्रण बिन्दुओं का सर्वेक्षण करने के लिये अनुपयुक्त हैं । जल्दी परिणाम प्राप्त करने की आवश्यकता ने कई तीव्र तरीकों का विकास करने में योगदान किया है जिनमें पी.सी.आर. खाद्योत्पन्न रोगाणुओं की विशिष्ट एवं संवेदनशील पहचान करने की एक विशिष्ट तकनीक है । इसलिये वर्तमान अध्ययन का उद्देश्य मछलियों में सालमोनेला प्रजातियों की विशिष्ट पहचान के लिये पी.सी.आर. पर आधारित तकनीक का विकास करना था । मौजूदा अध्ययन में बहुत सारे प्राइमर्स को स्क्रीन करने के उपरान्त हमने सालमोनेला सूक्ष्म जीवाणु के आई.एन.वी. ए जीन से आधरित एक विशिष्ट एवं उचित प्राइमर्स शोध करने के पश्चात तैयार किया । मुम्बई के मछली एवं मछली पकड़ने वाले वातावरण पर दर्शित सर्वेक्षण किया गया और 26 सालमोनेला स्ट्रेन अलग किये गये । शुद्ध ब्राथ कल्चर एवं सीडेड ऊत्तक होमोजिनेट को शीघ्र डी.एन.ए. प्रक्रिया द्वारा डी.एन.ए. तैयार किया । इसी डी.एन.ए. को पी.सी.आर के अनुकूल पाया गया । इसके अलावा, जैव रसायनिक एवं सिरोलोजिक्ली अटिपिकल स्ट्रेन्स भी इसी पी.सी.आर. तकनीकी द्वारा सालमोनेला ग्रुप मे ही सुःनिश्चित पाऐ गये जिनका न्यूक्लियोटाघ्ड अनुक्रमण भी जीन बैंक डेटाबेस के सामान पाया गया । मल्टीप्लेक्स पी.सी.आर. में 147, 302, 403, 450 बेसपेयर के डी.एन.ए. टुकडे क्रमशः इ. कोलाई, वि. कालरा, सालमोनेला एवं वि. पैराहिमोलाइटिकस को पहचान के लिये प्राप्त हुऐ । इनमें से हानिकारक वी. कालेरा एवं सालमेनेला के डी.एन.ए. बैंड खासतौर पर बहुत ही उत्साहजनक परिणाम है । इस तरह से वर्तमान अध्ययन में विकसित पी.सी.आर. तकनीकी मत्स्य परिसंस्करण उद्योगो में मत्स्य गुणवत्ता प्रवधन एवं जीवाणु परिक्षण हेतु एक सफल, शीघ्र, विशिष्ट एवं सटीक विधि के रुप में लागू करने योग्य है ।

कुंजी शब्द : सालमोनेला, पी.सी.आर., एच.ए.सी.सी.पी., मल्टीप्लेक्स पी.सी.आर.

ABSTRACT

Salmonella is considered as one of the major food-borne human pathogens and the alarming rate of increase of salmonellosis worldwide has become a growing concern among consumers, industry and regulatory agencies. The conventional detection methods of *Salmonella* spp. are labour intensive and time consuming and hence, are inefficient for the effective monitoring of critical control points (CCPs) for the implementation of hazard analysis critical control point (HACCP) based quality assurance system in the fish processing units. Polymerase chain reaction (PCR) has been proved to be a powerful technique for the rapid, specific and sensitive detection of food-borne pathogens. Therefore, development of a PCR-based method for the specific detection of *Salmonella* spp. from fish and fishery products was aimed in the present study. On the screening of many PCR primers, one new protocol based on the primers designed by us in the present study targeting *inv*A gene of *Salmonella* was found to be highly specific and accurate. A representative survey on the prevailing hygienic status of fish and fish handling environments of Mumbai was also attempted and 26 *Salmonella* strains were isolated from a total of 84 samplings made. Detailed biochemical characterisation and serotyping was performed and all the strains were grouped into 11 serovars. The PCR-based method developed in the present study detected all these isolates reproducibly from pure broth cultures as well as seeded tissue homogenates using a quick DNA preparation method, showing complete agreement of this PCR method with those of conventional culture based methods of isolation and identification of *Salmonella*. Further, some of the biochemically and/or serologically atypical *Salmonella* isolates also produced the *Salmonella* specific bands by this PCR method, which were confirmed as belonging to *Salmonella* genus by sequencing and sequence alignment using GenBank database. Further, performance of this PCR method was also evaluated in a multiplex PCR assay for simultaneous detection of few common seafood-borne human pathogens, yielding 147 bp, 302 bp, 403 bp, 450 bp distinct DNA bands specifically targeting *E. coli*, toxigenic *Vibrio cholerae*, *Salmonella* spp. and *V. parahaemolyticus,* respectively in a single tube reaction. Performance of the primers targeting toxigenic *V. cholerae* and *Salmonella* spp. in the specificity test was highly encouraging. The PCR methods developed in the present study can be successfully implemented in the fish processing industries for fast, specific, accurate and effective quality management of fish and fishery products with respect to *Salmonella* and *V. cholerae* contaminations.

Key words: *Salmonella*, PCR, HACCP, multiplex PCR

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INTRODUCTION

1. INTRODUCTION

Fish and fishery products, besides serving as an economic source of protein to millions, have carved their niche over the years as a billion-dollar export oriented industry in India. The quantum of export has grown significantly from a few million US dollars during early sixties to reach US $ 1.425 billion in the year 2002-03 (MPEDA, 2003a). Today export of fish and fishery products contributes to an extent of about 3.32% to the total export earnings of India. Processed fish, shrimp, squid and innovative value added products have found their market all over the world, thereby earning valued foreign exchange for our country.

Concomitant to its physical growth, there has been an increasing concern over the quality and safety of fish and fishery products. Because of the increased rate of incidence of food-borne diseases around the world in recent years, evaluation of microbiological safety of fish has become an important concern among the consumers, industries and regulatory agencies. Despite the fact that America's food supply is the safest in the world (Wang *et al.*, 1997), tens of million of cases of food-borne illnesses occur in the United States every year with a burden to the economy to the tune of 1-10 billion dollars. Similar impact of food-borne infections worldwide has been reported by many investigators (Lee *et al.*, 1996; Buzby and Roberts 1997; FAO, 1998; Wallace *et al.*, 2000). United States Food and Drug Administration (USFDA) has recognised about 14 food-borne bacteria, as revealed from its Bacteriological Analytical Manual (FDA, 2001), capable of causing food-borne diseases. The bacteria are *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *C. botulinum*. Infections due to many of these have been reported to have transmitted through fish, shellfish and other seafood products (Ries *et al.*, 1992; Okuda *et al.*, 1997).

Among all, the genus *Salmonella* stands out to be one of the leading cause of food-borne outbreaks and infections in many countries (Tirado and Schimidt, 2001). It is the leading cause of food-borne gastroenteritis in the United States (Olsen *et al.*, 2000) and is one of the most commonly reported causes of

food-borne disease outbreaks in the western world. *Salmonella* infection has resulted in an estimated annual cost of $ 846 million for Canada and $ 4 billion for the United States. Similarly, *Salmonella* was responsible for 68% of food-borne outbreaks in France (Lepoutre *et al.*, 1994) and 84.5% of the outbreaks in European Union (EU) nations (Schmidt, 1995). Typhoid fever caused by *Salmonella* Typhi[1] remains an important public health problem in India and other developing nations of the world, with 16 million cases and 600,000 deaths annually (Nasser *et al.*, 2002). The genus *Salmonella* comprises more than 2600 serovars and most, if not all, of these serovars are considered pathogenic to animals and humans (Jay, 1986, Kwang *et al.*, 1996). Being a major source of food-borne diseases, *Salmonella* also serves as an indicator of the safety of food and water supply of a country (Anon, 1993). Therefore, USFDA has strict guidelines that the fish and fishery products entering the US market should be *Salmonella* free.

Traditionally, serotyping, phase typing, biotyping, antibiotic resistance typing etc. are being used for typing *Salmonella* spp. Among the various methods, serotyping based on Kauffmann-White Scheme has become more popular and widely used method of classification and detection. According to this scheme, each serovar is regarded as a separate species. This has resulted in a nomenclatural nightmare to keep in order such a huge group of *Salmonella*. With the introduction of molecular biology, DNA became the target for molecular characterisation and classification of *Salmonella* in the last two decades. New DNA-based typing methods, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), ribotyping, multilocus enzyme electrophoresis (MLEE), pulse field gel electrophoresis (PFGE), have contributed to reclassification of serovars of *Salmonella* in a new subspecies group schemes (Reeves *et al.*, 1989; Baumler *et al.*, 1998). The genus *Salmonella* has now been divided into two species - *Salmonella enterica* and *S. bongori* (Le Minor and Popoff, 1987; Boyd *et al.*, 1996). Accordingly all the serovars, previously recognised as species, such as *Salmonella typhi* and *S. typhimurium* should now be designated as *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium (Salyers and Whitt, 2002) or simply *S.*Typhi and *S.* Typhimurium, respectively (Anon, 2000).

[1] *S. typhi* has now been designated as *S. enterica* serover Typhi or simply *S.* Typhi by the modern concept of *Salmonella* nomenclature (Anon, 2000, Salyers and Whitt, 2002). Therefore, this recent convention of *Salmonella* nomenclature (i.e., *S.* Typhi) has been used for all the serovars of *Salmonella* in this thesis.

The growing concern of microbiological safety of fish and fishery products worldwide has made a revolutionary change in the overall quality control regime. The traditional extensive end-point evaluation of quality, i.e., testing for failure, has been transformed to a longitudinally integrated quality assurance system of preventing failure. Ups and downs in the Indian seafood trade, with the constant upward trend of trade in one hand and repeated rejection of seafood consignments on the other, besides the EU ban during 1997 and the changes in regulations of the importing countries, have made implementation of Hazard Analysis Critical Control Point (HACCP)-based quality management system mandatory for processing of fish and fishery products meant for export.

HACCP is a structured approach of total quality management based on a systematic approach to hazard identification, assessment and control. Its sphere starts right from the environment involved with the procurement of raw material and other ingredients, passes through the different stages of processing till the product is finally consumed. In such an approach, it is required to take frequent samplings at the different stages of product movement for the assessment of hazards and also to assess the effectiveness of the corrective action. In such a scenario, the quality control laboratory of present day needs to be equipped with handling the bulk work load and should also be in a position to produce reliable diagnosis at the shortest possible time, so as to implement corrective action, if any.

Traditional culture-based methods are still the commonly used methods for the detection of pathogens in fish and fishery products. However, these methods are time consuming and labour intensive and, hence, are inadequate for the in-process monitoring of critical control points (CCPs). Thus, the availability of rapid, reliable and internationally accepted sensitive methods for the detection of pathogenic contaminants has become increasing necessity for effective implementation of HACCP in fish processing industries.

The last couple of decades have seen many developments in techniques and also dawning of new technologies, which were predicted to change our way of detecting pathogenic bacteria in food. A wide range of alternative approaches have been developed, such as enzyme-immunoassays, DNA-DNA hybridization, hydrophobic grid membrane filter methods, automated conductance

and antibody-antigen based detection methods (Blackburn, 1993; Olsen, 2000). However, many of these methods only replace the use of plating media and suffer from the lack of specificity and sensitivity. *In-vitro* amplification of DNA by PCR represents a major advancement in the development of rapid methods and has increasingly been used for rapid, sensitive and specific detection of food-borne pathogens. Very reliable PCR methods have been developed for the relevant bacteria and various formats of PCR have also been used to explore specific cases of microbial detection. However, their use in routine quality management in fish processing industries of India has not yet been achieved.

It is in this context, the present study on the development of a rapid method for the detection of *Salmonella* contamination in fish and fishery products using molecular techniques was carried out with the following objectives:

- To undertake a representative survey on the prevailing hygienic condition of fish landing centres and fish markets of Mumbai with special reference to *Salmonella* contamination
- To characterize the *Salmonella* isolates, if any, by biochemical, serological and antibiotic resistance typing techniques
- To perform molecular characterization of *Salmonella* isolates by PCR ribotyping
- To develop a PCR-based method for fast, accurate and specific detection of *Salmonella* spp. from fish and fishery products
- To evaluate the *Salmonella* spp. specific detection in a multiplex PCR-based assay for the simultaneous detection of few common pathogen in fish and fishery products.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Fish Processing Industry of India and Quality Issues

International trading of fish in India has been an old practice. It used to export marine products comprising dried items like dried fish, dried shrimp, shark fins, fish maws etc., mainly confined to neighbouring countries like SriLanka, Myanmar and Singapore (Bojan, 2001). Frozen products entered the export market with the shipment of a tonne of frozen shrimp from Cochin in 1953. This landmark achievement motivated many enthusiastic entrepreneurs to venture into shrimp processing (Iyer, 2000b). During the early sixties, the export of dried marine products declined while the export of processed items made a steady progress. With the devaluation of Indian currency in 1966, frozen and canned items increasingly figured in our exports and the sophisticated affluent markets like USA, France, Australia, Canada, Japan, etc. became important buyers. However, canned products disappeared from the export consignments during the eighties and diversified products like, cephalopods, frozen fish and value-added products gradually entered the export market. While all these items hold good prospects, live fish, chilled freshwater fish, etc. are promising items for the future.

The export of marine products has grown significantly as one of the important items of India's export from a few million US $ in 1961-62 to US $ 1.425 billion in 2002-03 (MPEDA, 2003a). Although 10 major and 30 minor items of marine products are exported from India, frozen shrimp is the major contributor to the export earnings to the tune of 67% contribution with only 29% of the total volume of marine products exported during 2002-03. USA emerged as the principal buyer of Indian marine products during the year relegating Japan to the second position with a share of 29.81% in value and 13.21% in quantity. China is the largest market of Indian seafood in terms of quantity, receiving 36.55% of total quantity exported, comprising mostly finfish items. The other principal markets for Indian seafood are the EU nations, Middle East, Southeast Asian countries, Australia and some African countries.

Over the years, there has been an increasing concern over the quality and safety of processed food including fish and other seafood. Increasing pollution in the aquatic environment, intensive production systems and improper handling during

processing and transport have increased the risk of fish and other seafood products. The safety concerns are on the rise due to the residues of pesticides, antibiotics, toxins, heavy metals and other harmful chemicals besides getting contaminated with pathogenic microorganisms that constitute health hazards. As a result, there has been a major shift of quality control of food items, from the end-point inspection to quality assurance by prevention, elimination and reduction in the levels of health hazards. This shift has been clearly reflected in the mandatory regulations of the EU and USA and thus, as an exporting country, India has been gradually adopting to such changes.

In the early stages, the quality of the products processed and exported by the fish processing industry in India was highly heterogeneous, as sufficient technical knowledge and transportation facilities were not available. Realising the importance of quality control in the fish processing industry, pre-shipment inspection of export consignments was introduced on a voluntary basis with effect from 1st September 1963 {The Export (Quality Control and Inspection) Act, 1963 (No. 22 of 1963)}. Under the aegis of this act, the Export Inspection Council of India (EIC) was set up for conducting quality control and pre-shipment inspection on 1st January 1964 (Thomas and Iyer, 2002). Frozen shrimps were the first to be brought under the purview of compulsory quality control and inspection scheme with effect from 15th March 1965. Subsequently, other seafood products of export importance were brought under the compulsory quality control and pre-shipment inspection scheme. In the beginning, consignments of fishery products were subjected to organoleptic examination alone. In the case of frozen fishery products, testing for certain bacteriological factors was introduced in the year 1973. The system of consignment-wise inspection continued until 1977, when in-process quality control (IPQC) of frozen fish and fishery products was introduced vide Gazette Notification No. S. O. 4007 & 4008 dated 31st December 1977. IPQC prescribed minimum requirements for raw material, manufacturing process, product testing, and the preservation and packaging of the final product. Thus, in 1978, in addition to the end-product specification, requirements were also introduced for processing, freezing, storage, transportation and in-house testing capabilities of the processing establishments. Subsequently, a modified system of IPQC (MIPQC) providing self-inspection facility was introduced and the necessary executive instruction for the systems (IPQC and

MIPQC) was issued separately vide Gazette Notification No. S. O. 1153 & 1153A, dated 9th April 1988. The earlier IPQC system was rechristened as "Quality Control Inspection in Approved Units (QCIA)" and the Modified In-Process Quality Control (MIPQC) Scheme as In-Process Quality Control (IPQC) (EIA, 1989).

The Government of India as per the notification dated 13.12.1990, recognized all the fish processing plants approved under IPQC system by the various Export Inspection Agencies (EIAs) in the country as an agency for pre-shipment inspection of the commodities produced by them for export. Gradually, the system described above started giving way to new quality concepts based on assessment of risks to human health, particularly the risks arising on account of harmful chemicals, pesticide residues, heavy metals, additives and disease-causing organisms. The European Economic Commission (EEC) vide its Directive No. 91/493/EEC of 22.7.1991 laid down health conditions for the production of fishery products and placing them on market (MPEDA, 2003b). It was the first to introduce a system called "own checks" to be followed during the course of processing, preserving and packaging of seafood. Subsequently, USFDA decided to introduce a regulation making HACCP-based seafood quality assurance system mandatory for its domestic industry as well as for the exporting countries. The regulation came into force on 18 December 1997 (Federal Register, 18 December 1995, 21 CFR Parts 123 and 1240) (FAO, 1998). As a consequence of these developments, it became imperative for all developing countries including India to design and implement export quality assurance programme that should meet the requirement of the importing countries. The Government of India, therefore, reviewed the existing export inspection system and introduced a new HACCP-based quality assurance and monitoring system (QAMS) for fresh, frozen and processed fish and fishery products with effect from 21st August 1995 in line with the requirements of major importing countries. The Export of Fresh, Frozen and Processed Fish and Fishery Products (Quality Control, Inspection and Monitoring) Rules, 1995, notified vide Gazette Notification No. S.O. 729 (E) and S.O. 730 (E) dated 21.8.1995 vest the primary responsibility on the processors to ensure that the fishery products intended for export are handled, processed, stored and transported under proper hygienic conditions, so as to meet the stipulated health conditions and that the products confirm to the prescribed specifications. The EIC, which was recognized as the

competent authority, has been vested with the responsibility to ensure by regular monitoring of the processing establishments that processors comply with the requirements (Thomas and Iyer, 2002).

Indian seafood export faced a serious setback subsequent to the imposition of quality norms by EEC as per the Directive No. 91/493. The seafood processors exporting to EU nations were required to meet various conditions under this directive latest by June 1997. However, subsequent to the inspection held by EU officials in June 1997, a ban was imposed on import of fish and fishery products from India. To overcome this situation, concerted efforts were made by the Government of India and an agreement was made between the Government of India and EU authorities in processing and trading of fish and fishery products. Accordingly, upgradation and renovation of fish processing units were undertaken on a war footing and based on the subsequent inspection by EU authorities, the ban on import of fish and fishery products from India was lifted in December 1997 vide Decision No. 97/876/EU dated 23.12.1997 (MPEDA, 1998). Since then, 138 fish processing plants and five freezer vessels have been approved for export to European countries (MPEDA, 2004). In the recent past, export to USA has also been affected by rejections of seafood consignments because of contamination with pathogenic organisms like *Salmonella* spp. and *V. cholerae*. This prompted several processors to introduce HACCP based quality assurance system in their production units.

HACCP is a management tool that provides a more structured approach to hazard identification, assessment and control than that available by traditional inspection and quality control procedures. As defined by World Health Organisation (WHO), 'HACCP is a systematic approach to the identification and assessment of the hazards and risks associated with a food operation and the defining of the means of their control' (WHO, 1993). The basic framework of HACCP was developed during the early 1970's by the Pillsbury Company with the co-operation and participation of US National Aeronautics and Space Administration (NASA) for the United States space programme that aimed at the production of zero defect food for the astronauts (Mukundan, 2001). It relies on managing the total quality of a production system starting from the place of origin of raw material through the stages of processing till it is finally consumed. The system not only deals

with the raw material and its ingredients, but also the environment involved during the process. Thus in the fish processing industry, the sphere of HACCP starts right from the fishing ground, the boat deck and the landing centre through the various steps of processing to get the final product, its packaging, storage and transportation. However, as Venugopal (1999) has rightly pointed out, the implementation of HACCP system in India faces problems because it does not cover the supplies of raw material and the shipping lines transporting the finished products to the destination. Hence, many processors are still facing the problems of contamination with food-borne pathogens and the possible risk of rejection of exported consignments.

2.2. Emerging Issues in Microbiological Safety of Fish and Fishery Products

Evaluation of the microbiological safety of foods has become an important concern of present-day consumers, industry and regulatory agencies. More than ever before, there is strong consumer awareness of food quality and safety, and this continues to increase. New risks and challenges are emerging as a result of changes in the method of food production at the farm and processing levels, emergence and re-emergence of food-borne pathogens (Tauxe, 1997), newer preservation technologies, change in eating habits and increased international transport (Archer and Young, 1988). The modern food-processing industry has to cope with the new generation of "ready to cook" or "ready to eat" convenience foods, which are minimally processed and contain little or no preservative (Sahu *et al.*, 1996). Further, many microorganisms previously unrecognized as food-borne or harmful are emerging as human pathogens transmitted by food. There are also cases of acquisition of key virulence factors particularly that encoded in jumping genes like plasmids or transposons, detected by newly developed isolation procedures or intelligent laboratory skills of microbiologists (Meng and Doyle, 1997). Expansion in international trade has facilitated the dissemination previously localised infectious agents to far-off places. Since the Uruguay Round Trade Agreement on the Application of Sanitary and Phytosanitary (SPS) Measures entered into force in 1995, microbiological risk assessment has become an integral part of Codex Alimentarius Commission (CAC) that has adopted a definite set of principles and guidelines to evaluate the microbiological safety issues in international food trade (CAC, 1999a). Subsequently, at its 32nd session in November 1999, Codex

Committee on Food Hygiene (CCFH) recognized that there are significant public health problems related to microbiological hazards in foods (CAC, 1999b). Accordingly, CCFH identified 21 pathogen-commodity combinations of concern and prioritised these according to the criteria like significance of public health problem, the extent of problem in relation to geographic distribution and international trade and the availability of data and other information with which to conduct a risk assessment.

Food-borne diseases remain a major public health problem all around the world. As estimated by Mead *et al.* (1999), in the United States there are as many as 9000 deaths per year due to food-borne diseases in general (estimates for 1994), and between 6.5 and 33 million illness. The annual cost of food-borne illness caused by *Salmonella* spp., *Campylobacter jejuni, Clostridium perfringens, Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Toxoplasma gondii* in the United States alone has been estimated at US $ 6.5-13.3 millions (Buzby and Roberts, 1997). Estimates of 1984 indicate that in the United States, the cost of food-borne diseases in which the vector was seafood, accounted for an equivalent of around 7.8% of the total value of fish and fish products traded that year. In Korea and Japan, the epidemiological evidence collected from 1971 to1990 incriminates seafood as the major cause of food poisoning (Lee *et al.*, 1996). The major bacterial groups implicated were *Vibrio* spp. followed by *Salmonella* spp., *Clostridium* spp., *E. coli* and *S. aureus*. Among all, *Salmonella* spp. are the leading cause of food-borne outbreaks and infections in many countries (Wallace *et al.*, 2000; Tirado and Schmidt, 2001). In general, it is accepted that the impact of food-borne diseases is more pronounced in developed countries. In developing countries, estimations are very difficult due to the lack of reliable epidemiological data. According to one WHO estimate, mortality due to food-borne and water-borne diseases could be responsible for between one-third and a half of the total number of deaths in some countries. Some studies also indicate that the economic impact of food-borne infections even if lower than in developed countries in absolute terms, is higher in relative terms, when compared to GDP and local incomes (FAO, 1998).

International trade of fish products expanded to US $ 152 billion in 1999. Developed countries accounted for nearly 84% of the total value of fishery products (FAO, 1998). Considering the quantum of export of fishery products, it is

essential to note the microbiological quality that has a realistic impact on the final safety criteria. It is noteworthy that the two largest recent outbreaks of food-borne diseases have been associated with the consumption of seafood. These are the epidemic hepatitis A in China with over 250,000 cases (Anon, 1988) and the tragic epidemic of cholera in Peru (Ries *et al.*, 1992). As documented by the National Academy of Sciences (NAS, 1991), pathogens that represent a potential public health risk to consumers of fresh and frozen finfish and shellfish products are from three main potential sources: (a) indigenous species from natural aquatic environment (b) non-indigenous species introduced into the aquatic environment from human and/or animal sewage or faecal wastes and (c) microorganisms associated with the processing, distribution and storage environments.

2.2.1. Indigenous pathogens in the aquatic environment

The aquatic environment is known to harbour several species of human pathogens as indigenous microflora (Garrett *et al.*, 1997). Many of the species associated with the freshwater environment are also found in estuarine and marine environments. Bacterial pathogens indigenous to the aquatic environment include *L. monocytogenes, Aeromonas hydrophila, Plesiomonas shigelloides, Erysipelothrix interrogans, Clostridium botulinum* type E and *Vibrio* spp. like *V. cholerae, V. parahaemolyticus* and *V. vulnificus* (Dalsgaard, 1998). Additionally the protozoan parasite *Giardia lamblia* has also been linked with fish-borne illness (CDC, 1989).

2.2.1.1. *Vibrio* spp.

Among the various pathogens that are important for the fish processing industry, *Vibrio* spp. are perhaps the most important ones (Karunasagar and Karunasagar, 1999). Substantial evidence is available showing that *Vibrio* spp. are natural inhabitants of marine aquatic environments in both tropical and temperate regions, with most human infections acquired by exposure to such environments or to food derived from them (Colwell, 1984; Jay, 1986; Kelly *et al.*, 1991). As of now, 12 species of *Vibrio* like, *V. alginolyticus, V. charchariae, V. cholerae, V. cicinatiensis, V. damsela, V. fluvialis, V. furnissi, V. hollisae, V. metschnikovii, V. mimicus, V. parahaemolyticus* and *V. vulnificus* are known to cause or to be associated with human infections

(Wong *et al.*, 1992; Wong *et al.*, 1993). But the most important pathogenic *Vibrio* spp., which have been associated with fish-borne illnesses include *V. cholerae, V. parahaemolyticus* and *V. vulnificus* (Karunasagar and Karunasagar, 2002).

2.2.1.1.1. *Vibrio cholerae*

Among all the members of *Vibrio* spp., *V. cholerae* has been responsible for the highest number of reported cases of food-borne epidemic. Outbreaks of cholera causing deaths are estimated at 120,000 annually worldwide (WHO, 1995). Between 1983 and 1986, shrimps valued at US $ 3 million from Southeast Asia were rejected and destroyed by the health authorities in Japan due to the presence of pathogenic *V. cholerae* (Reilly, 1987). Cholera has been categorised as one of the emerging and re-emerging infections (Satcher, 1995) threatening many developing countries. Several recent events that mark the epidemiological importance of the disease include the re-emergence of cholera in Latin America in 1991 (Levin, 1991; Ries *et al.*, 1992); the explosive outbreak of cholera among Rwandan refugees in Goma (Zaire), which resulted in 17,000 cases and 12,000 deaths in 1994 (Siddique *et al.*, 1995); and the emergence of *V. cholerae* 0139 in the Indian subcontinent during 1992-93, possibly marking the beginning of the eighth pandemic of cholera (ICDDR, 1993; Ramamurthy *et al.*, 1993; Swerdlow and Ries, 1993). In a recent review, Faruque *et al.* (1998) have summarized the epidemiology, genetics and ecology of toxigenic *V. cholerae*. *V. cholerae* has been divided into more than 150 serotypes on the basis of O somatic antigens and out of them, only serogroups O1 and O139 have been reported to cause cholera through the production of cholera toxin, a protein encoded by *ctx* gene (Mekalanos *et al.*, 1983; Finkelstein, 1988; Shimada *et al.*, 1994). Therefore, the presence of these two serotypes of *V. cholerae* in fish and fishery products indicates public health hazard.

V. cholerae belonging to non-O1 and non-O139 serotypes are widely distributed in the aquatic environment and are present even in environments that are not faecally contaminated, however, most of these do not cause human illness. Occasionally, some of them may be associated with mild gastroenteritis, but not the disease "cholera" (Karunasagar and Karunasagar, 2002). Unlike the non O1 serotypes, the establishment of *V. cholerae* in the aquatic environment is a

debatable issue (Islam *et al.*, 1996). Therefore, contamination of seafood with *V. cholerae* could be attributed to unhygienic landing centre besides improper handling and transport.

2.2.1.1.2. *Vibrio parahaemolyticus*

V. parahaemolyticus has been reported as an indigenous bacterium in a variety of marine and coastal environments including sea water and sediment, and in finfish and shellfish all over the world (Nair *et al.*, 1980; Twedt, 1989; Doyle, 1990). The organism has been recognized as the causative agent of gastroenteritis since its first report in 1950 (Fujino *et al.*, 1953). The *V. parahaemolyticus* gastroenteritis is contracted almost solely from seafood (Jay, 1986). It is one of the important food-borne pathogens in Taiwan, Japan and other coastal regions (Chiou *et al.*, 1991). Honda and Lida (1993), and Okuda *et al.* (1997) have reported the cases of food poisoning after consumption of seafood contaminated with *V. parahaemolyticus*. Recently with the emergence of the pandemic 03:K6 strain of *V. parahaemolyticus* causing acute gastroenteritis, this seafood-borne pathogen has assumed great significance (Matsumoto *et al.*, 2000).

Sakazaki *et al.* (1968), Miyamoto *et al.* (1969), and Thompson and Vanderzant (1976) have noted that the strains of *V. parahaemolyticus* associated with gastroenteritis in man produce β-haemolysin on a high-salt blood agar called Wagatsuma agar (Wagatsuma, 1968). However, only 1-2% of the environmental strains show this haemolysis. This phenomenon, known as the Kanagawa phenomenon, is induced by a thermostable direct haemolysin (TDH). TDH, the major virulence factor of *V. parahaemolyticus* (Nishibuchi and Kaper, 1985), is encoded by the *tdh* gene (Kaper *et al.*, 1984; Nishibuchi *et al.*, 1991; Okuda and Nishibuchi, 1998). Bandekar *et al.* (1982) had reported the occurrence of Kanagawa-positive *V. parahaemolyticus* strains in shrimp (*Penaeus indicus*) from Mumbai region. Studies on clinical strains that were Kanagawa-negative led to the discovery of a TDH-related haemolysin (TRH) carried by the *trh* gene (Nishibuchi *et al.*, 1989), which is also considered an important virulence factor of this organism (Honda *et al.*, 1988, Honda *et al.*, 1990).

The *trh* gene sequence is about 70% identical to the *tdh* gene sequence. There is much greater strain-to-strain divergence among the *trh* sequences than among the *tdh* sequences. The *trh* sequences among different strains, however, can be clustered into two groups represented by the *trh*1 and *trh*2 genes, which have 84% sequence identity (Kishishita *et al.*, 1992). Strains possessing the *tdh* gene, *trh* gene or both were shown to be strongly associated with gastroenteritis (Shirai *et al.*, 1990; Kishishita *et al.*, 1992). The surveillance for *V. parahaemolyticus* infection was initiated in January 1994 in Calcutta (India). A group of strains belonging to the serovar 03:K6 and possessing the *tdh* gene, but not the *trh* gene appeared suddenly in February 1996 and was shown to be responsible for the high incidence of *V. parahaemolyticus* infection since then in Calcutta (Okuda *et al.*, 1997). In addition to the production of *tdh* enterotoxin, it appears that *V. parahaemolyticus* also requires intestinal colonization factors to cause disease. The absence of *tdh* and *trh* genes in most *V. parahaemolyticus* strains isolated from the aquaculture environment suggests a low virulence of such strains. Although, there exists difference of virulence between environmental and clinical strains of *V. parahaemolyticus*, the bacterium remains an important cause of gastroenteritis associated with the consumption of seafood (Dalsgaard, 1998).

2.2.1.1.3. *Vibrio vulnificus*

V. vulnificus has been isolated from various raw seafood and is ubiquitous in the estuarine environment (Oliver *et al.*, 1983; O'Neill *et al.*, 1992; Wright *et al.*, 1996). The organism has been identified as the causative agent of food-borne diseases such as gastroenteritis and life-threatening septicaemia in immuno-compromised individuals (Blake *et al.*, 1980; Klontz *et al.*, 1988). *V. vulnificus* is responsible for three types of clinical manifestations in human, namely primary septicaemia leading to secondary lesions, gastroenteritis and wound infections (Thampuran, 2001). The bacteria have been isolated from shellfish and coastal waters of many countries (McLaughlin, 1995; Karunasagar and Karunasager, 1999). The bacteria have also been isolated worldwide in aquaculture environments with the highest densities being reported when the water temperature is high (De Poala *et al.*, 1994; Oliver, 1995; Hoi *et al.*, 1998).

2.2.1.2. *Clostridium botulinum*

C. botulinum is one of the dreaded pathogens capable of producing neurotoxins, which are the most potent natural toxins known to man, causing death by respiratory failure (Montecucco and Schiavo, 1994; Kessler and Benecke, 1997). Out of the seven types (types A to G) of the species available in nature, types A, B, E and F are the common types involved in human botulism (Lalitha, 2001a). Among all, *C. botulinum* type E is present in soil and sediments, and is mostly involved in botulinum poisoning in seafoods (Iyer, 1979). Cooked chilled fish that has been packed in airtight containers and canned fishery products that have been insufficiently processed are the potential causes of botulinum hazard (Balachandran, 2001).

2.2.1.3. *Giardia lamblia*

The protozoan parasite *G. lamblia* is one of the most common human parasites and causes a lengthy course of nonbacterial diarrhoea (Nash, 1992; Weiss *et al.*, 1992). *G. lamblia* is the only species of the genus that is known to cause disease in humans. The infectious stage of the parasite is the cyst and is usually transmitted among individuals by the faecal-oral route (Farthing, 1994). In the United States, infection is frequent among infants and children attending day care centres, and among travelers, campers and immuno-compromised patients. The disease outbreaks due to *G. lamblia* infections are often attributed to contaminated water supplies (Shaw *et al.*, 1977). Little information is available about the relevance of contaminated food, but infected person handling freshly prepared food has been reported as a transmission source (Adam, 1991).

2.2.2. Non-indigenous species of pathogens introduced into the aquatic environment

Potential pathogens associated with human and/or animal faecal pollution are enteric bacteria, protozoan parasites like *Cryptosporidium* and *Cyclospora,* and viruses such as Norwalk and Norwalk-like human enteric viruses (NAS, 1991; Kilgen, 2001). Important bacterial groups include *Salmonella* spp., *Shigella* spp., entero-toxigenic strains of *E. coli*, *Compylobacter* spp., *Yersinia enerocolitica, L. monocytogenes* and *C. botulinum* (Kvenberg, 1991).

2.2.2.1. *Salmonella* spp.

Salmonellae are a major cause of food-borne infections worldwide. *Salmonella* strains have been isolated from humans and almost all animals throughout the world, and are responsible for a wide range of infections including self-limiting gastroenteritis, severe gastroenteritis, typhoid fever, bacteraemia, abortion, meningitis, respiratory disease, cardiac disease, osteomyelitis and other local infections (Gray, 1995). Food-borne diarrhoeal diseases caused by *Salmonella* spp. affect more than 275 million humans worldwide (Archer and Kvenberg, 1985). According to Lepoutre *et al.* (1994), *Salmonella* was responsible for 68% of the food-borne outbreaks in France. The number of reported cases of salmonellosis in England and Wales almost trebled from 10,251 to 27,000 between 1981 and 1988; the number, however, remained almost static at about 30,000 between 1989 and 1995 (Lewis, 1997). In USA, the figures are almost double of these, and accounts for about half the reported cases and outbreaks of food-borne diseases (Hill, 1996). In many countries, myriad of the salmonellosis cases go unreported, indicating that the incidence based on reported cases represents only a fraction of the total number (Lewis, 1997; Stone *et al.*, 1998). There are several reports on estimates of devastation due to *Salmonella* spp. infection worldwide (Todd, 1989a, b; FAO, 1998). Non-typhoid salmonellosis accounts for 1.3 billion cases of acute gastroenteritis/diarrhoea with three million deaths (Pang *et al.*, 1995). Similarly, one particular serovar, *Salmonella enterica* serover Typhi (*S. typhi*) is the aetiological agent of typhoid fever, a serious invasive bacterial disease of humans with an annual global burden of approximately 16 million cases, leading to 600,000 fatalities (Pang *et al.*, 1995; Parkhill *et al.*, 2001).

Salmonellosis has become an increasing problem in industrialized countries during the last few decades (CDC, 1987; Rodrigue *et al.*, 1990; McClelland and Pinder, 1994; Waage *et al.*, 1999), where more than 80% of all non-typhoid salmonellosis cases might occur individually rather than in outbreaks (WHO, 1997). In recent years, a notable increase in cases related to a multi-drug resistant *S. enterica* serover Typhimurium strain, named DT104, has been reported in several European countries (WHO, 1997). In the developing countries, on the other hand, where large-scale farming and processing of food animals is not yet the norm, salmonellae are not as important causes of community-acquired diarrhoea (Lewis,

1997). However, typhoid fever is still a major public health problem in many developing countries including India. Human beings are the only reservoir and host of serovars Typhi and Paratyphi a and b, which are transmitted by contaminated water and food in endemic areas. From 1950, the progressive resistance of *S. enterica* serovar Typhi to first-line antibiotics has been reported (Pang *et al.*, 1995). However, multi-drug resistant strains are now emerging in India and Southeast Asia (Pang *et al.*, 1995; Rowe *et al.*, 1997).

Food samples usually contain very few salmonella organisms. However, the presence of a single bacterium in food is considered significant (Tetjen and Fung, 1995). A variety of food products, especially poultry and other types of meat products, are the most important sources of human infection, besides water (D'Aoust, 1989; Baird-Parker, 1990). Salmonella spp. are generally not recognized as being a part of normal microbial flora in the aquatic environment, and their presence in seafood is, therefore, seen as a sign of poor standards of process hygiene and sanitation (Dalsgaard, 1998). In many cases where fertilized with chicken manure, the pond water was found to contain Salmonella spp. (Llobrerra *et al.*, 1990; Reilly and Twiddy, 1992). Salmonella spp. could be isolated from highly polluted fresh and marine waters, and the fish often remain positive for Salmonella as long as 30 days after the initial contamination (Kvenberg, 1991). Baudart *et al.* (2000a) have evaluated the Salmonella spp. load in coastal waters from point versus non-point sources of pollution. Reilly et al. (1992) have reviewed the occurrence of Salmonella spp. in cultured tropical shrimp. Nambiar and Iyer (1991) worked out the prevalence of Salmonella serotypes in the retail trade in Kochi and isolated 16 different serotypes from 156 fresh and 127 frozen fish samples. Iyer and Shrivastava (1989) have reported the isolation of 34 different serotypes from different aqua-products with the highest number of serotypes isolated from frozen frog legs. Bandekar *et al.* (1995) have reported Salmonella strains in the chicken and fish samples of Mumbai market. Panda (2002) has reported the incidence of Salmonella in a processed shrimp sample, indicating bad manufacture practices of the processing plant. The pattern of Salmonella serotypes in India has been reported by National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh (Nath et al., 1966 and 1970; Saxena et al., 1983). Incidence of antibiotic resistant *S.* Typhimurium (Bhatacharya, 1997), *S.* Enteritidis (Bakshi, 1998)

and *S.* Gallinarum (Joseph, 1994; Gopinath, 1996) have also been reported. There are several reports on the incidence of *Salmonella* spp. in poultry, meat and other foodstuff worldwide (Thorberg and Engvall, 2001; Stock and Stolle, 2001; Bailey *et al.*, 2002; Tavechio *et al.*, 2002). Similarly, there are also several instances of rejection of shrimp from Southeast Asia due to the contamination with *Salmonella* spp. (Karunasagar and Karunasager, 1999). However, Rekhadevi (2001) did not find any salmonella contamination in her surveillance study taking raw material, water, ice, sanitary samples and finished products obtained from fish processing plants around Kochi (India).

Salmonella is a typical genus of Enterobacteriaceae, facultative anaerobic, intracellular parasite that invades the mucous membrane and is spread primarily by faecal-oral transmission (Cocolin *et al.*, 1998). The Gram-negative bacilli, are peritrichously flagellated (D'Aoust, 1989) and mostly motile with the exception of *S.* Pullorum and *S.* Gallinarum (Iyer, 2000b). These mesophillic organisms are distributed all over the world, but principally occurring in the gut of man and animals, and in environments polluted with human or animal excreta. A characteristic feature of this organism is its broad host spectrum, which comprises most animal species, including mammals, birds and cold-blooded animals, in addition to humans (Waage *et al.*, 1999). Besides, *Salmonella* spp. can withstand extreme conditions and isolation from dry pelleted chicken manure has also been reported by Dalsgaard and Olsen (1995).

The salmonellae may be divided into three groups based on host predilections (COS, 1969). They are: (1) primarily adapted to man – comprising the typhoid and paratyphoid agents, *S.* Typhi, *S.* Paratyphi a, b, c (2) primarily adapted to particular animal host – including *S.* Choleraesuis, *S.* Pullorum, *S.* Gallinarum, *S.* Dublin and so on, and (3) un-adapted - this group includes over 2000 serovars that may cause illness in man and other animals and generally do not show any host preference. Food-borne gastroenteritis is caused primarily by the un-adapted members of this genus (Jay, 1986).

In the early bacteriological literature, the organism now designated as '*Salmonella*', was referred to as 'Bacterium' and later as 'Eberthella' in the honour of Eberth. The present generic name "*Salmonella*" is in honour of Dr. Daniel Salmon,

an early American bacteriologist. The clinical importance of *Salmonella* spp. was recognized long before modern methods of species identification based on DNA-DNA homology or 16S rRNA sequence were developed. As a result of medical interest in this genus, serotyping based on Ewing (1986) and Kauffmann-White scheme of antigenic structure (Popoff and Le Minor, 1997, Popoff *et al.*, 1995) was used until recently as a way to identify bacterial groups, where each named serotype was regarded as a separate *Salmonella* species. The result was a nomenclatural nightmare to keep in order such a large group of bacteria like *Salmonella* spp., which comprises more than 2600 serovars (Portillo, 2000).

Several methods such as serotyping (Popoff and Le Minor, 1997), phase typing (Threlfall and Frost, 1990), biotyping (Christensen *et al.*, 1992), biochemical reactions, outer membrane protein profile and antibiotic resistance typing are being used for typing *Salmonella* serovars. Serotyping based on antigenic structures is the most common and widely used method of typing *Salmonella* strains. The typical *Salmonella* spp. possess two sets of antigens: the somatic or O antigens and flagellar or H antigens. Some *Salmonella* spp. also produce a surface polysaccharide, of which Vi antigen of *S.* Typhi is the most important (Lewis, 1997). The antigens are numbered and labeled, and the antigenic formula is written following a definite scheme, well known as Kauffmann-White scheme of antigenic structure. On the other hand, biotyping divides common *Salmonella* serotypes according to their biochemical characters. Biotyping is a more useful adjunct to phase typing, which subdivides *Salmonella* serotypes based on the bacteriophase associated with it. Analysis of outer membrane protein profile has been applied to *Salmonella* serovars, but it is not very useful for epidemiological studies (Helmuth *et al.*, 1985). Similarly the use of lipopolysaccharide analysis for the characterisation of *Salmonella* strains has not been found to be discriminative (Hitchock and Brown, 1983). Antibiogram studies have also been used to classify *Salmonella* serovars based on their antimicrobial drug resistance pattern. This study has its importance owing to the emergence of multi-drug resistant strains of *Salmonella* spp. and the spread of resistance through drug resistant plasmid "R" plasmid.

These traditional methods are being replaced by DNA-based techniques like plasmid profiling, restriction endonuclease analysis (REA), RFLP, PFGE, MLEE, etc. RAPD analysis is a method for analysis of variability in the

genome of strains. This is a faster, relatively simpler and economical method of genomic typing (Welsh and McClellan, 1993). Plasmid profile analysis is considered to be a very useful tool in epidemiological studies. The plasmid profile is used for strain differentiation within serovars and phase types (Threlfall and Frost, 1990) and demonstration of virulence. ERIC (enterobacterial repetitive intergenic consensus sequences) are repititive palindromic sequences having highly conserved central inverted repeat. Hence the inter-ERIC distances are useful for strain differentiation. In prokaryotes 16S, 23S and 5S rRNA genes are present in multicopy numbers. 16S rRNA gene is highly conserved and has been used for establishing evolutionary linkages. Ribotyping based on 16S rRNA gene has proved to be an efficient tool for molecular epidemiology and strain differentiations, and has been applied alone or in combination with other approaches for typing of *Salmonella* spp. (Esteban *et al.*, 1993). The 16S, 23S and 5S genes are separated by spacer regions which exhibit a large degree of sequence and length variation at genus and species levels. Typing of *Salmonella* spp. using the 16S-23S spacer region has been reported to have differentiation of serotypes and subtyping of serotypes (Jensen *et al.*, 1993; Jensen and Hubner, 1996; Lagatolla *et al.*, 1996).

With the advent of new methods of typing, the classification and nomenclature of *Salmonella* spp. are being debated for the last couple of decades (Le Minor *et al.*, 1970; Le Minor and Popoff, 1987; Euzeby, 1999; Ezaki *et al.*, 2000; Yabuuchi and Ezaki, 2000). The DNA-based typing has contributed to the reclassification of serovars of *Salmonella* in a new subspecies-groups scheme (Reeves *et al.*, 1989; Baumler *et al.*, 1998). The genus *Salmonella* contains two lineages that diverged early in its evolution: the species *S. enterica* and *S. bongori* (Le Minor and Popoff, 1987; Reeves *et al.*, 1989; Boyd *et al.*, 1996). Accordingly, the groups that were previously designated as species, such *S. typhimurium* and *S. typhi* should now be designated as *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi, or simply *S.* Typhimyrium and *S.* Typhi, respectively (Anon, 2000; Salyers and Whitt, 2002). Members of *S. enterica* are divided into seven subspecies groups (I, II, IIIa, IIIb, IV, VI, VII). Group I includes serovars causing diseases in human and other warm-blooded animals, such as *S. enterica* serovar Typhi, Paratyphi, Sendai, Typhimurium, Enteritidis, Choleraesuis, Dublin, Gallinarum,

Pullorum, and Abortusovis (Baumler *et al.*, 1998). Groups II to VII include *S. enterica* serovars frequently isolated from cold-blooded vertebrates (Baumler *et al.*, 1998). Group V includes *S. bongori* (Boyd *et al.*, 1996).

2.2.2.2. *Listeria monocytogenes*

Within the last few decades, *L. monocytogenes* has been recognized as an important food-borne pathogen. Although the disease is relatively rare and only affects certain groups within the population, namely young, old, immuno-compromised and pregnant women, it is considered to be serious because of the high mortality rate. The ability of *L. monocytogenes* to grow at refrigeration temperatures coupled with the appearance of the pathogen in raw and processed meats as well as poultry, vegetables and sea food makes this bacterium a serious threat to susceptible consumers and to the entire food industry (Ryser and Marth, 1989). The incidence of *L. monocytogenes* has significantly affected the seafood trade. The occurrence of this pathogen has been reviewed by Fuchs and Reilly (1992), and Karunasagar and Karunasagar (2000). In 1980, there was an outbreak of the disease in Auckland (New Zealand), involving 22 prenatal cases resulting in five deaths. Investigators suggested that the consumption of raw seafood might have been a contributory factor, although the evidence presented was epidemiological rather than microbiological (Lenon *et al.*, 1984). In India the incidence of *L. monocytogenes* in seafoods has been worked out by some research laboratories. Fuchs and Surendran (1989) examined fresh fish and found that 30% of the samples contained *L. innocua*, a species considered to be non-pathogenic, and no *L. monocytogenes* was isolated. Similarly, Kamat and Nair (1994) did not find any incidence of *L. monocytogenes* in seafood sold in retail markets of Mumbai. But, Jeyasekaran *et al.* (1996) detected *L. monocytogenes* in 17.2% of finfish and 12.1% of shellfish from the Mangalore region. Ben embarek (1994) has reviewed in detail the presence, growth and detection of *L. monocytogenes* in seafoods. There are a number of routes through which *Listeria* spp. can enter the aquatic environment and contaminate fish, the main ones being run-off from agricultural land, direct contamination by animals, and the release of sewage and processing effluents (Fuchs and Reilly, 1992).

2.2.2.3. *Escherichia coli*

E. coli is the most common aerobic organism in the gastrointestinal tract of man and other warm-blooded animals. The organism is a typical faecal coliform and maintains an excellent correlation with faecal contamination. Therefore, in food as well as water microbiology, *E. coli* is considered as an indicator of faecal pollution. Akhtar (2002) has worked on the evaluation of critical control points in seafood processing based on *E. coli* contamination. However, during recent years, *E. coli* is also being regarded as a specific pathogen involved in intestinal and extra-intestinal infections. Unlike *V. cholerae, Shigella dysenteriae and L. monocytogenes* which are one-disease organisms, *E. coli* can cause a variety of diseases including diarrhoea, dysentery, haemolytic uremic syndrome, bladder and kidney infections, septicaemia, pneumonia and meningitis (Karunasagar and Karunasagar, 1999). In fact, different strains of *E. coli* are associated with different diseases and the food processing industry is mainly interested in pathogenic *E. coli* strains, which may be infective through the gastrointestinal route. Most strains of *E. coli* are not pathogenic and a food quality control laboratory should be able to differentiate such strains from pathogenic ones. The pathogenic *E. coli*, also referred to as enterovirulent *E. coli* (EEC), has been classified (Salyers and Whitt, 2002) into atleast six subgroups, viz.,

- Enterohaemorrhagic *E. coli* (EHEC)
- Enteroaggregative *E. coli* (EAggEC)
- Enterotoxigenic *E. coli* (ETEC)
- Enteropathogenic *E. coli* (EPEC)
- Enteroinvasive *E. coli* (EIEC)
- Diffusely adhering *E. coli* (DAEC)

Each of these groups can produce a different toxin or cause a different kind of infection (Karunasagar and Karunasagar, 1999; Hitchins *et al.*, 2001). Among the various pathogenic *E. coli*, EHEC has been recognized as a cause of serious diseases like haemorrhagic colitis and haemolytic uremic syndrome, which may lead to death due to acute kidney failure, and *E. coli* O157:H7 is the principal serotype of EHEC (Feng, 1993; Neil *et al.*, 1994). Outbreaks due to EHEC O157:H7 were first reported in 1982 and since then, the strain has caused, in the United States and

many other countries worldwide, a series of food-borne outbreaks with severe manifestations of illness (Griffin, 1995). Between 1982 and 1987, EHEC organisms have been associated with at least 20 outbreaks in the United States and Canada and the lone serotype O157:H7 is responsible for most of the outbreaks (Karmali, 1989). In a recent review, Park *et al.* (1999) have summarized in detail the emergence of this pathogen and its epidemiological significance. Kumar *et al.* (2001) studied the incidence of Shiga-toxigenic *E. coli* (STEC) in fresh seafood and meat marketed in Mangalore (India), and have reported that 5% of clam and 3% of fresh fish samples were positive for non-O157 STEC. Asai *et al.* (1999) have reported isolation of Shiga toxin-producing *E. coli* (STEC) O157 from processed salmon roe that was responsible for sporadic infections in Japan during 1998.

In contrast to the general belief that *E. coli* strains pathogenic by gastrointestinal route are of human origin, some ETEC strains may be carried in the gut of animals. Cattle, sheep, pigs and chicken are thought to be reservoirs of *E. coli* O157:H7, and the zoonotic transmission of pathogenic strains from animal to man is thought to occur. This could be a problem with aquaculture products where animal dung/waste is used to fertilize culture ponds. Water appears to be the main source of pathogenic *E. coli* and, therefore, it is important that fish processors take care of the processing water so that it is free from pathogenic *E. coli*. As of now, there have been no reports of major outbreaks of *E. coli* gastrointestinal infection transmitted through fish except for an outbreak of diarrhoea in children near Mangalore, in which enteropathogenic *E. coli* were involved (Stephen *et al.*, 1975).

2.2.2.4. *Yersinia enterocolitica*

Y. enterocolitica, a psychrotropic pathogenic microorganism, has been associated with sporadic outbreaks of food-borne illness all over the world. The capability of the organism to grow at refrigeration temperature (Palumbo, 1986) has brought to limelight the importance of this pathogen surviving in refrigerated foods. The incidence of *Y. enterocolitica* in seafoods has been reported by many workers (Lee, 1977; Pexiotto *et al.*, 1979; Gerard and Leland, 1992). In India, Khare *et al.* (1996) have reported the presence of avirulent strains of *Y. enterocolitica* in dry, fresh and frozen seafood available in the local markets of Mumbai. Kamat and

Bongirwar (2001) reported that among the seafood available in the local markets of Mumbai, pomfret, oyster and crab harboured *Y. enterocolitica* at 14.28, 20.00 and 10.00% levels, respectively.

2.2.3 Pathogenic bacteria associated with processing of fishery products

Pathogenic bacteria often associated with contamination, recontamination or cross contamination of seafood products during processing, distribution and storage include *V. parahaemolyticus, C. perfringens, C. botulinum, Salmonella* spp., *Shigella* spp., *B. cereus, S. aureus* and *L. monocytogenes* (NAS, 1991). As Iyer and Varma (1990) have pointed out, the main source of contamination with *Salmonella* spp. during processing of shrimp are process water, ice, shrimp contact surfaces, floor and lizard droppings apart from the water of harvest sources and beach sand. The incidences of *Listeria* spp., *Salmonella* spp. and *C. botulinum* have also been traced during processing of smoked fish and shellfish products (Heinitz and Johnson, 1998).

2.2.3.1. *Clostridium perfringens*

C. perfringens is probably the most extensively studied anaerobic bacterial pathogen that is known to cause gas gangrene disease. The first indication of the organism's association with food poisoning was established in 1940 by Knox and Mac Donald in England and McClung in USA. Today *C. perfringens* ranks second to *Salmonella* spp. as a cause of bacterial food poisoning in UK, although the incidence is much lower (Lalitha, 2001b). *C. perfringens* is classified into five types, A to E, based on the production of four major and eight minor toxins.

2.2.3.2. *Staphylococcus aureus*

Staphylococcal food poisoning is one of the most commonly occurring food poisonings (Wieneke *et al.*, 1993; Schmidt, 1995), caused by the ingestion of the enterotoxin formed in food during the growth of certain strains of *S. aureus*. The enterotoxins can be categorised into eight types – A, B, C1, C2, D, E, F, G, out of which, types A and D are the major cause of food poisoning (Sanjeev, 2001). The primary habitat of *S. aureus* is man and is present in sweat, ear gum, tear, throat,

wounds, etc. and to a greater extent, in the post-nasal drips of persons suffering from cold. Freshly caught fish does not contain any coagulase-positive staphylococci, but contamination can take place on subsequent handling. Therefore, its incidence in fish is indicative of poor personal hygiene of fish handlers (Balachandran, 2001).

2.2.3.3. *Shigella* spp.

The genus *Shigella* consists of four species and the virulent strains are often found associated with intestinal diseases like bacillus dysentery in man (Ward and Hart, 1996). Man is the only known natural host for *Shigella* spp. and fish gets contaminated with the organism through contaminated water, ice, contact surfaces, fish handlers, etc. (Balachandran, 2001). Food material gets contaminated by *Shigella* spp. mostly by man and flies. All virulent strains harbour a 120 – 230 kbp virulence plasmid (Hale *et al.*, 1983), which was first described for *S. flexneri* (Kopecko *et al.*, 1980). Sansonetti *et al.*, (1982) have demonstrated that the loss of this plasmid resulted in the loss of virulence in *Shigella* spp.

2.3. Recent Trends in the Detection of Pathogenic Microbes in Food

Food-borne diseases are mainly caused by pathogenic bacteria, which are either transmitted to humans from the animal reservoir or which contaminate the food process line. Isolation and detection of such bacteria in order to ensure quality and safety of products are of paramount importance in the quality assurance programme, and hence, the principal objectives of a seafood quality control laboratory.

A number of regulatory agencies like CAC, USFDA, EEC, ICMSF, government agencies of various countries (Japan, Canada, etc.), etc. have recommended the tolerance limit of these human pathogens in fish and fishery products. In India, EIC has stipulated the threshold limit of various pathogens in ensuring quality in the export-oriented seafood industry. Fish and fishery products meant for export must meet the quality standard stipulated by EIC as well as that of the importing country. Table 1 summarises the microbiological specifications for fish and fishery products by various agencies.

Table 1. Bacteriological guidelines for fish and fishery products

Parameter	Item	Specification (Max. limit)	Source/Agency
Aerobic plate count (cfu/g)	Fresh/Chilled/Frozen Shrimp, Lobster, Cephalopod and Finfishes (whole, dressed, fillet)	5×10^5	EIC, India
	Cooked/Boiled Shrimp, Lobster, Cephalopods and Finfishes (whole, dressed, fillet)	1×10^5	
	Fresh/Chilled/Frozen Crab & Crab Meat, Clam/Mussel Meat	10×10^5	
	Cooked/Boiled Crab & Crab Meat, Clam/Mussel Meat	1×10^5	
Vibrio cholerae (cfu/g)	Ready to eat fishery products	Nil	USFDA Canadian Food Inspection Agency
V. parahaemolyticus (cfu/g)	Ready to eat fishery products	1×10^4	USFDA
Escherichia coli (cfu/g)	Fresh, Chilled, Frozen Products	20	EIC, India
	Cooked or Boiled Products	Nil	
Salmonella spp. (cfu/25 g)	All	Nil	EIC, India, USFDA Canadian Food Inspection Agency
L. monocytogenes	All	Nil	USFDA
Staphylococcus aureus (coagulase positive) (cfu/g)	All	100	EIC, India
		10^4	Canadian Food Inspection Agency

Traditional microbiological quality-control methods were focused on end-product testing. This is being replaced by HACCP approach of food quality management, which relies on: the assessment of hazards associated with growing and harvesting of raw materials and ingredients; processing, marketing, and the preparation and consumption of food; determination of CCPs required to control the identified hazards; establishment of procedures to monitor CCPs; and corrective action to be taken when there is a deviation identified while monitoring a CCP (Lakshmanan, 2001). Therefore, in the HACCP framework, there is a shift from the end-product microbiological testing to in-process and environmental monitoring. In such a system, microbiological testing will be required for: assessing the hazard throughout the production chain, for determining specific CCPs required to control each specified hazard, for monitoring CCPs, and for validating and verifying the effectiveness of the HACCP plan.

Traditional microbiological techniques for the isolation and identification of bacteria from foods depend on pure cultures. These techniques involve enrichment, selective plating, purification and biochemical characterization, all of which may take several days. Besides, detection and isolation of the pathogenic bacteria from food are often difficult due to the high number of contaminating and indigenous bacteria, and the low number of the pathogenic bacteria of concern. In order to obtain even a modest sensitivity, sometimes, traditional isolation methods include two-stage enrichment, one pre-enrichment step followed by selective enrichment, both of which add to the time and labour required to produce a diagnosis and thus, becomes inadequate for the in-process monitoring of CCPs. There is, therefore, a vast scope for the improvement of detection and isolation methods, especially with respect to the changing scenario of quality management in seafood industry.

During the last few decades, a number of techniques have been developed as alternatives to the conventional culture based methods for the detection of pathogenic bacterial contamination in foods. A list of such techniques, which have been applied to improve methods for the detection of pathogenic bacteria in food, has been given in Table 2. Some of these techniques became very popular and put into wider use: colony hybridization (Grundstein and Hogness,

Table 2. Alternative methods for the detection and/or isolation of food-borne bacterial pathogens

Principle	Example of methodology	Remarks
♦ Improved (automatic) viable count	- Spiral plate count - Hydrophobic grid-membrane - Filter technique	
♦ Microscopy technique	- Direct epifluorescence filter technique (DEFT) - Flow cytometry - Fluorescence antibody technique	
♦ Electric charge	- Impedence methods	Subjective
♦ Electronic device	- Biosensors	Subjective
♦ Estimation of metabolic activity	- ATP assay	Subjective
♦ Antibody/antigen reaction	- Latex agglutination - Immuno-magnetic separation - Enzyme linked immunosorbent assays (ELISA)	Very simple tests
♦ DNA-based methods	- Colony hybridization - Single phase hybridization assay - Polymerase chain Reaction (PCR) - Oligonucleotide arrays (DNA-disc)	Widely used Increasingly used Has lot of potential

1975), PCR (Saiki *et al.*, 1988) and different antibody-based techniques such as immunomagnetic separation (IMS) (Olsvik *et al.*, 1994). In general, molecular methods such as DNA hybridization and PCR have significantly shortened the time required for analysis, besides being highly sensitive and accurate (Singh and Akhtar, 2001). These nucleic acid-based methods have been the focus of a number of recent reviews (Sall *et al.*, 1988; Walker and Dougan, 1989; Hill and Keasler, 1991; Jones, 1991; Wolcott, 1991a; Olsen *et al.*, 1995; Hill, 1996). The reviews unanimously praise the potential of these techniques to overcome some of the inherent problems in detection and isolation of bacterial pathogens in food. Olsen (2000) has reviewed the DNA-based methods for the detection of food-borne bacterial pathogens and had put forth the advantages of DNA-based methods over the traditional culture-based methods (Table 3). The potential of DNA-based methods for the detection of microbial resistance to antimicrobial agents has been reviewed by Fluit *et al.* (2001). On this backdrop, the potential of molecular methods, like nucleic acid hybridization and PCR, for seafood quality assurance are reviewed.

2.3.1. Nucleic acid hybridization

Nucleic acid hybridization refers to the formation of a double-stranded structure from two single-stranded homologous nucleic acid molecules. A high degree of complementarity required to produce a stable double-stranded molecule is the key factor for its specificity (Hanes and Higgins, 1985). For the purpose of diagnosis, one should have a unique and specific nucleotide strand called "DNA probe", which can be used for hybridization with the test sample. DNA probes or nucleic acid probes are segments of single-stranded DNA or RNA, varying in length between twenty and thousands bases that have been labeled with enzymes, antigenic substances, chemiluminescent moieties or radioisotopes and can bind with high specificity to complementary sequences of nucleic acids. When the probe DNA comes in contact with a complementary single-stranded DNA or RNA of any organism (i.e., the target molecule), it forms a stable double-stranded molecule, which can then be observed by various means. The radioactive signal of ^{32}P can be read by exposing to X-ray films, and the colour signals resulting due to biotin or digoxigenin or any other chemiluminescent substrate can be read by naked eye. Nucleic acid hybridization can be performed in three different ways such as Southern

Table 3. Advantages of DNA-based methods for the detection and characterisation of food-borne bacterial pathogens over traditional culture based methods (Olsen, 2000)

Application	DNA-based method	Culture-based method
♦ Direct detection of pathogens in food	Does not (in theory) require cultivation	Requires cultivation in enrichment and selective media
♦ Culture confirmation on enrichment broths	Rapid and sensitive	Series of biochemical tests are essential and time consuming
	Not affected by high concentrations of indigenous microflora	
	Automated detection possible	Automated detection not possible
♦ Characterization of pathogenic microbe from food samples	Can separate virulent from non-virulent microbes by targeting virulence genes	Separation of virulent strain of microbe requires lengthy serological characterisation after usual microbiological and biochemical analysis
	Simultaneous detection of several pathogens is possible by multiplex PCR	Multiplex characterization not possible
♦ Other application	Can specifically detect genetically modified organisms (GMOs)	May not distinguish GMO or mutated organisms

hybridization, colony hybridization and liquid hybridization (Datta, 1990). Southern hybridization (Southern, 1975) involves the transfer of DNA bands separated by gel electrophoresis on to nitrocellulose or nylon membrane under vacuum or by capillary action to produce faithful replica (Gibbs and Kazazian, 1989). The DNA bands blotted on the membrane are denatured, baked or exposed to UV light, and hybridized with the probe, whereas, in colony hybridization (Grundstein and Hogness, 1975) bacterial colonies or cultures are directly grown on a suitable membrane (like nitrocellulose, nylon or filter paper) or transferred from an agar surface by colony lift. The colonies on the membrane are then lysed, DNA denatured and hybridized (Datta *et al.*, 1988). Liquid hybridization (Enns, 1987; Klinger *et al.*, 1988) is conducted by lysing cells in liquid, denaturing DNA/RNA and then hybridizing with a radio-labeled probe. Hybridized molecules (target::probe) are captured and separated from the unhybridized probe, and the signal is quantified.

Selection of a proper probe is the most vital aspect in nucleic acid hybridization or DNA probe technology. Ideally, a DNA probe should be specific, highly sensitive, easy to prepare and easy to use. While there are many different approaches for the preparation of an appropriate probe, the selection of one is subjected to the type of information required. One of the direct approaches is to target specific genes of organisms. For a pathogen, it is advantageous to use genes involved in the virulence mechanism (Karunasagar and Karunasagar, 1999) because, different pathogens use different strategies and toxins to establish infection, and the genes controlling these functions will be specific. Similarly, to identify a particular taxonomic group, the probe must be directed towards a gene or region of the gene that is conserved throughout a particular species or genus. Identification of genes which code for 16S rRNA can be very useful for the development of probes, because 16S rRNA possesses both highly conserved and variable regions. It is possible to select regions to tailor probes of required specificity (Klinger *et al.*, 1988; Romaniuk and Trust, 1989).

Nucleic acid hybridization methods have been developed for a number of food-borne bacterial pathogens (Tenover, 1988). Some of the widely used probes for the detection of food-borne bacterial pathogens have been summarized in Table 4. Nucleic acid hybridization methods for the detection of toxigenic *E. coli* (Hill and

Table 4. DNA probes for detection of food-borne bacterial pathogens

Target pathogen	Probe* derived from	Reference
Campylobacter jejuni	a: Cryptic DNA b: 16S rRNA sequence	Taylor and Hiratsuka (1990) Romaniuk and Trust (1989)
Clostridium perfringens	b: Phospholipase C	Van Damme-Johgsten *et al.* (1990)
E. coli ETEC	a: LT and ST genes b: LT and ST gene	Moseley *et al.* (1980) Olsvik *et al.* (1991)
E. coli VTEC	a: Verotoxin-genes b: Verotoxin gene	Thomas *et al.*, (1991) Thomas *et al.* (1991)
E. coli EPEC	a: Plasmid-borne adhesion factor b: Adhesion factor	Nataro *et al.* (1985) Jerse *et al.* (1990)
Listeria monocytogenes	a: Haemolysin gene b: Invasion associated gene	Chenevert *et al.* (1989) Bohnert *et al.* (1992)
Salmonella spp.	a: Cryptic DNA b: Cryptic DNA a: Chromosome	Olsen *et al.* (1991) Olsen *et al.* (1995) Fitts *et al.* (1983)
Staphylococcus aureus	b. Enterotoxin A, B, C, D	Ewald *et al.* (1990)
Vibrio cholerae	a: Cholera toxin A-subunit b: Cholera toxin A-subunit	Wachsmuth *et al.* (1993) Wright *et al.* (1992)
V. parahaemolyticus	a: Thermostable direct haemolysin	Nishibuchi *et al.* (1986)
V. vulnificus	a: Cytolysin b: Cytolysin	Morris *et al.* (1984) Wright *et al.* (1993)
Yersinia enterocolitica	a: Virulence plasmid b: Invasion-associated gene	Hill *et al.* (1985) Feng (1992)

* a : polynucleotide probe; b : oligonucleotide probe

Payne, 1984), *Salmonella* spp. (Flowers *et al.*, 1987a) and *Listeria* spp. (Andrews, 1995) have been approved by the Association of Official Analytical Chemists (AOAC). For the detection of *Salmonella* spp., several restriction endonuclease fragments of chromosomal DNA selected randomly are used as probes. Some of these molecules were found to be specific for *Salmonella* spp. Commercial Gene-Trak Systems *Salmonella* Assay uses a fragment of about 2000 bases from *S. typhimurium* genomic DNA, which is propagated in bacteriophase fd (Fitts, 1985; Curiale *et al.*, 1986). A colorimetric DNA hybridization method for the detection of *Salmonella* spp. has also been described (Andrews, 1995; Mazola 2000). In this assay, *Salmonella* ribosomal RNA-specific DNA probe is used.

Nucleic acid probe-based assays would be very useful for the detection of pathogenic *Vibrio* spp. associated with seafood. In the case of *V. cholerae*, the cholera enterotoxin is the major virulence factor, encoded by *ctx*A and *ctx*B genes (Mekalanos *et al.*, 1983). Two sequences based on the structural gene of A subunit have been used as probes for the detection of *V. cholerae* (Hill *et al.*, 1992). An oligonucleotide probe based on the sequence of the gene encoding thermostable direct haemolysin (*tdh* gene) has been described for the detection V. *parahaemolyticus* (Nishibuchi *et al.*, 1986). Similarly, a cytotoxin/haemolysin has been implicated as a virulence factor of *V. vulnificus*, another important pathogen associated with seafood (Gray and Kreger, 1985). An oligonucleotide probe based on the sequence of the gene encoding cytotoxin has been found to be specific for environmental strains of *V. vulnificus* (Hill *et al.*, 1992). Wright *et al.* (1993) noted that the alkaline phosphatase-labeled oligonucleotide probe would be very useful for the rapid identification of *V. vulnificus* on non-selective media. Dalsgaard *et al.* (1996) reported that only one-third of strains identified as *V. vulnificus* using an oligonucleotide probe reached the identification threshold when the biochemical kit API 29E was used. This suggests that the nucleic acid probe would be very useful for detecting biochemically atypical strains of *V. vulnificus.*

The nucleic acid hybridization technology provides more sensitivity, specificity and rapidity than conventional methods. The DNA-based procedures have specific advantages over the culture-based method, especially while dealing with atypical phenotypic characteristics (Sall *et al.*, 1988). Further, as a means of culture

confirmation test, colony hybridization offers several advantages, as it is possible to screen a large number of enrichment broths on one filter and process many filters in the same hybridization solution (Kaysner *et al.*, 1988), besides, it is very cost effective to screen out negative broths in this method. Colony hybridization assays have been demonstrated to be equally good or superior to biochemical and serological methods in terms of sensitivity and specificity for the confirmation of *Salmonella* spp. and *L. monocytogenes* positive cultures (Flowers *et al.*, 1987b; Kim *et al.*, 1991).

2.3.2. Polymerase chain reaction (PCR)-based methods

PCR is a rapid procedure for *in-vitro* enzymatic amplification of specific segments of DNA (Saiki *et al.*, 1985, 1988; Mullis and Faloona, 1987). PCR-based techniques are used increasingly these days in research in food microbiology, because they offer specific and sensitive detection of pathogens and like nucleic acid probe hybridization, PCR can even separate virulent bacteria from avirulent members of the same species (Trost *et al.*, 1993). Although the basic ingredients for *in-vitro* nucleic acid amplification method were described in 1971, in which the extensive synthesis of a tRNA gene by primer directed DNA synthesis was postulated, this work failed to result in an exponential amplification process, which is the hallmark of PCR. Kary Mullis, and Saiki and co-workers developed the concept of PCR (Mullis and Faloona, 1987) during the early 1980s. The development of nucleotide sequencing methods, storage of this information in a computer researchable database, invention of automated oligonucleotide synthesis methods and the discovery of thermostable DNA polymerase (Saiki *et al.*, 1988) have all contributed to the rapid implementation and widespread use that PCR enjoys today.

In PCR, the region of DNA to be amplified (the target DNA) is defined by a pair of primers, the 3′ end of which serves as the initiation point for DNA replication. PCR is basically a cyclic reaction, involving three temperature schedules namely, denaturation, annealing and extension. In the first cycle, the template DNA strands are separated (denatured) and then, cooled in the presence of a billion-fold excess of primers, the four dNTPs and a thermally stable DNA polymerase. When the strands are cooled, the primer anneals at the complementary sequence of target DNA forming target DNA-primer hybrid complex. DNA replication is then initiated in

the presence of appropriate salts and pH, and at the end of the extension step two copies of target region are found. The sequence of the primer and the temperature of the annealing step are critical in controlling the accuracy (stringency) of the amplification process. In the second cycle, the two copies of the target region serve as the template and results in four copies of the target DNA. The thermo-cycling is usually repeated for 25 to 40 cycles, doubling the number of amplicon in each cycle, thus, resulting in millions of copies of the target DNA at the end of all the cycles (Gibbs and Kazazian, 1989).

Since its introduction in the mid 1980s, PCR technology has proved to be an invaluable method for the detection of pathogens in food. Beyond simple detection of a pathogen, the PCR technology can potentially provide genetic information relating to the virulence, chemosensitivity and epidemiological spread of a pathogen (Martin, 1994). PCR has many advantages over methods using nucleic acid probes. PCR reactions are very fast and sensitive enough to detect as low as less than 10 target molecules compared to the 10^5-10^6 copies of target sequence required to yield a clear and positive result in case of nucleic acid probe hybridization (Hill *et al.*, 2001). The sensitivity of PCR reaction can be further increased by targeting molecules that are present in multiple copies in a single cell such as rRNA sequence (Pickup, 1991). The other advantage of PCR over culture-based methods is that the PCR method can be suitably used for certain types of microorganisms that cannot be grown in culture media.

2.3.2.1. Application of PCR for the detection of seafood pathogens

2.3.2.1. 1. *Salmonella* spp.

Salmonella spp. are recognized as major zoonotic pathogens for animals and human (Humphrey, 2000). In many countries, *Salmonella* spp. are the leading cause of food-borne outbreaks and infections (Lacey, 1993; Wallace *et al.*, 2000; Tirado and Schmidt, 2001). In order to minimize the risk of infection for consumers, microbiological control of the food chain is being increasingly applied. Thus, the availability of reliable, rapid and internationally accepted test systems for the determination of the presence or absence of food-borne pathogens has become increasingly important for the agricultural and food industries.

Conventional culture-based method is still the most commonly used method for the detection of *Salmonella* spp. in foods or clinical samples, which may require 5-7 days to confirm the presence of the pathogen (Andrews, 1985; Flowers *et al.*, 1992; Miescier *et al.*, 1992). These culture-based methods are too time consuming and cumbersome to be used in legislative and food production controls. Moreover, if the suspected samples are not processed immediately, microbial contaminants may become unculturable and undetectable by standard microbiological methods (D'Aoust, 1989; Hill *et al.*, 1991). A wide range of alternative approaches, as already reviewed by Blackburn (1993) and as described in Table 2 such as enzymeimmunoassays (Becker *et al.*, 1998; Sherrod *et al.*, 1994), DNA-DNA hybridization (Curiale *et al.*, 1990; Rose *et al.*, 1991), antibody-based methods (Anderson and Hartman, 1985; Swaminathan *et al.*, 1985; Flowers *et al.*, 1992), HGMF methods (Entis and Boleszczuk, 1991) and automated conductance (Gibson *et al.*, 1992) have been developed. Many of these methods only replace the use of plating media and suffer from a lack of specificity and result in high incidence of false positives (Chen *et al.*, 1997b). DNA probe technology, particularly DNA-DNA hybridization, has been widely used to detect *Salmonella* spp. in contaminated food (Fitts, 1985; Gopo *et al.*, 1988; Tsen *et al.*, 1991; Aabo *et al.*, 1992; Papapetropoulou and Moschopoulos, 1996). The detection of *Salmonella* spp. by DNA-DNA hybridization methods is relatively rapid compared to the conventional microbiological methods, but hybridization requires the presence of at least 10^3-10^4 cells of target microbial pathogens in the sample to elicit a positive signal (Steffan and Atlas, 1991; Bej *et al.*, 1991a; Wolcott, 1991b; Bej and Mahbubani, 1992; Bej, 1993). Several reports suggest that the consumption of contaminated food containing <10 *Salmonella* cells can cause salmonellosis (Blaser and Newman, 1982; Greenwood and Hooper, 1983; D'Aoust 1985). Without the pre-enrichment of the suspected sample, DNA-DNA hybridization method does not provide the required sensitivity to detect this pathogen. There is therefore an urgent need for a rapid, comprehensive method to detect *Salmonella* with required specificity and sensitivity.

PCR represents a major advancement in the development of rapid methods and has increasingly been used in research for rapid, sensitive and specific detection of food-borne pathogens (Harris and Griffiths, 1992; Hill, 1996; Malorny *et al.*, 2003). Several workers worldwide have come up with different PCR primers and

protocols for the specific detection of *Salmonella* spp. Among these, many PCR protocols have targeted specific gene sequences such as *ori*C, which encodes the origin chromosomal replication (Widjojoatmodjo *et al.*, 1991); *sef*A, which codes for the SEF14 fimbrial antigen (Woodward and Kirwan, 1996); *omp*C that codes for outer membrane protein fraction (Kwang *et al.*, 1996); *agf*A, the structural gene for thin aggregative fimbriae (Doran *et al.*, 1992, 1993); *inv*A, which enables the bacteria to invade cells (Rahn *et al.*, 1992; Stone *et al.*, 1995, 1998; Chen *et al.*, 1997a; Cocolin *et al.*, 1998; Abouzeed *et al.*, 2000; Feder *et al.*, 2001; Carli *et al.*, 2001); *hns* gene that codes for a DNA binding protein (Jones *et al.*, 1993); 16S DNA (Lin and Tsen, 1996) and other *Salmonella* virulence genes (Chiu and Ou, 1996; Cohen *et al.*, 1996; Swamy *et al.*, 1996), while some other PCR protocol targeted random sequences (Aabo *et al.*, 1993, Tsen *et al.*, 1994). PCR protocols have also been developed to specifically detect a particular serotype such as *S. enteritidis* targeting the virulence plasmid profile (Wood *et al.*, 1994; Doran *et al.*, 1996); *S. typhi*, targeting *Via*B gene (Hasimoto *et al.*, 1995) and flagellin gene (Song *et al.*, 1993); *S. typhimurium* targeting *mdh* gene (Lin and Tsen, 1999) and *fliC* gene (Joys, 1985; Soumet *et al.*, 1999) and so on. However, the uneven distribution of several of these genetic markers within this bacterial population makes tests based on them less useful in screening for *Salmonella* spp. (Swamy *et al.*, 1996; Baumler *et al.*, 1997). Only PCR tests involving the invasion gene sequence(s) have proved successful as universal tests for *Salmonella* spp. because of the uniform distribution of the *inv* genes among various *S. enterica* serotypes (Galan and Curtiss, 1991; Swamy *et al.*, 1996). Therefore, as already mentioned, there are a number of PCR tests based on the *Salmonella inv*A DNA sequence, a type III secretion pathway component, that is strongly conserved but unique to *Salmonella* spp. (Galan and Curtiss, 1991, Galan *et al.*, 1992). The *inv*A gene seems to be present in all serovars of *S. enterica* (Chiu and Ou, 1996; Malorny *et al.*, 2003).

2.3.2.1.2. ***Vibrio cholerae***

Vibrio spp. are perhaps the most important among all the pathogens of significance to the seafood industry. Several instances of outbreaks of gastrointestinal illnesses due to the consumption of seafood contaminated with vibrios have been reported (Janda *et al.*, 1988, West, 1993). The severity of infection

and incidence of the epidemic due to *V. cholerae* has already been discussed. The findings that many environmental strains of *V. cholerae* O1 could be non-toxigenic (Sakazaki and Donovan, 1984) and that some non-pathogenic environmental vibrios could cross-react with polyvalent O1 antiserum (Shimada *et al.*, 1987) show that conventional biochemical identification and serotyping are inadequate to determine the *V. cholerae* hazard in seafoods. The emergence of *V. cholerae* O139 as a toxigenic epidemic strain in 1992 (Morris and CLTF, 1994) has added a new dimension to the problem of determining seafood safety. The discovery by Colwell *et al.* (1985) that some *V. cholerae* O1 present in environmental samples may not grow on laboratory media routinely used for isolation of this organism has revolutionized the concepts in the ecology of this organism. It has further been demonstrated that such "viable but non-culturable (VBNC)" forms can produce clinical symptoms of cholera in human volunteers confirming that non-culturable *V. cholerae* maintain pathogenic potential (Colwell and Huq, 1994). Traditional microbiological culture based method is the conventional method in vogue to detect the presence of *V. cholerae* in fish and fisheries products, which is very time consuming and takes minimum 3-4 days. Further, these methods are inadequate to address the intriguing aspects of *V. cholerae*, its virulence and the associated public health hazard.

PCR is being widely used in the identification of food-borne pathogens and it has become an invaluable diagnostic tool in clinical microbiology (Kapperud *et al.*, 1993). Since cholera toxin encoded by the *ctx* gene is the most important virulence factor of *V. cholerae* (Finkelstien, 1988), PCR amplification targeting this gene can be suitably applied for differentiating virulent strains of *V. cholerae* from avirulent ones in foods including seafood (Kock *et al.*, 1993). Fields *et al.* (1992) developed a PCR-based method for the detection of *V. cholerae* in clinical samples based on the amplification of *ctx* gene. Karunasagar *et al.* (1995, 1997b) could detect both *V. cholerae* O1 and O139 contamination of seafood using *ctx* gene-based PCR and had determined its sensitivity. PCR-based techniques have also been applied to generate DNA fingerprint of *V. cholerae* O1, O139 and non-O1 by Riviera *et al.* (1995). They studied the polymorphism in the ERIC sequence in *V. cholerae* strains and a found common fingerprint for toxigenic *V. cholerae* O1 and O139, and a different fingerprint for non-toxigenic *V. cholerae* O1.

2.3.2.1.3. *Vibrio parahaemolyticus*

Among the non-cholera vibrios causing gastrointestinal infections, *V. parahaemolyticus* appears to be the most important one. Though the organism has been reported worldwide as a constituent of indigenous flora in marine and coastal environments, only a small portion (1-2%) of the environmental strains is virulent. Conventional culture-based methods rely on enrichment, selective plating and biochemical identification of pure culture and the Kanagawa reaction can be detected using Wagatşuma agar, an ELISA method or by a DNA probe hybridization method. These are cumbersome and time consuming. Besides, there are lots of problems associated with selective enrichment as discussed by Karunasagar *et al.* (1986). Further, there is neither a suitable culture medium nor a commercially available immunological kit for the detection of Kanagawa-negative strains of *V. parahaemolyticus*, which exhibit virulence by producing TRH. The virulence factors TDH and TRH are coded by the *tdh* and *trh* genes, respectively, and the strains incapable of producing haemolysin lack these genes (Nishibuchi and Kaper, 1995). The clinical significance of the strains carrying the *tdh* gene, the *trh* gene or both was established by molecular epidemiological studies (Nishibuchi *et al.*, 1985; Shirai *et al.*, 1990; Kishishita *et al.*, 1992). Accordingly, PCR-based methods to detect the presence of *tdh* and *trh* genes have been developed as an effective method for the high-sensitive detection of the virulent strains of *V. parahaemolyticus* (Tada *et al.*, 1992; Lee and Pan, 1993; Karunasagar *et al.*, 1996). The detection of *V. parahaemolyticus* irrespective of TDH production in seafood has been described by Karunasagar *et al.* (1997a), which would be useful for the detection of contamination in a seafood quality control laboratory. Dileep *et al.* (2003) have used the PCR method targeted to a regulatory gene, *tox*R, which is present in all the strains of *V. parahaemolyticus*, irrespective of their ability to produce TDH or TRH (Kim *et al.*, 1999), as a method for identification at the species level with special emphasis to evaluate its ability to identify biochemically-atypical strains.

2.3.2.1.4. *Vibrio vulnificus*

V. vulnificus is a significant pathogen generally associated with septicaemias and the infection generally occurs through the intestinal route. Methods for the detection of *V. vulnificus* in seafood are similar to those for *V. parahae-*

molyticus involving enrichment, selective plating and biochemical identification of pure cultures, which are labour intensive and time consuming. Infection due to *V. vulnificus* can cause septicaemia, the mortality from which is quite high (> 50%) and death may occur as soon as 1 to 2 days after the first sign of illness (Tacket *et al.*, 1984; Oliver and Kaper, 1997). Therefore, rapid identification of *V. vulnificus* in natural samples is essential to reduce the potential fatal effects. A PCR-based assay targeting the cytolysin gene has been developed (Hill *et al.*, 1991), but the application of this assay for the detection of contamination of oysters has been a problem due to a PCR-inhibiting substance in oyster. Coleman *et al.* (1996) reported a protocol for DNA extraction and PCR amplification, which could detect the *V. vulnificus* naturally present in oysters. Lee *et al.* (1999) have developed a two-stage nested PCR method for direct detection of *V. vulnificus* in natural samples. Brauns *et al.* (1991) have reported the use of PCR for the detection of culturable and nonculturable *V. vulnificus* cells.

2.3.2.1.5. *Listeria monocytogenes*

L. monocytogenes hazard in seafood has become the focus of attention recently following sporadic cases of seafood-borne listeriosis (Lenon *et al.*, 1984; Facinelli *et al.*, 1989; Fredriksen, 1991; Baker *et al.*, 1993; Riedo *et al.*, 1994). The application of PCR for the rapid detection of *L. monocytogenes* in various food products has been investigated by a number of workers (Hill, 1996). The *hly* gene encoding the production of listeriolysin, a major virulence factor of *L. monocytogenes*, has been the popular target for PCR amplification. However, Bubert *et al.* (1992) showed that the conserved and variable regions in the *iap* gene, encoding the production of invasion-associated protein, could be useful targets for designing genus-specific and species-specific PCR amplifications. Jeyasekaran *et al.* (1996) utilized this PCR for the differentiation of *L. monocytogenes* and *L. innocua* isolated from tropical seafoods. Border *et al.* (1990) and Wang *et al.* (1992b) used the information on nucleotide sequence of *Listeria* spp. 16s rRNA to create genus-specific and species-specific primers.

2.3.2.1.6. *Escherichia coli*

E. coli is the most common aerobic organism with ubiquitous distribution, generally found in the gastrointestinal tract of man and therefore, considered as an

indicator of faecal contamination. However, during recent years, *E. coli* is also being regarded as a specific pathogen involved in intestinal and extra-intestinal infections. Different strains of *E. coli* are responsible for different diseases and the food-processing industry is mainly interested in pathogenic *E. coli* strains, which may be infective through the gastrointestinal route. Only a few strains of *E. coli* are pathogenic and a food quality control laboratory should be able to differentiate pathogenic strains from the non-pathogenic ones. Traditionally, strain differentiation was being done through serological reactions based on antigenic differences in highly variable bacterial surface molecules. However, with the advent of molecular biology, classification based on virulence factor (virotyping) is becoming popular.

The detection and enumeration of pathogenic *E. coli* in foods is quite difficult. In contaminated foods, the pathogenic *E. coli* may be found in small numbers compared to the background flora. This makes the recovery from the complex food matrix difficult. Some pathogenic forms do not have typical characteristics of *E. coli* and may be anaerogenic and may either fail to, or be late to, ferment lactose. Others are inhibited by bile salts at elevated temperature. For example, EHEC (O157:H7) strains grow poorly or not at all at 44.0-44.5°C, do not produce β-glucuronidase (MUG – negative) and fail to ferment sorbitol within 24 hours (Doyle and Schoeni, 1984; Doyle and Padhye, 1989). All these anomalies added to the problem in recovery of viable isolates in pure culture make the detection of pathogenic *E. coli* from food quite difficult. Recently developed DNA-based methods circumvent these problems to a great extent. PCR-based methods have been applied for the detection of pathogenic *E. coli* in foods. ETEC could be detected by PCR in surface water and in soft cheese without the need for a growth step (Furrer *et al.*, 1990; Meyer *et al.*, 1991). The detection of EIEC by amplifying gene encoding invasion factor has been reported by Keasler and Hill (1992). PCR-based assay targeting Shiga-like toxin (SLT) genes that code for Shiga-like toxin has been found useful for the detection and identification of EHEC (Gannon *et al.*, 1992). Lang *et al.* (1994) described a multiplex PCR for the detection of heat-labile toxin gene (LT), and SLT I and II genes in *E. coli* isolated from natural waters.

2.3.2.1. Critical aspects to address before use of PCR for routine analysis

PCR represents a simple procedure with both high sensitivity and specificity for immediate detection and identification of specific pathogens from

various food matrices. Earlier methodology had been standardized to get the result of PCR reaction within one day (Soumet *et al.*, 1997; Bailey, 1998; Gouws *et al.*, 1998; Fach *et al.*, 1999). Ferretti *et al.* (2001) came up with the development of a 12-hour PCR-based method for the detection of *Salmonella* spp. from food. On the other hand, very reliable PCR methods have been developed for the relevant pathogenic bacteria, but still the use in routine analysis is modest. This is not due to the lack of good PCR methods, but due to the lack of methods that can release the pathogenic bacteria from the complex food matrix into a suitable small test volume (Olsen, 2000). Similarly, there are several other practical problems to be addressed before employing the methodology for routine analysis.

Direct detection of pathogens in food samples is a major goal for PCR technology, but this is difficult to achieve. The PCR method can produce a positive reaction from nucleic acid obtained from one organism, but the small volume of 5–10 µl culture of sample that can be used in one reaction sets the detection limit as high as approximately 10^3 cells per milliliter (Olsen, 2000). Direct detection by PCR has, therefore, been performed in very few cases (Wang *et al.*, 1992a; Kock *et al.*, 1993). However, retrial of some of these experiments failed to confirm the result.

The recovery of amplifiable DNA from complex food matrices is a great challenge. Often, food contains substances that inhibit PCR reaction (Lantz *et al.*, 1994; Romero and Lopez-Goni, 1999) and partial or total inhibition may also be encountered due to compounds in selective media or chemicals from DNA extraction procedures (Wernars *et al.*, 1991; Rossen *et al.*, 1992). Kock *et al.* (1993) used primers to amplify a 779 bp fragment of *ctx* gene to detect *V. cholerae* in various food samples by direct PCR. They found positive results with seeded fruits and vegetables, but seeded shellfish homogenates (10% blends of oyster or shrimp) often inhibited the PCR reaction. The inhibition, however, could be removed by reducing the proportion of oyster or shrimp homogenates to 1%. Similarly, in a study on detection of *Listeria* spp. in cheese, Wernars *et al.* (1991) noted that the efficiency of PCR amplification was dependant on the type of cheese being analysed. The development of extraction methods to concentrate the target organism in a small volume while reducing inhibitory substances is, therefore, a high priority. Rossen *et al.* (1992) proposed dilution of sample as one of the simplest approaches to remove inhibitory substances, but this procedure lowers the sensitivity of detection. In an

attempt to reduce PCR-interfering substances, Fluit *et al.* (1993a, 1993b) used magnetic-immuno-PCR to concentrate *Listeria* cells out of the enrichment cultures. Similarly, Widjojoatmodjo *et al.* (1995) used *Salmonella*-specific antibody-coated magnetic particles to concentrate bacteria in a magnetic immuno-PCR assay (MIPA). The sensitivity of immuno-magnetic separation (IMS)-PCR could be improved by incorporating a brief pre-enrichment step of 6 to 24 hours (Fluit *et al.*, 1993b; Rasmussen *et al.*, 1995).

One of the serious drawbacks with the PCR methodology is that PCR fails to distinguish live and dead cells. Thus, a positive result obtained by using PCR on particularly processed food samples does not conclusively demonstrate that viable cells are present. To counter this problem, it is advised to draw samples at two time points several hours apart and check for the difference in the yield of the PCR product. Alternatively, an enrichment step could be included to avoid amplification of DNA from non-viable cells. The alternative methods that have been used for the purpose include reverse transcriptase PCR (RT-PCR) (Klein and Juneja, 1997; Vaitilingom *et al.*, 1998; Sheridan *et al.*, 1998); nucleic acid sequence-based amplification (NASBA) (Malek *et al.*, 1994; Birch *et al.*, 2001), etc. Mukhopadhyay and Mukhopadhyay (2002) have devised DNAase-treated DNA-PCR strategy for the elimination of exogenous DNA during PCR amplification.

Sometimes, the sensitiveness of PCR may become the serious drawback for its routine use. Small concentrations of contaminating DNA may result from cross-contamination due to improper laboratory handling, particularly during repeated amplification of same-target sequence (Persing, 1991) and may yield false positive results. Following specific precautions, the use of disposable materials, positive displacement pipettes, and the analysis of amplification products in an area separate from that of pre-amplification and sample preparation site are some of the helpful tips to minimize contamination (Kwok and Higuchi, 1989).

The main advantage of PCR is the sensitivity and specificity of the assay. Small concentrations of target DNA, corresponding to low numbers of bacteria cells, can be detected in a short time. The sensitivity can be further increased by the use of ribosomal RNA (rRNA) as the target sequence (Pickup, 1991). Because of its high copy number in cells, rRNA is naturally amplified

(>10^4 copies of target sequence in rRNA/cell compared to 1 or 2 copies for rDNA) and contains sequences that are specific for certain bacterial groups (Hill and Keasler, 1991; Wolcott, 1991a). While using targets other than rRNA, Lee *et al.* (1999) and Liu *et al.* (2002) performed two-stage nested-PCR using a pair of primers internal to the primary PCR product to increase sensitivity besides verifying the specificity of the first-round product. Similarly, selection of primers that amplify sequences of DNA from genes encoding virulence factor or toxins allow identification of only those organisms that are potentially pathogenic. Several pathogens can be detected simultaneously in one step by multiplex PCR. Such multiplex PCR methods of relevance to food microbiology have been used to detect variants of enteropathogenic *E. coli* (Franck *et al.*, 1998), to detect three pathogenic *Vibrio* spp., *V. cholerae, V. parahaemolyticus* and *V. vulnificus* (Trost *et al.*, 1993), to perform species differentiation within *Listeria* spp. (Wesley *et al.*, 2002), and to simultaneously detect *E. coli*, *Salmonella typhimurium* and three pathogenic *Vibrio spp.* (Brasher *et al.*, 1998). Such methods show the potential for practical everyday use of PCR methods in food microbiology.

To sum up, PCR methods are well developed and when applied as culture confirmation tests, they are reliable, fast and sensitive. The ability to concentrate the target organisms from food without co-concentration of inhibitory substances still needs to be improved before the detection directly from processed food becomes a realistic goal.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Samples and Sampling Sites

Three stations comprising two fish landing centres (Versova fish landing centre and Sasoon Dock fish landing centre) and one fish market (Four Bungalows fish market) were selected as the representative sampling sites to assess the prevailing hygienic condition of fish and fish handling environments of Mumbai. The samples for the study comprised fish, shrimp, water and swab from fish contact surfaces. Besides these, processed shrimp samples were also collected from a fish processing plant (Hindustan Lever Limited, Rabale, Navi Mumbai) involved in export of fish and fishery products. The samplings were done on a monthly interval during September to December (post-monsoon season) and all the analysis were carried out in the fish molecular biology laboratory of the institute.

In total eighty four samples were collected as per the details given in Table 5. It was observed that, Versova fish-landing centre is located at the mouth of Versova Creek, which brings sewage and polluted water from Mumbai suburb to the sea. Similarly, at the time of sampling the Sasoon Dock fish landing centre had high tide at its peak. The designated fish market is a regular market near the residential place of Four Bungalows area and receives fish from both of these landing centres.

3.2. Sampling Procedure

Fish and shrimp samples were collected randomly in aseptic condition with utmost precautions to prevent cross contamination. Polyethylene zip bags sterilized by overnight fumigation with 40% formaldehyde and $KMnO_4$ (at 1:1 ratio) were used for carrying of fish, shrimp and swab samples. Similarly, autoclaved polypropylene bottles were used for carrying water samples from the sampling site to the laboratory.

One sampling unit consisted of a random mixture of approximately 100 g of fish and/or shrimp from three different containers/stacking areas. Approximately 100 ml water was collected from the designated area at random and regarded as one water sample. Samplings from fish contact surfaces were made using sterile cotton swab (HiMedia Laboratories, Mumbai). Swabs from three different sorting surfaces/containers/boat decks/dressing surfaces each on an area of 25 cm^2 formed one sampling unit. Samples were brought to the laboratory in insulated containers under ice and processed within six hours of sampling.

Table 5. Descriptions of the samples from fish and fish handling environments of Mumbai

Sampling station	Sample type	Number of samples	Sample description
Sasoon Dock fish landing Centre	Marine fish	3	a) *Herpedon nehereus* b) *Coilia dussumarie* c) *Trichiurus* spp.
	Shrimp	3	a) *Metapenaeus monocerus* b) *Parapenaeopsis stylifera* c) *Metapenaeus dobsonii*
	Swab	3	a) Fish sorting platform surface b) Container to carry fish c) Boat deck
	Water	3	a) Water from fish sorting/auction surface b) Creek water on East side of harbour c) Creek water on West side of harbour
Versova fish landing centre	Marine fish	9	a) *Herpedon nehereus* b) *Coilia dussumarie* c) *Trichiurus* spp.
	Shrimp	9	a) *Metapenaeus monocerus* b) *Parapenaeopsis stylifera* c) *Metapenaeus dobsonii*
	Swab	6	a) Container to carry fish b) Boat deck
	Water	6	a) Fish washing water b) Creek water
Four Bungalows fish market	Marine fish	9	a) *Herpedon nehereus* b) *Coilia dussumarie* c) *Trichiurus* spp.
	Freshwater fish	9	*a) Catla catla* *b) Labeo rohita* c) *Clarius batrachus*
	Shrimp	9	a) *Metapenaeus monocerus* b) *Parapenaeopsis stylifera* c) *Metapenaeus dobsonii*
	Swab	6	a) Fish dressing platform b) Containers carrying fish
	Water	6	a) Marine fish washing water b) Freshwater fish washing water
Fish processing Plant	Processed product, ready for export	3	a) Peeled undeveined raw IQF shrimp b) Peeled deveined cooked IQF shrimp c) Peeled undeveined blanched IQF shrimp
Total		84	

3.3. Bacteriological Analysis

The bacteriological analysis involved determination of total bacterial load and screening for the presence of human pathogenic organisms, taking *Salmonella* spp. as the representative group. All the bacteriological analyses were performed as per the standard methods of AOAC adopted by the United States Food and Drug Administration (USFDA) as outlined in Bacteriological Analytical Manual *Online* (FDA, 2001).

3.3.1. Aerobic plate count

Aerobic plate count (APC) or total plate count (TPC) was done by the pour plating method following USFDA guideline (Maturin and Peeler, 2001). Twenty five gramme each of fish and shrimp sample was homogenized in a small amount of total 225 ml sterile physiological saline (0.85% NaCl) using pestle and mortar under aseptic condition. Similarly, 25 ml of water sample was poured into 225 ml physiological saline. Swab samples drawn in triplicate of 25 cm^2 each were individually put into 10 ml saline and later pooled to get 30 ml saline suspension for 75 cm^2 and considered as neat in APC enumeration. After appropriate serial dilution in the same diluent, they were plated in duplicate on plate count agar (HiMedia Laboratories, Mumbai). The plates were incubated at 37^0C for 48 hours. Plates having colonies in the range of 25 - 250 were selected for counting the number of colonies and enumeration was done as per USFDA guidelines.

Enumeration was done by the following formula

$$N = \frac{\Sigma C}{[(1 \times n_1) + (0.1 \times n_2)]} \times d$$

Where

N = Number of colonies per ml or g of the product

ΣC = Sum of all colonies on all plates counted

n_1 = Number of plates in first dilution counted

n_2 = Number of plates in second dilution counted

d = Dilutions from which the first counts were obtained

Reporting: Finally the APC was reported as colony forming units (CFU) per gramme or millilitre. To get APC (cfu cm^{-2}) for swab samples the value obtained by the above formula was multiplied by a factor of 30/75, so as to take care of the dilutions.

3.3.2. Isolation and identification of *Salmonella* spp.

The *Salmonella* spp. isolation and identification protocol of USFDA (Andrews and Hammack, 2001) was used in the present study with little modification following International Organization of Standardization (ISO, 1991). The media and reagents used in this experiment were prepared following the specifications of Official Methods of Analysis of AOAC international (AOAC, 2000).

I. **Pre-enrichment:** Twenty five gramme each of fish and shrimp sample was homogenized in a small amount of total 225 ml buffered peptone water (HiMedia Laboratories, Mumbai). Similarly, 25 ml each of water and reconstituted swab sample was mixed with 225 ml buffered peptone water in 500-ml conical flask. The contents were mixed properly and incubated at 37^0C for 24 hour.

II. **Selective enrichment:** Wet compositing of the pre-enriched samples was done by mixing equal quantities of all the fish, shrimp, water and swab samples of one sampling day separately (Panda and Nayak, 2001). After thorough mixing, 1 ml each of the wet-composited pre-enriched sample was transferred to 10 ml Tetrathionate (TT) broth (DIFCO Laboratories, Detroit Michigan, USA) and 0.1 ml of each to 10 ml Rappaport-Vassiliadis (RV) medium (DIFCO Laboratories, Detroit Michigan, USA). The TT broths were incubated at 43^0C and RV medium at 42^0C for 24 hour in thermostatically controlled water bath (Julabo, Germany).

III. **Selective plating:** A loopful (3 mm) from each of the incubated TT broth and RV medium was plated separately on the Bismuth Sulfite Agar (BSA), Brilliant Green Agar (BGA), Xylose Lysine Desoxycholate Agar (XLDA) and Hektoen Enteric Agar (HEA) (DIFCO Laboratories, Detroit Michigan, USA). The plates were incubated at 37^0C for 24 hour.

IV. **Examination of colonies:** The plates were examined for the presence of typical *Salmonella* colonies. Characteristics to pick up the colonies on the selective plates were as follows:

BSA : Brown, grey or jet black colonies with or without metallic sheen

BGA : Pink colonies with or without black centres

XLDA : Pink colonies with or without black centres

HEA : Blue-green to blue colonies with or without black centres

Few serovars of *Salmonella* spp. often produce atypical reaction on the selective plates as follows:

HEA & XLDA: Yellow colonies with or without black centres

BSA : Green colonies with little or no darkening of the surrounding medium

V. **Biochemical and serological tests:** Two typical and two atypical colonies were picked from each plate and transferred to plate count agar slants for purification and identification. They were subjected to different biochemical reactions and serological tests (Test No. 1 through 10, Table 6) following Andrews and Hammack (2001), to obtain a presumptive diagnosis. Additional biochemical reactions (Test No. 11 through 15, Table 6) were performed for the cultures that were unable to give conclusive *Salmonella* or non-*Salmonella* reactions. *Salmonella* Typhi (MTCC 734) was used as reference strain for these tests.

Final interpretation of the biochemical and serological tests were as follows:

- Cultures that showed typical biochemical and serological reactions specific for *Salmonella* were considered as *Salmonella* spp.
- Cultures that did not show typical biochemical reactions and also did not give positive serological reactions were discarded.
- Cultures that showed typical biochemical reactions but was negative for serological reactions were maintained as probable *Salmonella* spp.
- Cultures with atypical biochemical reactions but showed positive serological tests were also maintained as probable *Salmonella* spp.

3.4. Biochemical Characterisation and Serotyping of *Salmonella* Isolates

The presumptive *Salmonella* isolates were subjected to further biochemical reactions (Table 7) for further biochemical characterisation following the methods of Harrigan and McCance (1976), Ewing (1986) and Jeyasekaran (1996).

All the presumptive *Salmonella* spp. isolates were sent to the National *Salmonella* Centre (Vet.), Indian Veterinary Research Institute (I.C.A.R.), Izatnagar, Bareilly, Uttar Pradesh (India) (NSC, IVRI), for confirmation and serological classification (i.e., serotyping) by establishing the definitive antigenic formula following the Kauffmann-White Scheme (Popoff and Le Minor, 1997).

Table 6: Biochemical and serological reactions to identify *Salmonella* spp.

Test No.	Test or substrate	Result		*Salmonella** spp. reaction
		Positive	**Negative**	
1	Glucose (TSI)	Yellow butt, Pink slant	Red butt	+
2	Lysine iron agar (LIA)	Purple butt	Yellow butt	+
3	H_2S production (TSI & LIA)	Blackening	No blackening	+
Note: Cultures that gave both acid (yellow) butt in LIA test and acid (yellow) butt and acid (yellow) slant in TSI were discarded				
4	Urease test	Purple-red colour	No colour change	-
5	Serological polyvalent flagellar (H) antigen test	Agglutination	No agglutination	+
Note: Cultures giving both urease positive reaction and flagellar (H) agglutination negative reaction were discarded				
6	Lysine decarboxylase test	Purple colour	Yellow colour	+
7	Phenol red dulcitol broth	Yellow and/or gas	No gas, no colour change	+
8	Malonate utilization test	Blue colour	No colour change	-
9	Indole production test	Violet colour at surface	Yellow colour at surface	-
Note: Cultures that gave both positive indole test and negative flagellar (H) agglutination test were discarded				
10	Serological polyvalent somatic (O) antigen test	Agglutination	No agglutination	+
Additional biochemical reactions				
11	Phenol red lactose broth	Yellow and/or gas	No gas, no colour change	-
12	Phenol red sucrose broth	Yellow and/or gas	No gas, no colour change	-
13	Methyl Red (MR) test	Diffuse red colour	Diffuse yellow colour	+
14	Voges-Proskauer (VP) test	Pink-to-red colour	No colour change	-
15	Citrate utilisation test	Growth; blue colour	No growth, no colour change	+
Note: (i) Cultures that were positive in test No. 11 or 12 were discarded (ii) Cultures that gave both positive VP test and negative MR test were discarded				

* Standard reaction for more than 90% of *Salmonella* spp. (Andrews and Hammack, 2001)

Table 7. Further Biochemical characterisation of *Salmonella* isolates

Test No.	Test or substrate	Result		*Salmonella* spp. reaction*
		Positive	Negative	
1	Motility	Motile	Non-motile	+
2	Morphology	-	-	Rod/ bacilli
3	Statement of acid and gas			
	a) Glucose	Acid production (yellow) and gas	No gas; no colour change	+[a]
	b) Inositol	Acid production (yellow) and gas	No gas; no colour change	Variable
	c) Maltose	Acid production (yellow) and gas	No gas; no colour change	+
	d) Mannitol	Acid production (yellow) and gas	No gas; no colour change	+
4	Nitrate reduction test	Red colour	No colour change	+
7	Gelatin liquefaction test	Liquefaction (clear patches)	No colour change	-
8	H_2S production in TSI	H_2S production (black colour)	No colour change	+
9	Galactosidase test	Yellow colour	No colour change	-
10	Oxidase test	Purple colour	No colour change	+
11	Phenylalanine deaminase test	Dark green	No colour change	-

* Standard reaction for more than 90% of *Salmonella* spp.

[a] *S.* Typhi shows negative reaction

3.5. Antibiotic Susceptibility Profile of the *Salmonella* Isolates

Resistance of the *Salmonella* spp. isolated in the study to various antibiotics was determined by the standard disc diffusion method (Bauer *et al.*, 1966). The set of antibiotics to be used for the purpose was established by selecting representative members from different families of antibiotics. A total of 22 different antibiotics were used for the purpose as described in Table 8. Young culture (18-24 h) of the isolates were swabbed using sterile cotton wool on to Muller Hinton Agar (HiMedia Laboratories, Mumbai) plates in duplicate and the antibiotic impregnated discs (HiMedia Laboratories, Mumbai) were evenly placed after 30 min of pre-diffusion time. The plates were incubated at 37^0C for 24 hour. The diameter of zone of inhibition was measured and the interpretation was done in accordance to performance standards for antimicrobial disc susceptibility tests of the National Committee of Clinical Laboratory Standards (NCCLS, 2000). The isolates were classified as resistant, sensitive and intermediate for each antibiotic taking the average diameter of zone of inhibition into consideration.

Multiple Antibiotic Resistance Index (MAR Index) for each isolate was calculated by dividing the number of antibiotics to which the particular isolate is resistant to the total number of antibiotics tested. A MAR Index of more than 0.2 is considered as the isolate has been originated from high-risk source of contamination (Krumperman, 1983, Harish *et al.*,2003).

3.6. Development of a *Salmonella* spp. Specific PCR Reaction

3.6.1. Bacterial reference strain and cultural characteristics

Twenty-one standard *Salmonella* strains belonging to different serovars and 12 different species of non-*Salmonella* strains generally encountered in fish and fishery environment were used as reference strain in the present experiment. The detailed description of the *Salmonella* spp. and the non-*Salmonella* strains along with their source of collection has been delineated in Table 9 and 10, respectively.

The standard *Salmonella* spp. strains were maintained in Nutrient Agar (NA) medium and the non-*Salmonella* strains were maintained in the medium suggested by the supplier of the particular strain. Al the strains were maintained aerobically in room temperature.

Table 8. Antibiotics used to study the resistance of *Salmonella* isolates

Antibacterial group	Antibiotics used in the study	Remarks	Mode of action
β-Lactam ring compounds	Penicillin G	Natural penicillin	Inhibit cell wall synthesis
	Ampicillin	Semi-synthetic penicillin	
	Cephalexin Cephotaxime	Semi-synthetic cephalosporin	
Glycopeptide	Vancomycin	Large molecule	Inhibit cell wall synthesis
Aminoglycosides	Streptomycin Kanamycin Amikacin Neomycin Gentamycin	Bactericidal compounds	Inhibit protein synthesis
Tetracycline	Tetracycline Oxytetracycline	Broad spectrum antibacterial agents, bacteriostatic	
Chloramphenicol	Chloramphenicol		
Macrolide	Erythromycin	Bacteriostatic	
Quinolone	Nalidixic acid	Narrow spectrum	Inhibit nucleic acid synthesis
Fluoroquinolone	Ciprofloxacin Levofloxacin Norfloxacin	Second generation fluoroquinolone, broad spectrum	
Sulfonamide	Co-trimoxazole	Antimetabolites and other antibacterial agents	Indirectly act on DNA synthesis
Nitrofuran	Furazolidone Nitrofurantoin		
Polymyxin	Colistin	Miscellaneous antibacterial agent	Disrupters of cell membrane

Table 9. Reference strains of *Salmonella* spp. used in the study

Sl. No.	Name of the strain	Source code *	Source*
1	*Salmonella* Typhi	MTCC 734	IMTECH, Chandigarh
2	*Salmonella* Paratyphi	-	CRI, Kasauli
3	*Salmonella* Pàratyphi A	MTCC 735	IMTECH, Chandigarh
4	*Salmonella* Paratyphi B	E 120	NSC, IVRI, Izatnagar
5	*Salmonella* Enteritidis	-	CRI, Kasauli
6	*Salmonella* Typhimurium	NCIM 2501	NCL, Pune
7	*Salmonella* Newport	E 112 b	NSC, IVRI, Izatnagar
8	*Salmonella* Abony	NCIM, 2257	NCL, Pune
9	*Salmonella* Virchow	MTCC 1163	IMTECH, Chandigarh
10	*Salmonella* Infantis	MTCC 1167	IMTECH, Chandigarh
11	*Salmonella* Brunei	MTCC 1168	IMTECH, Chandigarh
12	*Salmonella choieraesuis* subsp. Arizona	MTCC 660	IMTECH, Chandigarh
13	*Salmonella* Weltevreden	MTCC 1169	IMTECH, Chandigarh
14	*Salmonella* Kentucky	Isolate from fish poisoning cases	NSC, IVRI, Izatnagar
15.	*Salmonella* Paratyphi	- do -	NSC, IVRI, Izatnagar
16	*Salmonella* Typhimurium	- do -	NSC, IVRI, Izatnagar
17	*Salmonella* Gallinarum	STD. IVRI. IZAN 9R/83	NSC, IVRI, Izatnagar
18	*Salmonella* Typhimurium	MTCC 1253	IMTECH, Chandigarh
19	*Salmonella* I.	NSC 3642	Old collection of the lab, confirmed by NSC, IVRI
20	*Salmonella* I. monophasis	NSC 3643	- do -
21	*Salmonella* Javiana	NSC 3646	- do -

* IMTECH : Institute of Microbial Technology (CSIR), Chandigarh
NCL : National Chemical Laboratory (CSIR), Pune
CRI : Central Research Institute (Govt. of India), Kasauli
NSC : National Salmonella Centre (Vet.), IVRI (ICAR), Izatnagar
MTCC : Microbial Type Culture Collection
NCIM : National Collection of Industrial Microorganisms

Table 10. Reference strains of non-*Salmonella* spp. used in the study

Sl. No.	Name of the strain	Source code *	Source *
1	*Escherichia coli*	MTCC 1687	IMTECH, Chandigarh
2	*Vibrio cholerae* O1	-	NICED, Kolkata
3	*Vibrio parahaemolyticus*	MTCC 451	IMTECH, Chandigarh
4	*Vibrio vulnificus*	MTCC 1145	IMTECH, Chandigarh
5	*Proteus mirabilis*	NCIM 2241	NCL, Pune
6	*Proteus morganii* (*Morganella morganii*)	NCIM 2860	NCL, Pune
7	*Staphylococcus aureus*	NCIM 5021	NCL, Pune
8	*Aeromonas hydrophila*	NCIM 2319	NCL, Pune
9	*Klebsiella pneumoniae*	NCIM 2719	NCL, Pune
10	*Hafnia alvei*	NCIM 2351	NCL, Pune
11	*E. coli* EHEC O157:H7	-	NICED, Kolkata
12	*Vibrio cholerae* – non pathogenic	-	College of Fisheries (UAS), Mangalore
13	*Vibrio parahaemolyticus*	-	Dr. S. K. Panda (Panda, 2002)

* IMTECH : Institute of Microbial Technology (CSIR), Chandigarh
NCL : National Chemical Laboratory (CSIR), Pune
CRI : Central Research Institute (Govt. of India), Kasauli, H.P.
NSC : National Salmonella Centre (Vet.), IVRI (ICAR), Izatnagar, U.P.
NICED : National Institute of Cholera and Enteric Diseases
MTCC : Microbial Type Culture Collection
NCIM : National Collection of Industrial Microorganisms

3.6.2. Isolation of bacterial genomic DNA

Total Genomic DNA from all pure cultures of bacterial strains was purified by following the CTAB-NaCl method described by Ausubel *et al.* (1987). The method has been briefly described as follows:

I. The strain of interest was inoculated into 5 ml Luria Bertani (LB) broth (HiMedia Laboratories, Mumbai) and incubated with shaking at 170 rev/min (Pelican Bio Innovations, Chennai) at 37°C for 12 – 14 hour.

II. The culture (1.5 ml) was centrifuged (Jouan, France) at 10,000 X g for three minutes in a microcentrifuge tube to get a compact pellet of bacterial cells.

III. The supernatant was discarded and the pellet was resuspended in 567 µl Tris-EDTA buffer [10 mM Tris – Cl (pH 8.0), 1 mM EDTA] by repeated pipetting. Then, 30 µl of 10% (w/v) sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, Mo., USA) and 3 µl of proteinase K (Sigma-Aldrich, USA) solution (20 mg/ml) were added, and the mixture was incubated for 1 hour at 37°C.

IV. The samples were treated with 100 µl of 5 M NaCl and 80 µl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl (Sigma-Aldrich, St. Louis, Mo., USA) solution (10% CTAB in 0.7 M NaCl) and the mixture was incubated at 65°C for 10 minutes.

V. After the incubation, the samples were first extracted with an equal volume of chloroform-isoamyl alcohol (24:1, v/v) and then with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v).

VI. The extract was transferred to a fresh tube and the DNA was precipitated with 0.6 volume of 100% isoproponal (E. Merck, Mumbai), pelleted by brief spinning at room temperature and washed with 1 ml of 70% cold ethyl alcohol.

VII. The DNA pellet was dried in a DNA concentrator (Heto, Denmark) for 10 min and resuspended in 50 µl Tris-EDTA buffer (pH 8.0).

3.6.3. Qualitative and quantitative analysis of the isolated DNA

An aliquot (typically 1-2 µl) of the sample DNA was subjected to spectrophotometric analysis at optical densities of 260 and 280 nm wavelengths to determine the quantity and purity of the isolated genomic DNA. The reading at 260

nm was used for calculation of the concentration of nucleic acid in the sample, as an OD_{260} of 1.0 corresponds to approximately 50 μg/ml for double-stranded DNA. The ratio between the readings at 260 and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. The DNA sample with OD ratio < 1.7 were re-extracted with phenol and precipitated in ethanol. Qualitative evaluation of the isolated DNA was also done by running gel electrophoresis (Ausubel *et al.*, 1987) at a concentration of 0.8% agarose and accordingly some of the samples were treated with RNase to get rid of RNA contamination.

3.6.4. PCR primers

Three pairs of primers were used for testing the specificity of *Salmonella* spp. detection. One pair of primers based on *inv*A gene of *Salmonella* spp. was published previously (Brasher *et al.*, 1998) and two pairs of primers were newly designed for the purpose. The detailed specifications of the primers along with their source have been given in Table 11.

3.6.5. Optimisation of PCR protocol

Genomic DNA of both target and non-target strains were amplified in 0.2 ml sterile PCR tubes in a reaction volume of 25 µl. One pair of primers was taken at a time and a series of standardization reactions was carried out for optimising the composition of reaction mixture and thermocycler programming (Sambrook and Russel, 2001). *S. typhi* (MTCC 734) and *E. coli* (MTCC 1687) were used as positive and negative controls, respectively. Heated lid thermocycler (Mastercycler®, Effendorf, Germany) with block sensor was used for all the standardisation reactions. Table 12 describes various components of the reaction mixture, both in terms of quantity and final concentration, which worked out to be common for all the three sets of primers used in the present study. The optimised temperature schedule for different primers used in the study has been described in Table 13.

3.6.6. PCR screening to test the specificity of primers (phase I)

In order to evaluate the specificity of primers to detect *Salmonella* spp., PCR amplification reactions were carried out taking one pair of primers at a time. Each pair of primers was taken for amplification of seventeen *Salmonella* spp. and ten non-*Salmonella* strains using the optimised PCR protocol (Table 12 and 13). The

Table 11. Description of the primers used for the test of specificity of *Salmonella* spp. detection

Primer set		Primer Sequence (5′ – 3′)	Melting temp. Tm (^{0}C)	Target sequence	Amplicon size (bp)	Reference
A	L-INVA R-INVA	CTC TAC TTA ACA GTG CTC GTT TAC TTG ATA AAC TTC ATC GCA CCG TCA	61 61	*inv* A gene	571	Brasher *et al.* (1998)
B	BS1-F BS1-R	TGA CGC AAA AGA GGA AGG AT ATA ACC GTG CGG AAG TTG AC	58 60	*S. typhi* specific probe	201	Designed in this study using gene sequence U78640
C	BS2-F BS2-R	Sahu and Singh (2004)	57 56	*inv* A gene	403	Designed based on *Salmonella* spp. *inv* A gene sequence

Table 12. Components of PCR reaction mixture used in the study

Sl. No.	Component	Concentration of stock	Quantity (μl)	Final Conc.
1.	Template DNA	50 ng/μl	1	50 ng
2.	dNTP mix	10 mM each	0.5	200 μM each
3.	10x PCR buffer (750 mM Tris-HCl, pH 8.8; 200 mM $(NH_4)_2SO_4$; 0.1% Tween 20)	10x	2.5	1x
4.	$MgCl_2$	25 mM	1.5	1.5 mM
5.	Primer (Forward)	10 pmol	1	0.4 μM
6.	Primer (Reverse)	10pmol	1	0.4 μM
7.	Taq Polymerase	(1Unit/μl)	1	1 U
8.	Autoclaved Milli 'Q' water	(To make up)	16.5	
Total			25 μl	

Table 13. Optimised temperature schedule for the primers used in specificity testing

Step No	Step	A (L-INVA, R-INVA)		B (BS1-F, BS1-R)		C (BS2-F, BS2-R)	
		Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (min)
1	Initial denaturation	94	5	94	3	94	3
2	Denaturation	94	1	94	1	94	1
3	Annealing	56	1.5	55	1	55	1
4	Extension	72	2	72	1	72	1
	Step 2 to 4	Repeat 29 times		Repeat 29 times		Repeat 34 times	
5	Final Extension	72	10	72	10	72	7
6	Hold at 4^{0}C						

pair of primers showing good performance was short-listed and subjected to further evaluations as described else where in this chapter. The best performing pair of primers was short-listed and subjected to further evaluations as described subsequently.

3.6.7. Analysis of PCR product by agarose gel electrophoresis

Agarose gel electrophoresis was performed to analyse the PCR products using the standard protocol of Ausubel *et al.* (1987). Briefly, the PCR products (10-µl aliquot) were analysed by electrophoresis in a 1.8% agarose (low EEO, Sigma-Aldrich, USA)) gel incorporated with ethidium bromide (0.5 µl/ml) in 0.5x Tris-Borate-EDTA buffer (0.045 M Tris-Borate, 0.001 M EDTA). The gel was run at a constant voltage of 5 V/cm in the presence of tracking dye (6x gel loading dye; 0.25% bromophenol blue, 0.25% xylene cynole and 40% sucrose in water; MBI Fermentas, Germany). The size of the DNA bands were established by DNA molecular weight marker (100 bp ladder plus, MBI Fermentas, Germany) separated concurrently in the same gel. The DNA bands were viewed under UV light and documented using Gel documentation system (Syngene, Cambridge, UK) and Thermal printer (Sony, Japan).

3.7. Evaluation of a Simple and Rapid DNA Preparation Method for PCR Assay

3.7.1. Rapid-boiling lysis preparation of template DNA

Template DNA preparation by rapid-boiling lysis method, described by Mukhopadhyay and Mukhopadhyay (2002) and Medici *et al.* (2003), were followed with little modification for the preparation of bacterial cell lysate for use in PCR reaction as the source of template DNA.

I. 1.5 ml of the over-night culture of the bacterial strain of interest was centrifuged at 10,000 X g for three minutes at room temperature to get a compact pellet of bacterial cells.

II. The supernatant was discarded carefully and the pellet was resuspended in 300 µl autoclaved Milli Q water by vortexing.

III. The cell suspension was centrifuged at 10,000 X g for three minutes at room temperature and the supernatant was discarded carefully.

IV. The pellet was resuspended in 100 µl autoclaved Milli Q water by repeated pipetting and the cell suspension was incubated at 95^{0}C for 10 min and immediately chilled on ice for 5 min.

V. The suspension was centrifuged at 4^{0}C and the supernatant was carefully transferred to a fresh microcentrifuge tube.

VI. The tube was incubated again at 95^{0}C for 7 min and chilled immediately on ice.

VII. The tube was centrifuged again for three minutes at 10,000 X g at 4^{0}C and the supernatant cell lysate was transferred to a fresh microcentrifuge tube. An aliquot of 5 µl of the bacterial cell lysate was used for PCR reaction.

3.7.2. PCR evaluation of bacterial cell lysate

The *Salmonella* spp. specific PCR reaction short-listed as the ideal one in the present study was repeated for thirteen representative standard *Salmonella* strains by replacing 1 µl purified genomic DNA with 5 µl bacterial cell lysate. All other conditions in the PCR protocol (Table 12 and 13) remained the same.

3.7.3. Evaluation of the PCR method by conventional culture based method

Bacterial cell lysates of all the *Salmonella* spp. isolated and characterised, in the present study were used to evaluate both the *Salmonella* spp. specific PCR reaction and use of bacterial cell lysate as template. All conditions in the PCR protocol (Table 12 and 13) remained the same, with the only exception of replacing 1 µl purified genomic DNA with 5 µl bacterial cell lysate.

3.7.4. Evaluation of the PCR method by artificial seeding

The efficiency of the PCR protocol was further evaluated for its ability in detecting *Salmonella* spp. contamination in fish and fishery products by artificial seeding and DNA preparation by rapid boiling lysis method.

One strain from each serotype isolated and characterised in the present study was taken for this evaluation. Bacterial suspension was prepared by inoculating the strains in LB medium and incubating overnight at 37^{0}C. The bacterial suspensions were adjusted to a concentration of 10^7cells/ml in sterile physiological saline using spectrophotometer, considering OD_{650nm}= 0.2 is equivalent to10^8 cells/ml (Waage *et al.*, 1999). Processed shrimp, ready for export, procured form a

fish processing plant in Mumbai and a type of fish Kamaboko obtained from retail market at Tokyo, Japan were used to prepare homogenate in sterile physiological saline at 1:9 (w/v) ratio. One millilitre each of the appropriately diluted bacterial suspension was added to 99 ml of the shrimp or Kamaboko homogenate. The contents were mixed thoroughly by repeated swirling.

Fifty millilitre of the artificially seeded homogenate was centrifuged at 100 X g for 10 min to pellet debris. 1.5 ml of the suspension from each was taken for preparation of bacterial cell lysate following the methods delineated in section 3.7.1. Five micro litre of the bacterial cell lysate was taken for PCR evaluation as described previously.

3.7.5. PCR screening to test the specificity of primers (phase II)

Bacterial cell lysates of additional four *Salmonella* spp. and two non-*Salmonella* strains were taken for second phase screening of the specificity of the PCR method developed in this study. The entire protocol remained the same as described above in section 3.7.2. The robust nature of the PCR protocol developed was also evaluated by repeating all the PCR reactions in a heated lid gradient thermocycler (PCR Express, Thermo Hybaid, USA) with tube sensor.

3.8. PCR Analysis of Biochemically and/or Serologically Atypical Isolates

Few presumptive *Salmonella* spp. that failed to give the typical *Salmonella* spp. specific reactions by only one or two tests were subjected to PCR analysis using the method developed in the present study. Purified genomic DNA of these isolates were used for PCR reaction, since the use of bacterial cell lysate for PCR amplification in these cases failed to produce repeatability in one of the isolates. The isolates producing the specific band in *Salmonella* spp. specific PCR assay were considered as 'atypical *Salmonella* spp'.

3.9. Analysis of Atypical *Salmonella* Isolates by Sequencing

For confirmation and characterisation of the PCR result obtained with the so called atypical *Salmonella* spp., one of the PCR amplicons was subjected to sequencing reactions at molecular biology unit, National Centre for Cell Science (NCCS), Pune. Nucleotide sequencing of the DNA band of interest was tried both with cloning and sequencing as well as direct sequencing of PCR product.

3.9.1. Cloning of PCR products

The PCR product obtained with one of the atypical *Salmonella* spp. was directly cloned to T/A cloning vector (pTZ57R/T) and then transformed into *E. coli* (DH5α) using "InsT/Aclone™ PCR product cloning kit" (MBI Fermentas).

PCR reaction was specifically run for the purpose in the pre-standardised reaction conditions with slight modification in the final extension step of 30 min instead of the usual 7 min at 72°C. The PCR product was purified using Microcon PCR centrifugal devices (Millipore, USA) following the manufacturer's instructions. Cloning of the purified PCR product was then performed, following the protocol provided by the manufacturer, through the following steps:

3.9.1.1. Ligation of PCR product

Following components were added into an autoclaved microcentrifuge tube for the ligation reaction:

▪ Plasmid vector pTZ57R/T DNA	(0.165μg, 0.18 pmol ends)	3μl
▪ Purified PCR product	(approx. 0.54 pmol ends)	4μl
▪ Ligation buffer	(10 X)	3μl
▪ PEG 4000-solution		3μl
▪ Deionized water	(dH_2O)	16μl
▪ T4 DNA ligase	(5U/μl)	1μl

The mixture was then incubated at 22°C over night (i.e., about 12 hours) and kept at -20°C until transformation.

3.9.1.2. Transformation of the ligated product

Transformation of the ligated product into *E. coli* (DH5α) cells was done using TransformAid™ Bacterial Transformation System (MBI Fermentas, Germany) following the protocol provided by the manufacturer.

3.9.1.2.1. Preparation of competent cell of from *E. Coli* (DH5α)

I. 2 ml TransformAid™ C - Medium was taken in a sterile test tube and was inoculated with *E. coli* DH5α colony.

II. Culture was incubated overnight at 37°C with shaking at 200 rpm.

III. 1.5 ml of TransformAid™ C-Medium was pre-warmed to 37°C in a test tube and inoculated with 1/10th volume of the overnight culture (i.e., 0.15 ml)

IV. Freshly inoculated C-Medium was incubated for 20 min at 37°C with vigorous shaking and cells were taken for transformation.

3.9.1.2.2. Transformation procedure

I. LB-Ampicillin agar plates were over layered with X-Gal and IPTG solutions and were pre-warmed at 37°C for 30 minutes.

II. TransformAid™ T-solution was prepared by mixing 250 µl each of T-solution (A) and T- solution (B) and was kept on ice.

III. 1.5 ml of fresh culture was taken in a microcentrifuge tube and was centrifuged at 4°C for 1 minute at 10,000 X g.

IV. Supernatant was discarded and the pelleted cells were resuspended in 300µl Transform Aid-T solution. The tube was incubated on ice for 5 minutes.

V. The cells were then pelleted down by spinning at 10,000 X g for 1 minute at 4°C and supernatant was discarded

VI. The cell pellet was again resuspended in 120 µl of TransformAid™ T-solution and incubated on ice for 5 minutes

VII. 2.5 µl each of the ligation mixture was dispensed into two fresh microcentrifuge tubes and the tubes were held in ice for 2 minutes.

VIII. 50µl each of the resuspended competent cells was added to the tubes containing ligated plasmid DNA and incubated in ice for 5 min.

IX. Transformed cells were then spread plated on pre-warmed LB-Ampicillin agar plates containing X-Gal and IPTG and incubated overnight at 37°C.

3.9.1.3. Selection of recombinant clones and its confirmation

Atleast five isolated white colonies were picked per plate and inoculated into LB tube containing Ampicillin (@ 50µg/ml). The tubes were incubated overnight at 37°C with vigorous shaking. Tubes showing good growth were used for plasmid isolation.

3.9.1.3.1. Isolation of plasmid from the positive clones

Plasmid mini-prep from the positive clones was undertaken using alkaline lysis method (Sambrook and Russel, 2001).

I. 1.5 ml of the overnight culture was taken in a microcentrifuge tube and centrifuged at 10,000 X g at 4°C for three minutes.

II. Supernatant was discarded and the bacterial cell pellet was resuspended in 100µl of ice-cold solution I [50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)] by vigorous vortexing.

III. 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the contents were mixed by gently inverting the tubes few times and placed on ice for 5-10 minutes.

IV. 150 µl of ice-cold solution III (60 ml, 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml water)) was added to the tube and vortexed in an inverted position at maximum speed for 10 seconds to disperse solution III through viscous bacterial lysate and stored in ice for 5 minutes.

V. Mixture was centrifuged at 4°C and supernatant transferred to a fresh tube

VI. The sample was then extracted with equal volume of chlorophorm-isoamyl alcohol (24:1) at 4°C.

VII. The double stranded DNA was precipitated with 2.5 volume of chilled absolute ethanol, allowed to stand for 5 minutes at 4°C and then centrifuged at 14,000 X g for 5 minutes at 4 °C.

VIII. Supernatant was drained and DNA pellet was rinsed with 1 ml 70 % ethanol at room temperature.

IX. The pellet was dried in DNA concentrator (Heto, Germany) for 10 min and dissolved in 20 µl Tris-EDTA buffer (pH 8.0).

X. Analysis of the plasmid DNA was done by running an aliquot (2 µl) in 1.0% agarose gel electrophoresis in 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA) buffer (Ausubel *et al.*, 1987).

3.9.1.3.2. Restriction endonuclease analysis (REA) of recombinant plasmids

Approximately 1μg of isolated recombinant plasmid DNA was double digested with restriction enzymes *Bam*H I and *Xba* I (New England Biolab, UK) following the instructions of the manufacturer. Briefly, the plasmid DNA was digested with *Xba* I at 37°C for 1 hour followed by heat inactivation of the enzyme at 65°C for 20 min. It was then treated with *Bam*H I at 37°C for 1 hour followed by inactivation of the enzyme by adding 1 μl 0.5 M EDTA. The digested plasmid DNA was electrophoresed on 1% agarose gel in 1x TAE buffer. The DNA bands were analysed and documented as described above.

3.9.2. Purification of PCR product for direct sequencing

About 20 μl of the PCR product was mixed with 0.6 volumes of PEG-NaCl solution (20% PEG 4000, 2.5 M NaCl) and incubated at 37°C for 30 min. The precipitate was collected by centrifugation at 10,000 X g for 30 min. The pellets were washed twice with 70% ethanol and dried under vacuum. The DNA pellet was then resuspended in triple distilled water.

3.9.3. Sequencing of the PCR product

Nucleotide sequencing of the purified PCR product and the cloned plasmid DNA was carried out using BigDye® Terminator v 3.1 cycle sequencing kit (PE Applied Biosystems, USA) at NCCS, Pune. The cycle sequencing PCR was set up with the reverse primer (BS2-R) (Table 11) for direct sequencing of PCR product and M 13 universal primer for sequencing the insert in the clone. Other ingredients added to the mixture were: big dye terminator RR mix which contains the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, FS, rTth pyrophosphatase, magnesium chloride and buffer.

The sequencing PCR reaction was optimized and run on PE GeneAmp 9700. The sequencing was done on ABI PRISM 310 automated DNA sequencer (ABI PRISM CE-1 Sequencer version 3.3.2, Applied Biosystems International, Foster City, CA, USA).

3.9.4. Analysis of the sequence

The sequence obtained was put for random nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) in the GenBank (http://www.ncbi.nlm.

nih.gov/blast) to check for the random matching of the sequence with the sequence available with the GenBank database. Multiple alignment of the deduced sequence was performed in ClustralW (1.82) multiple sequence alignment software (www.ebi.ac.uk/ClustralW) with the matching sequences of many *Salmonella* serotypes, as obtained by random BLAST, to establish the extent of similarity.

3.10. Molecular Characterisation of *Salmonella* Isolates by PCR Ribotyping

An attempt was made for the molecular subtyping of *Salmonella* serotypes by PCR amplification of the spacer sequence between 16S and 23S genes in the rRNA transcriptional units. The primers used were designed based on the homologous region flanking the heterogeneous intervening sequence in the rRNA gene.

Forty *Salmonella* strains comprising 19 standard reference strains and 21 isolates were used for the study. Purified DNA prepared by standard CTAB:NaCl method (Ausubel *et al.*, 1987) and that by rapid boiling lysis method were used as template for PCR amplification. Oligonucleotide primers used in the experiment, L17 (5' CAA GGC ATC CAC CGT GT-3') and G17 (5' GTG AAG TCG TAA CAA GG 3') were previously described by Jensen and Straus (1993) and Baudart *et al.* (2000b). Amplification was performed in a final volume of 25 µl with a reaction mixture containing 1µl dNTPs (each at 400µM, MBI Fermentas), 2.5µl 10x PCR buffer [750mM Tris-Cl (pH 8.8), 200mM $(NH_4)_2SO_4$, 0.1% Tween 20; MBI Fermentas], 1µl $MgCl_2$ (at 1.5mM, MBI Fermentas) and 1µl of each primer (at 12.5 µM, Operon Inc., USA). Programmable heated lid thermocycler (PCR Express, Thermo Hybaid, USA) was used for the PCR reaction in the following programme: (i) initial denaturation at 94^0C for 3 min. (ii) Intermediate step 80^0C for 1min to add 1µl Taq DNA Polymerase (1U, MBI Fermentas) (iii) 30 reaction cycles comprising of denaturation at 94^0C for 30 s, annealing at 55^0C for 3 min and extension at 72^0C for 1 min, and (iv) final extension at 72°C for 30 min.

Ten micro litre of the PCR products were separated on agarose gel electrophoresis using 2% Metaphor® Agarose (Biowhittaker, USA). The gel was cast in 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA) and run at 0.5x TAE at 5 V/cm with

temperature constantly maintained at 15°C. After the separation of PCR products the results were viewed and documented as described previously.

The molecular weights of the polymorphic bands for each of the strains were noted. Based on the similarity in the DNA bands, in terms of the number of bands and their molecular weight, PCR ribotype pattern was established.

3.11. Evaluation of the *Salmonella* spp. Specific PCR Protocol in a Multiplex PCR Assay

The multiplex PCR method involved four pair of primers (Table 14), each one specifically targeting *uid*A gene of *E. coli*, an indicator of faecal contamination; *inv*A gene of *Salmonella* spp., one of the leading agent of food-borne infections; *ctx* gene of *V. cholerae*, the causative agent of enteric cholera and the *tl* gene of *Vibrio parahaemolyticus,* a leading cause of seafood-borne gastroenteritis. All the primers were standardized to work in a common reaction conditions.

Purified genomic DNA was isolated and quantified as per the protocol described in section 3.6.2 and 3.6.3, respectively. The reaction components as per the description in Table 15 were added into a 0.2 ml sterile PCR tube sequentially. The PCR temperature schedule included initial denaturation at 95°C for 3 min followed by 30 cycles at 94°C for 1 min, 56°C for 1.5 min and 72°C for 2 min and a final extension at 72°C for 7 min. A 10 µl aliquot of amplified DNA was examined by electrophoresis as described earlier.

Specificity of the participating pairs of primers was established by performing cross-reactions of each pair of the primers with twelve non-target strains usually encountered in fish and fishery environment (Table 9 and 10) while using the target strains as positive control.

Table 14. Specifications of the primers used in the multiplex PCR

Target pathogen	Primer set	Primer Sequence (5′ – 3′)	Target gene	Amplicon size (bp)	Reference
Salmonella spp.	BS2-F BS2-R	Sahu and Singh (2004)	*inv* A	403	Designed based on *Salmonella* spp. *inv* A gene sequence
Escherichia coli	L – *uid* A R – *uid* A	TGG TAA TTA CCG ACG AAA ACG GC ACG CGT GGT TAC AGT CTT GCG	*uid* A	147	Bej *et al.* (1991b) Bej *et al.* (1991c) Brasher *et al.* (1998)
Vibrio cholerae	L – *ctx* R – *ctx*	After modification based on Brasher *et al.* (1998)	*ctx*	302	Shirai *et al.* (1991) Bej *et al.* (1996) Brasher *et al.* (1998)
V. *parahaemolyticus*	L – *tl* R – *tl*	AAA GCG GAT TAT GCA GAA GCA CTG GCT ACT TTC TAG CAT TTT CTC TGC	*tl*	450	Taniguchi (1986) Brasher *et al.* (1998) Bej *et al.* 1999

Table 15. Components of reaction mixture used in multiplex PCR

Sl. No.	Component	Concen-tration of stock	**Multiplex PCR** (four pair of primers vs. four pathogen)		**Cross reaction I** (one pair of primer vs. four pathogen)		**Cross reaction II** (one pathogen vs. four pairs of primers)		**Cross reaction III** (one pair of primers vs. one pathogen)	
			Quantity added (µl)	**Final Conc.**	**Quantity added** (µl)	**Final Conc.**	**Quantity added** (µl)	**Final Conc.**	**Quantity added** (µl)	**Final Conc.**
1	Template DNA	100 ng/µl	4 x 1.0	4 x 100 ng	4 x 1.0	4 x 100 ng	1.0	100 ng	1.0	100 ng
2	dNTP mix	10 mM each	2.0	800 µM each	0.5	200 µM each	0.5	200 µM each	0.5	200 µM each
3	10x PCR buffer *	10 x	2.5	1 x	2.5	1 x	2.5	1 x	2.5	1 x
4	$MgCl_2$	25 mM	1.5	1.5 mM	1.5	1.5 mM	1.5	1.5 mM	1.5	1.5 mM
5	Primer (Forward)	10 pmol/µl	4 x 1.0	4 x 0.4µM	1.0		4 x 1.0	4 x 0.4µM	1.0	0.4 µM
6	Primer (Reverse)	10 pmol/µl	4 x 1.0	4 x 0.4µM	1.0	0.4 µM	4 x 1.0	4 x 0.4µM	1.0	0.4 µM
7	Taq Polymerase	(1Unit/µl)	2.0	2 U	1.0	1 U	1.0	1 U	1.0	1 U
8	Autoclaved Milli 'Q' water		5.5 µl		13.5		10.5		16.5	
Total			25 µl		25 µl		25 µl		25 µl	

* 10 X PCR buffer = 750 mM Tris-Cl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20

RESULTS

4. RESULTS

4.1. Hygienic Status of Fish and Fish Handling Environments of Mumbai

4.1.1. Total bacterial load

Aerobic Plate Counts (APC) were enumerated to assess the overall microbiological quality of the samples. The results have been shown in Table 16. APC of fish and shrimp sample ranged from 3.7 $x10^4$ to 1.7$x10^7$ cfu/g; while that for water, swab and processed shrimp samples were 1.3$x10^4$ to 6.9$x10^7$ cfu/ml, 4.8 x 10^3 to 2.0 x 10^6 cfu/cm^2 and 6.2$x10^2$ to 3.1$x10^3$ cfu/g, respectively.

4.1.2. Isolation and identification of *Salmonella* spp.

Twenty six *Salmonella* spp. were isolated in the present study (Table 17). Maximum number of isolates, totalling 18, were from Four Bungalows fish market followed by 8 isolates from Versova fish landing centre. No *Salmonella* spp. was isolated from Sasoon Dock fish landing centre and the fish processing plant. The isolates were confirmed and sero-grouped into 11 serovars by NSC, IVRI. The assigned serogroup to the *Salmonella* isolates along with the respective antigenic formula has been listed in Table 17. The detailed biochemical characteristics of all the *Salmonella* isolates have been delineated in Table 18.

4.2. Antibiotic Susceptibility Profile of the *Salmonella* Isolates

All the twenty six *Salmonella* isolates were subjected to disc diffusion test against 22 commonly used antibiotics as per the description in Table 8. Table 19 describes antibiotic susceptibility profile of individual *Salmonella* isolates. Level of resistance of the *Salmonella* isolates to each antibiotics tested has been represented in Table 20. More than 90% of the isolates were resistant to vancomycin, nitrofurantoin, penicillin and erythromycin. On the other hand, almost all the *Salmonella* isolates were susceptible to ciprofloxacin, norfloxacin, chloramphenicol, co-trimoxazol, and levofloxacin.

4.3. Qualitative and Quantitative Analysis of Bacterial Genomic DNA

The OD_{260}/OD_{280} ratio for all the purified bacterial genomic DNA was approximately 1.8, indicating that the genomic DNA was of good quality. Agarose gel electrophoresis of the isolated DNA from standard *Salmonella* and non-*Salmonella* strains was performed to assess the quality of isolated DNA. Fig. 1 depicts the genomic DNA bands of few representative bacterial strains.

Table 16. Bacteriological load of various samples of fish and fish handling environments of Mumbai

Sampling Station	Sample category	Number of Samples	Aerobic Plate Count (APC)*	
			Range	Average
Sasoon Dock fish landing centre	Marine fish	3	$1.2x10^6$ - $4.3x10^6$	3.1×10^6
	Shrimp	3	$3.3x10^6$(est.) - $1.7x10^7$	1.1×10^7
	Swab	3	$3.2x10^5$ - $2.0x10^6$	1.2×10^6
	Water	3	$1.2x10^5$ - $6.9x10^7$ (est)	2.4×10^7
Versova fish landing centre	Marine fish	9	$2.1 \times 10^5 - 3.4 \times 10^5$	2.7×10^5
	Shrimp	9	3.7×10^4 - 1.2×10^5	7.8×10^4
	Swab	6	4.8×10^3- 5.2×10^3	5.0×10^3
	Water	6	1.4×10^4 - 1.3×10^4	1.3×10^4
Four Bungalows fish market	Marine fish	9	6.3×10^5 - 8.7×10^5	7.5×10^5
	Freshwater fish	9	9.5×10^6 - 1.2×10^7	1.1×10^7
	Shrimp	9	1.1×10^6 - 1.4×10^6	1.3×10^6
	Swab	6	5.1×10^4 - 5.4×10^4	5.2×10^4
	Water	6	8.2×10^5 - 1.1×10^6	9.4×10^5
Fish processing plant	IQF shrimp	3	$6.2 x10^2 - 3.1 \times 10^3$	1.9×10^3
Total		81		

* APC of fish, shrimp and IQF shrimp is in cfu/g, while that for water and swab sample are in cfu/ml and cfu/cm^2, respectively

Table 17. Description of the *Salmonella* serovars isolated in the study

Sl. No.	Isolate code	Salmonella serovar	Antigenic formula	Source of Isolation*
1.	BSCC1	*S.* Senftenberg	1,3,19:g,s,t:-	FW fish, FB market
2.	BSCC2	*S.* Saintpaul	4,5,12:e,h:1,2	FW fish, FB market
3.	BSCC3	*S.* Reading	4,5,12:e,h:1,5	FW fish, FB market
4.	BSCC4	*S.* Saintpaul	4,5,12:e,h:1,2	FW fish, FB market
5.	BSCC5	*S.* Reading	4,5,12:e,h:1,5	FW fish, FB market
6.	BSCC6	*S.* Reading	4,5,12:e,h:1,5	FW fish, FB market
7.	BSCC7	*S.* Senftenberg	1,3,19:g,s,t:-	FW fish, FB market
8.	BSCC8	*S.* Stanley	4,5,12:d:1,2	Marine fish, FB market
9.	BSCC9	*S.* Stanley	4,5,12:d:1,2	Marine fish, FB market
10.	BSCC10	*S.* Sarajane	4,5,12:e,n,x	Marine fish, FB market
11.	BSCC11	*S.* Saintpaul	4,5,12:e,h:1,2	Swabs, FB fish market
12.	BSCC12	*S.* Sandiego	4,5,12:e,h(r):e,n,z_{15}	Swabs, FB fish market
13.	BSCC13	*S.* Saintpaul	4,5,12:e,h:1,2	Water, FB fish market
14.	BSCC14	*S.* Stanley	4,5,12:d:1,2	Marine fish, FB market
15.	BSCC15	*S.* Stanley	4,5,12:d:1,2	Shrimps, FB market
16.	BSCC16	*S.* Heidelberg	4,5,12:r:1,2	Shrimps, FB market
17.	BSCC17	*S.* Reinickendorf	4,12:1,z28:e,n,x	Water, FB fish market
18.	BSCC18	*S.* Bradford	4,5,12:r:1,5	Swabs, FB fish market
19.	BSCC19	*S.* Typhimurium	4,5,12:i:1,2	Creek water, Versova LC
20.	BSCC20	*S.* Ohio	6,7:b:1,w	Creek water, Versova LC
21.	BSCC21	*S.* Ohio	6,7:b:1,w	Swab, Versova LC
22.	BSCC22	*S.* Ohio	6,7:b:1,w	Swab, Versova LC
23.	BSCC23	*S.* Ohio	6,7:b:1,w	Creek water, Versova LC
24.	BSCC24	*S.* Ohio	6,7:b:1,w	Wash water, Versova LC
25.	BSCC26	*S.* Ohio	6,7:b:1,w	Wash water, Versova LC
26.	BSCC27	*S.* Ohio	6,7:b:1,w	Creek water, Versova LC

* FB market = Four Bungalows fish market; FW fish = Freshwater fish and Versova LC = Versova fish landing centre

Table 18. Detailed biochemical and serological characterisation of *Salmonella* serovars

Test \ Isolate code	BSCC 1	BSCC 2	BSCC 3	BSCC 4	BSCC 5	BSCC 6	BSCC 7	BSCC 8	BSCC 9	BSCC 10	BSCC 11	BSCC 12	BSCC 13
***Salmonella* serovar**	*Salmonella* Senftenberg	S. Saintpaul	S. Reading	S. Saintpaul	S. Reading	S. Reading	*Salmonella* Senftenberg	S. Stanley	S. Stanley	S. Sarajane	S. Saintpaul	S. Sandiego	S. Saintpaul
1. Statement of acid (a) & gas (g)													
a. Dulcitol	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
b. Glucose	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
c. Inositol	-, -	a, g	a, g	a, g	a, g	a, -	a, g	-, -	-, -	-, -	a, g	a, g	a, g
d. Lactose	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
e. Maltose	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
f. Mannitol	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
g. Sucrose	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -	-, -
2. Nitrate reduction test	+	+	+	+	+	+	+	+	+	+	+	+	+
3. Indole production test	-	-	-	-	-	-	-	-	-	-	-	-	-
4. MR test	+	+	+	+	+	+	+	+	+	+	+	+	+
5. VP test	-	-	-	-	-	-	-	-	-	-	-	-	-
6. Citrate utilisation test	+	+	+	+	+	+	+	+	+	+	+	+	+
7. Urease test	-	-	-	-	-	-	-	-	-	-	-	-	-
8. Gelatin liquefaction test	-	-	-	-	-	-	-	-	-	-	-	-	-
9. H_2S in TSI slant	+	+	+	+	+	+	+	+	+	+	+	+	+
10. Galactosidase test	-	-	-	-	-	-	+	-	-	-	-	-	-
11. Oxidase test	+	+	+	+	+	+	+	+	+	+	+	+	+
12. Phenyalanine test	-	-	-	-	-	-	-	-	-	-	-	-	-
13. Lysine test	+	+	+	+	+	+	+	+	+	+	+	+	+
14. Malonate test	-	-	-	-	-	-	-	-	-	-	-	-	-
15. Poly O antiserum	+	+	+	+	+	+	+	+	+	+	+	+	+
16. Poly H antiserum	+	+	+	+	+	+	+	+	+	+	+	+	+

Contd...

Test \ Isolate code	BSCC 14	BSCC 15	BSCC 16	BSCC 17	BSCC 18	BSCC 19	BSCC 20	BSCC 21	BSCC 22	BSCC 23	BSCC 24	BSCC 26	BSCC 27
***Salmonella* serovar**	S. Stanley	S. Stanley	S. Heidelberg	*Salmonella* Reinickendorf	S. Bradford	*Salmonella* Typhimurium	S. Ohio	S. Ohio	S. Ohio	S. Ohio	S. Ohio	S. Ohio	S. Ohio
1. Statement of acid (a) & gas (g)													
a. Dulcitol	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
b. Glucose	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
c. Inositol	-, -	-, -	-, -	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
d. Lactose	-, -	-, -	-, -	-, -	a, g	a, g	-, -	-, -	-, -	-, -	-, -	-, -	-, -
e. Maltose	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
f. Mannitol	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g	a, g
g. Sucrose	-, -	-, -	-, -	-, -	a, g	a, g	-, -	-, -	-, -	-, -	-, -	-, -	-, -
2. Nitrate reduction test	+	+	+	+	+	+	+	+	+	+	+	+	+
3. Indole production test	-	-	-	-	-	+	-	-	-	-	-	-	-
4. MR test	+	+	+	+	-	+	+	+	+	+	+	+	+
5. VP test	-	-	-	-	+	-	-	-	-	-	-	-	-
6. Citrate utilisation test	+	+	+	+	+	+	+	+	+	+	+	+	+
7. Urease test	-	-	-	-	-	-	-	-	-	-	-	-	-
8. Gelatin liquefaction test	-	-	-	-	-	-	-	-	-	-	-	-	-
9. H_2S in TSI slant	+	+	+	+	+	-	+	+	+	+	+	+	+
10. Galactosidase test	-	-	-	-	±	-	-	-	-	-	-	-	-
11. Oxidase test	+	+	+	+	+	+	+	+	+	+	+	+	+
12. Phenylalanine test	-	-	-	-	-	-	-	-	-	-	-	-	-
13. Lysine test	+	+	+	+	+	+	+	+	+	+	+	+	+
14. Malonate test	-	-	-	-	-	-	-	-	-	-	-	-	-
15. Poly O antiserum	+	+	+	+	+	+	+	+	+	+	+	+	+
16. Poly H antiserum	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 19. Antibiotic susceptibility profile of the *Salmonella* isolates

Isolate code	Salmonella serovar	No. of antibiotics			MAR Index	Remarks*
		Resistant	Intermediate	Susceptible		
BSCC1	S. Senftenberg	7	3	12	0.32	a
BSCC2	S. Saintpaul	5	7	10	0.22	a
BSCC3	S. Reading	6	8	8	0.27	a
BSCC4	S. Saintpaul	7	5	10	0.32	a
BSCC5	S. Reading	7	4	11	0.32	a
BSCC6	S. Reading	5	4	13	0.22	a
BSCC7	S. Senftenberg	5	8	9	0.22	a
BSCC8	S. Stanley	5	7	10	0.22	a
BSCC9	S. Stanley	7	5	10	0.32	a
BSCC10	S. Sarajane	4	7	11	0.18	
BSCC11	S. Saintpaul	5	8	9	0.22	a
BSCC12	S. Sandiego	4	5	13	0.18	
BSCC13	S. Saintpaul	5	3	14	0.22	a
BSCC14	S. Stanley	3	6	13	0.14	
BSCC15	S. Stanley	4	6	12	0.18	
BSCC16	S. Heidelberg	4	7	11	0.18	a
BSCC17	S. Reinickendorf	7	6	9	0.32	a
BSCC18	S. Bradford	7	6	9	0.32	a
BSCC19	S. Typhimurium	7	5	10	0.32	a
BSCC20	S. Ohio	12	3	7	0.54	a, MAR
BSCC21	S. Ohio	11	5	6	0.50	a, MAR
BSCC22	S. Ohio	12	3	7	0.54	a, MAR
BSCC23	S. Ohio	12	4	6	0.54	a, MAR
BSCC24	S. Ohio	13	3	6	O.59	a, MAR
BSCC26	S. Ohio	13	3	6	0.59	a, MAR
BSCC27	S. Ohio	13	1	8	0.59	a, MAR

* a : Originated from high risk source of contamination
MAR : Multiple Antibiotic Resistant strain

Table 20. Prevalence of antibiotic resistance among *Salmonella* spp. in fish and fishery environment

Antibiotic	Code	Content (mcg)	Resistant isolates (%)[a]	Intermediate isolates (%)	Sensitive isolates (%)
Penicillin G	P	10 units	100	--	--
Ampicillin	A	10	30.8	50.0	19.2
Cephalexin	Cp	30	30.8	69.2	--
Cephotaxime	Ce	30	26.9	7.7	65.4
Vancomycin	Va	30	92.3	--	7.7
Streptomycin	S	10	7.7	30.8	61.5
Kanamycin	K	30	30.8	38.4	30.8
Amikacin	Ak	30	26.9	7.7	65.4
Neomycin	N	30	--	69.2	30.8
Gentamycin	G	10	30.8	15.4	53.8
Tetracycline	T	30	26.9	61.5	11.5
Oxytetracycline	O	30	3.8	65.4	30.8
Chloramphenicol	C	30	3.8	--	96.2
Erythromycin	E	10	100	--	--
Norfloxacin	Nx	10	--	--	100
Nalidixic acid	Na	30	--	11.5	88.5
Ciprofloxacin	Cf	10	--	--	100
Levofloxacin	Le	5	--	--	100
Co-trimoxazole	Co	25	--	--	100
Furazolidone	Fr	50	80.5	19.2	--
Nitrofurantoin	Nf	300	96.2	3.8	--
Colistin	Cl	10	42.3	57.7	

[a] % = Percentage of the total No. of *Salmonella* isolates tested (i.e., 26)

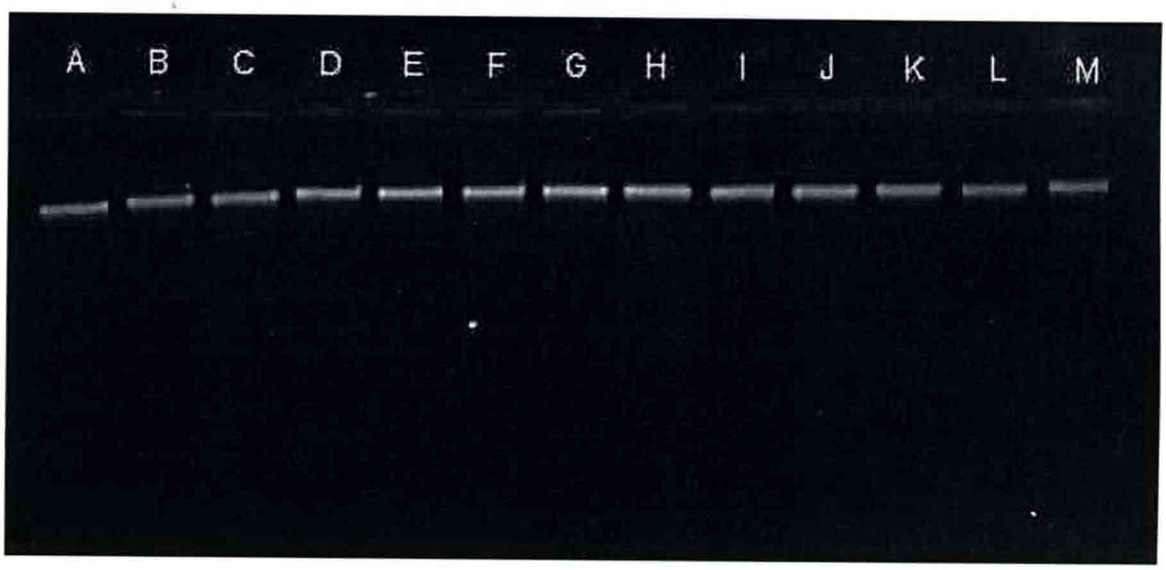

Fig. 1. Genomic DNA (approx. 100 ng each) isolated from few bacteria and run on 0.8% agarose gel

A – *S.* Typhi
B – *S.* Paratyphi
C – *S.* Typhimurium
D – *S.* Enteritidis
E – *S.* Abony
F – *E. coli*
G – *V. cholerae*
H – *V. vulnificus*
I - *V. parahaemolyticus*
J – *A. hydrophila*
K – *P. mirabilis*
L – *S. aureus*
M – *M. morganii*

4.4. Development of *Salmonella* spp. Specific PCR Reaction

4.4.1. PCR screening of primers for the test of specificity (Phase I)

Results of the test for specificity of *Salmonella* spp. detection with primer set A (L-INVA & R-INVA), set B (BS1- F & BS1-R) and set C (BS2-F & BS2-R) have been presented in Fig. 2, Fig. 3 and Fig. 4, respectively. As it is seen in Fig. 4, the performance of primer set C in the first phase screening was satisfactory. The pair of primers repeatedly produced the *Salmonella* spp. specific 403 bp band with all the 17 *Salmonella* spp. while there were no false positives with any of the non-*Salmonella* strains tested. Therefore, this pair of primers was short-listed as the best pair of primers tested so far.

4.4.2. Evaluation of bacterial cell lysate for PCR reaction

The PCR reaction carried out with primer set C by taking bacterial cell lysate of 13 standard *Salmonella* strains have been given in Fig. 5. Since all the bacterial cell lysate produced the specific 403 bp PCR band repeatedly even after the change of Thermocycler, all further *Salmonella* spp. specific PCR was carried out taking bacterial cell lysate as the template DNA.

4.4.3. Evaluation of the PCR method using *Salmonella* isolates

The PCR reaction carried out with primer set C using bacterial cell lysate of the *Salmonella* isolates as the template has been given in Fig. 6. All the twenty six *Salmonella* spp. isolated in the present study produced consistently the specific 403 bp PCR product. This shows the total agreement between the conventional culture-based method and the PCR-based method for detecting the *Salmonella* strains.

Evaluation of the PCR method was also done using the cell lysate prepared from shrimp and kamaboko homogenates that were artificially seeded with one representative of each serovars isolated in the present study. Fig. 7 depicts that the PCR method could detect all these serovars for the presence of *Salmonella* in complex tissue homogenate

4.4.4. PCR screening of primers for the test of specificity (Phase II)

The results of the PCR reaction carried out with primer set C using the bacterial cell lysate of additional *Salmonella* and non-*Salmonella* strains are given in Fig. 8. The specific 403 bp band was obtained with all the additional *Salmonella*

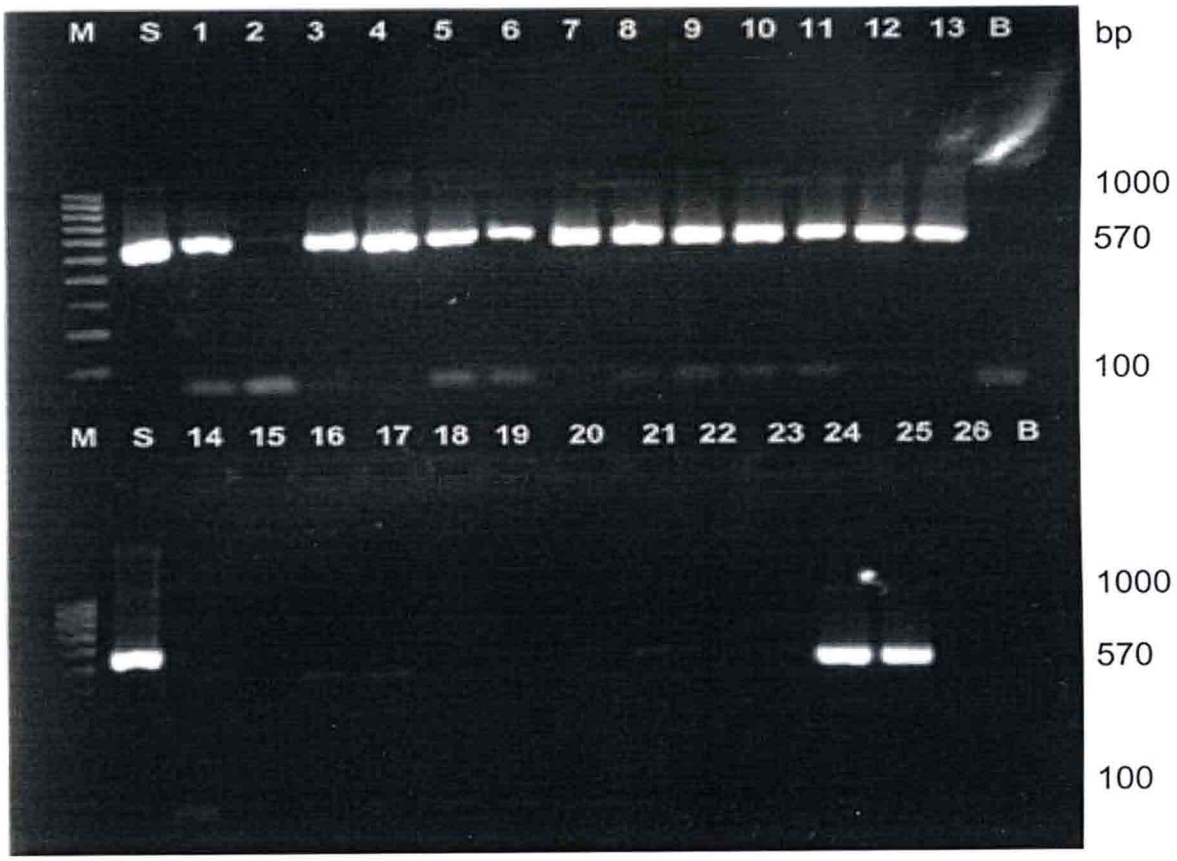

Fig. 2. Evaluation of primer set A for *Salmonella* spp. specific PCR detection

M - 100 bp DNA ladder
S - *S.* Typhi (std.)
1 - *S.* Paratyphi
2 - *S.* Paratyphi A
3 - *S.* Paratyphi B
4 - *S.* Typhimurium
5 - *S.* Enteritidis
6 - *S.* Kentuky
7 - *S.* Abony
8 - *S.* Virchow
9 - *S.* Infantis
10 - *S.* Brunei
11 - *S.* Arizonae
12 - *S.* Weltevreden
13 - *S.* Newport
14 - *E. coli*
15 - *V. cholerae*
16 - V.*parahaemolyticus*
17 - *V. vulnificus*
18 - *A. hydrophilla*
19 - *P. mirabilis*
20 - *K. pneumonae*
21 - *M. morganii*
22 - *H. alvei*
23 - *Staph. aureus*
24 - *S.* Paratyphi
25 - *Salmonella* I.
26 - *S.* I. monophasic
B - Blank

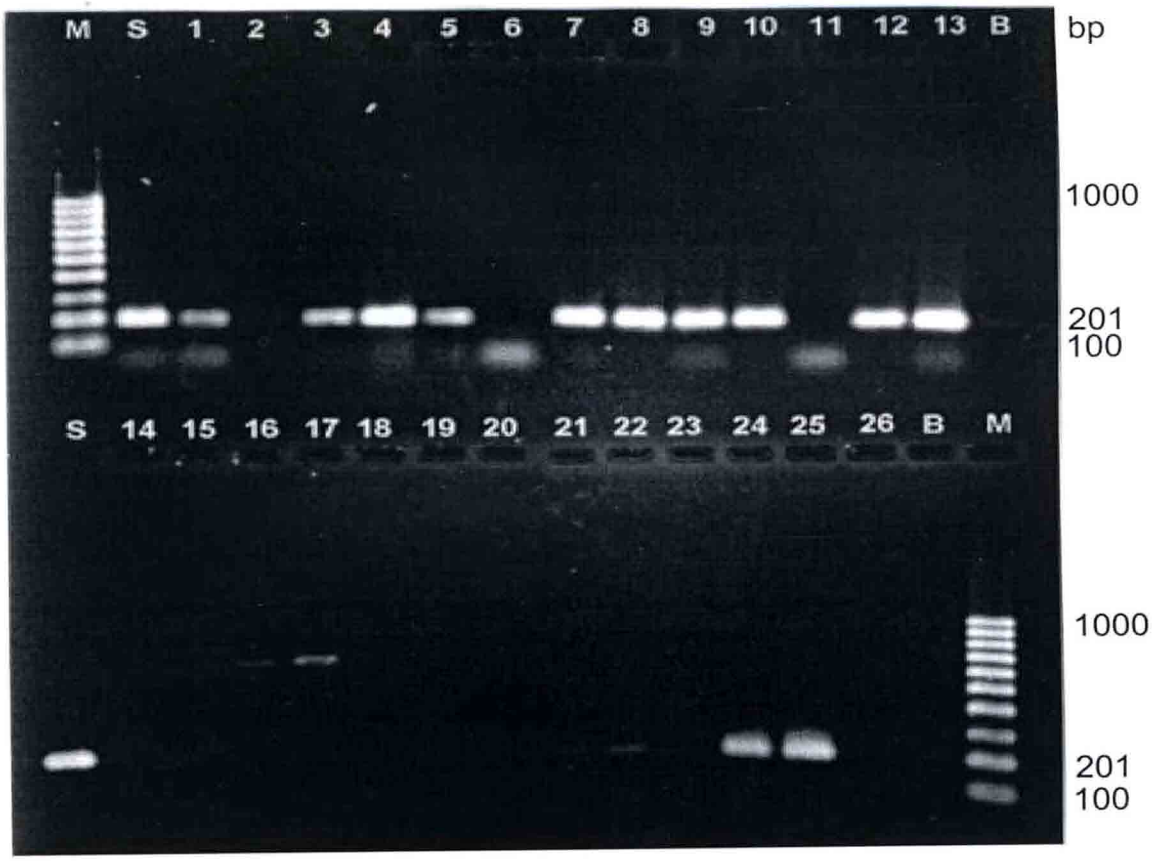

Fig. 3. Evaluation of primer set B for *Salmonella* spp. specific PCR detection

M - 100 bp DNA ladder
S - *S.* Typhi (std.)
1 – *S.* Paratyphi
2 - *S.* Paratyphi A
3 - *S.* Paratyphi B
4 - *S.* Typhimurium
5 - *S.* Enteritidis
6 - *S.* Kentuky
7 - *S.* Abony
8 - *S.* Virchow
9 - *S.* Infantis
10 - *S.* Brunei
11 - *S.* Arizonae
12 - *S.* Weltevreden
13 - *S.* Newport
14 - *E. coli*
15 - *V. cholerae*
16 - V.*parahaemolyticus*
17 - *V. vulnificus*
18 - *A. hydrophilla*
19 - *P. mirabilis*
20 - *K. pneumonae*
21 - *M. morganii*
22 - *H. alvei*
23 - *Staph. aureus*
24 - *S. paratyphi*
25 - *Salmonella* I.
26 - *S.* I. monophasic
B - Blank

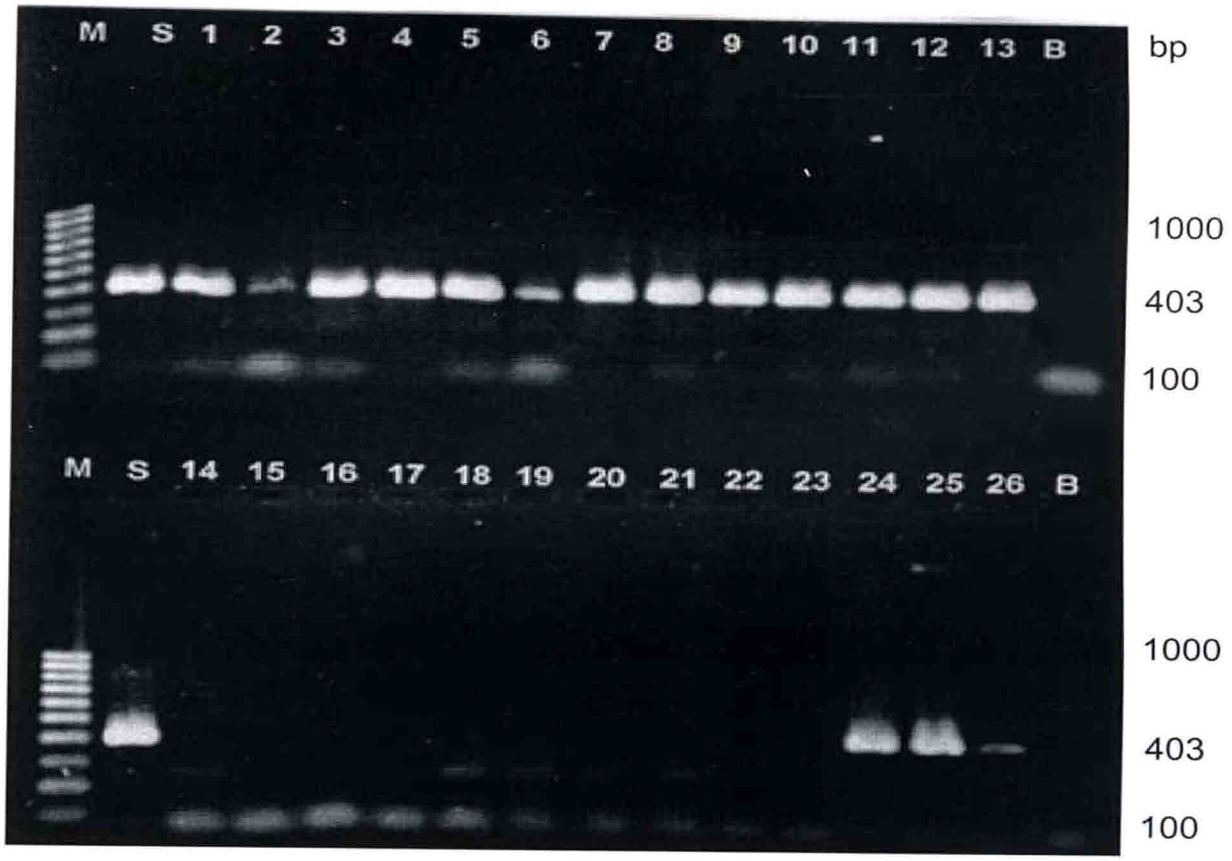

Fig. 4. Evaluation of primer set C for *Salmonella* spp. specific PCR detection

M - 100 bp DNA ladder
S - *S.* Typhi (std.)
1 – *S.* Paratyphi
2 - *S.* Paratyphi A
3 - *S.* Paratyphi B
4 - *S.* Typhimurium
5 - *S.* Enteritidis
6 - *S.* Kentuky
7 - *S.* Abony
8 - *S.* Virchow
9 - *S.* Infantis
10 - *S.* Brunei
11 - *S.* Arizonae
12 - *S.* Weltevreden
13 - *S.* Newport
14 - *E. coli*
15 - *V. cholerae*
16 - V.*parahaemolyticus*
17 - *V. vulnificus*
18 - *A. hydrophilla*
19 - *P. mirabilis*
20 - *K. pneumonae*
21 - *M. morganii*
22 - *H. alvei*
23 - *Staph. aureus*
24 - *S. paratyphi*
25 - *Salmonella* I.
26 - *S.* I. monophasic
B - Blank

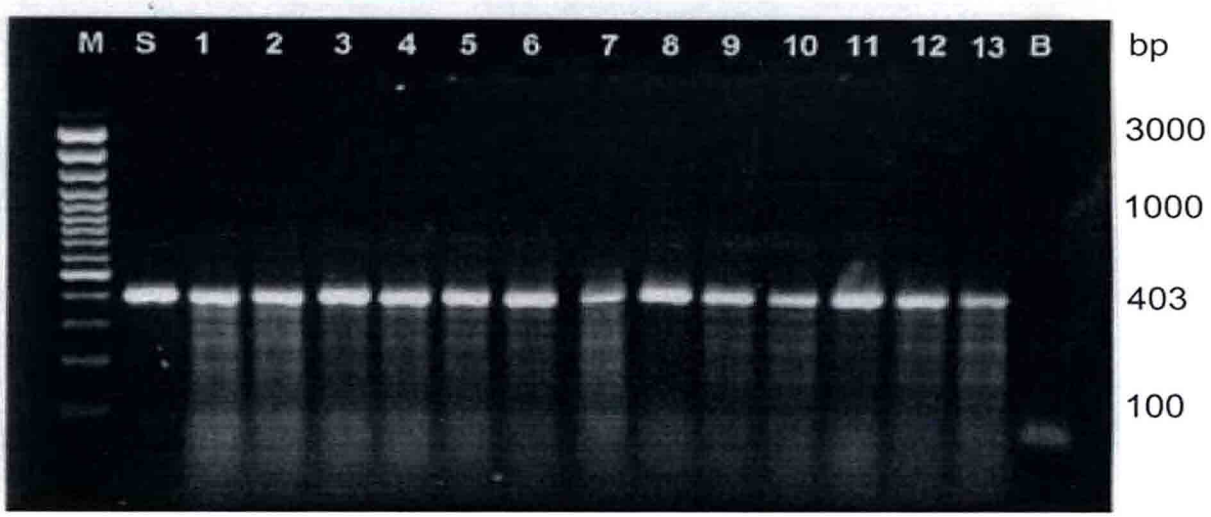

Fig. 5. Evaluation of bacterial cell lysate as template DNA in PCR reaction using primer set C

S - *S.* Typhi (std.)	5 - *S.* Enteritidis	10 - *S.* Brunei
1 - *S.* Paratyphi	6 - *S.* Kentuky	11 - *S.* Arizonae
2 - *S.* Paratyphi A	7 - *S.* Abony	12 - *S.* Weltevreden
3 - *S.* Paratyphi B	8 - *S.* Virchow	13 - *S.* Newport
4 - *S.* Typhimurium	9 - *S.* Infantis	B. Blank

M - DNA molecular weight marker

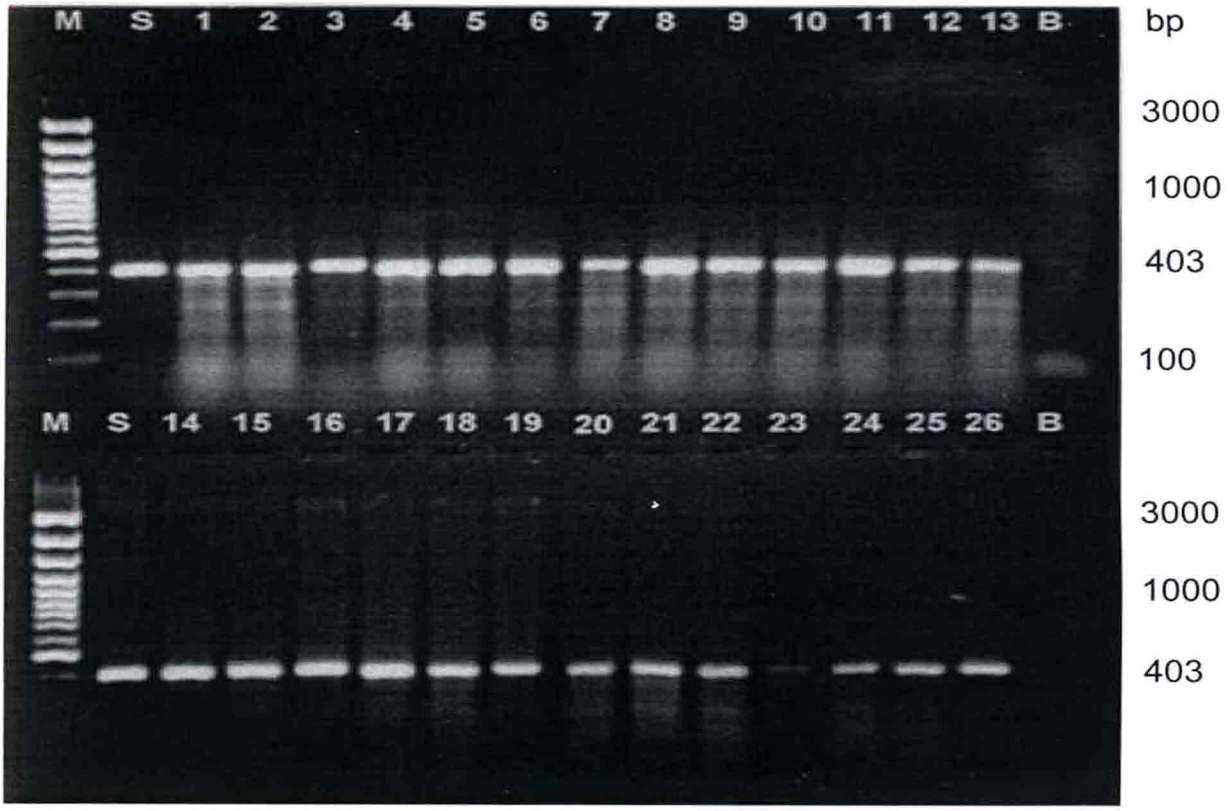

Fig. 6. PCR-based detection of the *Salmonella* spp. isolated in the present study using primer set C

S. *S.* Typhi (Std)
M. Mol. weight marker
1. *S.* Senftenberg
2. *S.* Saintpaul
3. *S.* Reading
4. *S.* Saintpaul
5. *S.* Reading
6. *S.* Reading
7. *S.* Senftenberg
8. *S.* Stanley
9. *S.* Stanley
10. *S.* Sarajane
11. *S.* Saintpaul
12. *S.* Sandiego
13. *S.* Saintpaul
14. *S.* Stanley
15. *S.* Stanley
16. *S.* Heidelberg
17. *S.* Reinickendorf
18. S. Bradford
19. *S.* Typhimurium
20. *S.* Ohio
21. *S.* Ohio
22. *S.* Ohio
23. *S.* Ohio
24. *S.* Ohio
25. *S.* Ohio
26. *S.* Ohio
B. Blank

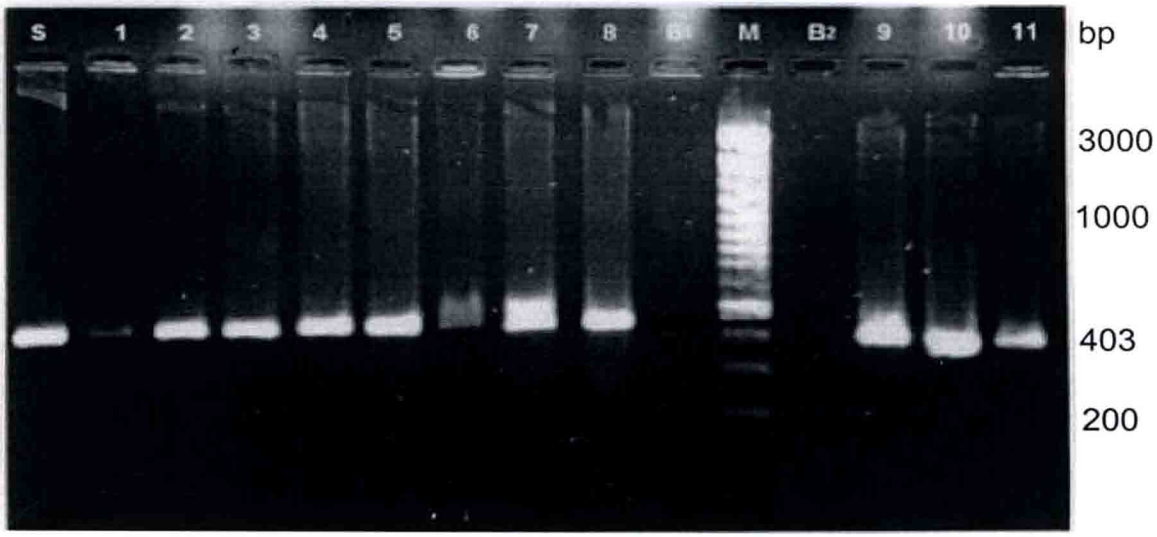

Fig. 7. PCR-based detection of *Salmonella* spp. in artificially seeded shrimp (Well No. 1 to 8) and kamaboko (Well No. 9 to 11) homogenate

S - *S.* Typhi (std.)
1 - *S.* Senftenberg
2 - *S.* Saintpaul
3 - *S.* Reading
4 - *S.* Stanley
5 - *S.* Sarajane
6 - *S.* Sandiego
7 - *S.* Heidelberg
8 - *S.* Reinickendorf
B_1- Shrimp homogenate
M - Mol. weight marker
B_2- Kamaboko homogenate
9 - *S.* Bradford
10 - *S.* Typhimurium
11 - *S.* Ohio

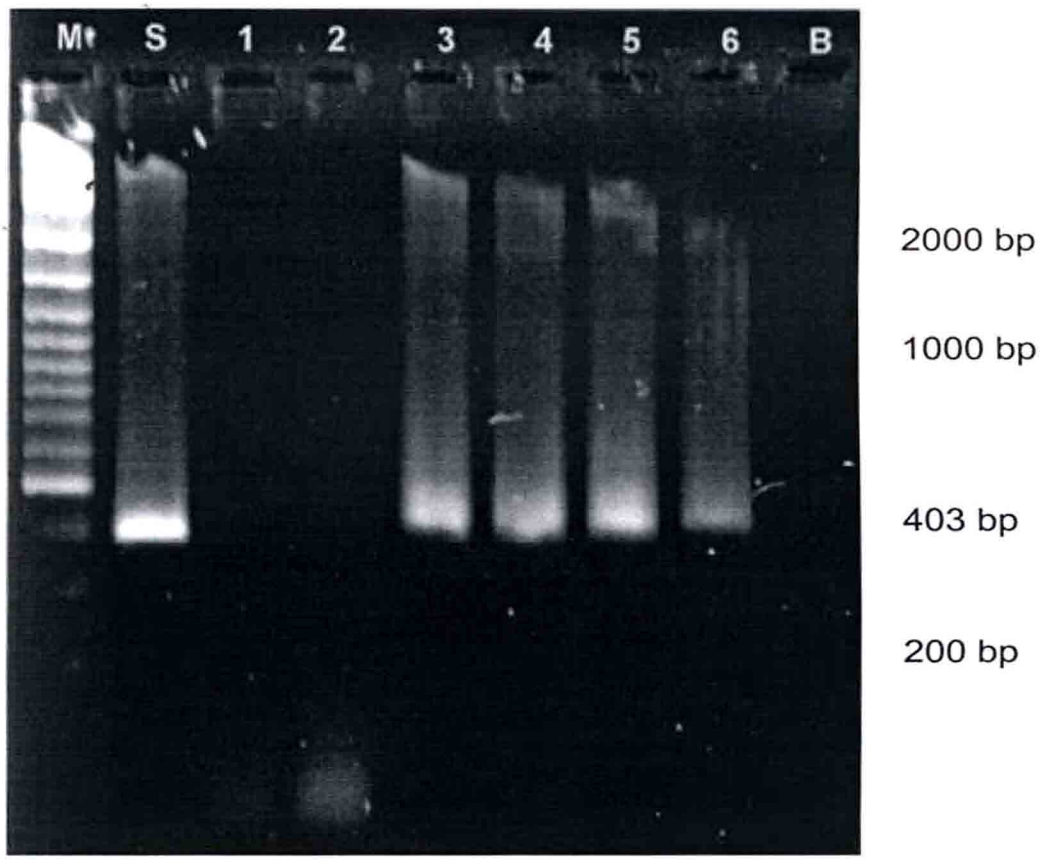

Fig. 8. Evaluation of primer set C (Phase II) for *Salmonella* spp. specific PCR detection using additional standard strains

S - *S.* Typhi (std.)
1 - *V. cholerae* (non-pathogenic)
2 - *E. coli* EHEC O157:H7
3 - *S.* Paratyphi
4 - *S.* Typhimurium
5 - *S.* Gallinarum
6 - *S.* Typhimurium
B - Blank
M - DNA molecular weight marker

strains while there were no false positives. All the above evaluations for primer set C were repeated in duplicate in the second thermocycler used and the results were reproducible without any ambiguity (data not shown)

4.5. PCR Analysis of Biochemically and/or Serologically Atypical Isolates

Three presumptive *Salmonella* isolates (BSCC25, BSCC28 and BSCC29) that were atypical in terms of *Salmonella* specific biochemical and/or serological reactions by one or two tests only, were cross-checked by the *Salmonella* spp. specific PCR method developed in the present study. Results of the detailed biochemical and serological analysis have been presented in Table 21. PCR analysis of these isolates using purified genomic DNA has been presented in Fig. 9. The *Salmonella* specific 403 bp band was produced with all the three isolates and the isolates BSCC 25, BSCC 28 and BSCC 29 were designated as *Salmonella* atypical I (AT I), *Salmonella* atypical II (AT II) and *Salmonella* atypical III (AT III), respectively.

4.6. Analysis of Atypical *Salmonella* Isolates by Sequencing

4.6.1. Cloning of PCR amplicon

The PCR product obtained with one of the atypical *Salmonella* isolates (AT III) was directly cloned to T/A cloning vector (pTZ57R/T) and then transformed into *E. coli* (DH5α). A good transformation efficiency of $1x10^4$ transformants/µg plasmid was found. Appearance of the recombinant clones as white and non-recombinant clones as blue colonies on LB-Ampicillin plate containing IPTG and X Gal, has been shown in Fig. 10.

4.6.2. Analysis of recombinant clones

Few recombinant clones (isolated white colonies) were picked, grown in LB-ampicillin medium and subjected to plasmids isolation followed by restriction endonuclease analysis (REA) (Fig. 11). The figure depicts that the uncut cloned plasmid (3.29 kb) (well code U) was running behind the plane vector plasmid (2.86 kb) (well code P). After double digestion with *Bam*H I and Xba I, the linearised plasmid vector (2.86 kb) and the insert (403) bp are seen in well C.

Table. 21. Biochemical and serological characteristics of atypical *Salmonella* isolates

***Salmonella* isolate** / **Biochemical tests**	**BSCC 19**	**BSCC 25**	**BSCC 28**	**BSCC 29**
	***Salmonella* (reference isolate)**	***Salmonella* atypical I** (AT I)	***Salmonella* atypical II** (AT II)	***Salmonella* atypical III** (AT III)
1. Statement of acid & gas				
a. Dulcitol	a, g	a, g	a, g	a, -
b. Glucose	a, g	a, g	a, g	a, g
c. Inositol	-, -	-, -	a, g	-, -
d. Lactose	-, -	a, g *	-, -	-, -
e. Maltose	a, g	a, g	a, g	a, g
f. Mannitol	a, g	a, g	a, g	a, -
g. Sucrose	-, -	a, g *	a, g *	-, -
2. Nitrate reduction test	+	+	+	+
3. Indole production test	-	-	-	-
4. MR test	+	- *	- *	+
5. VP test	-	+ *	+ *	-
6. Citrate utilization test	+	+	+	+
7. Urease test	-	-	-	-
8. Gelatin liquefaction test	-	-	-	-
9. H_2S in TSI	+	-	+	+
10. Galactosidase test	-	+	+	-
11. Oxidase test	+	+	+	+
12. Phenylalanine test	-	-	-	-
13. Lysine test	+	-	-	+
14. Malonate test	-	-	+	-
15. Motility test	-	±	-	-
16. Poly O antiserum	+	+ *	+	+
17. Poly H antiserum	+	±	+	+
18. Detail serological analysis at NSC, IVRI	*Salmonella* Typhimurium	Not *Salmonella*	Not *Salmonella*	Not * *Salmonella*

* Test result differing from the normal *Salmonella* reactions

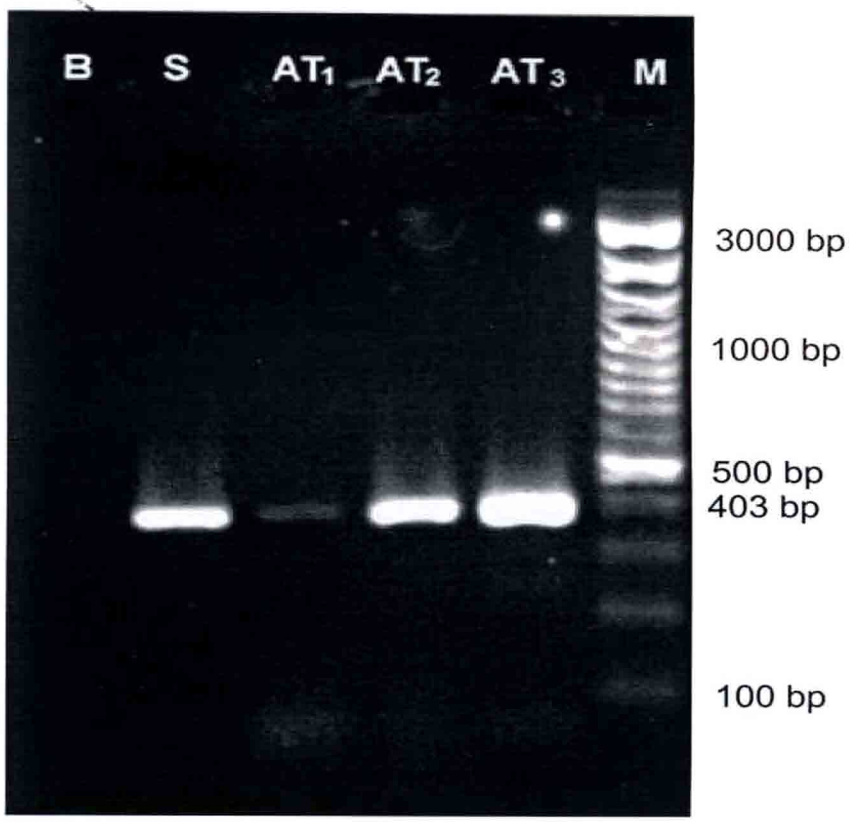

Fig. 9. PCR analysis of biochemically and/or serologically atypical *Salmonella* isolates

B – Blank

S – *S.* Typhi (std)

M – Molecular weight marker

AT_1 – *Salmonella* atypical I (ATI)

AT_2 – *Salmonella* atypical II (ATII)

AT_3 – *Salmonella* atypical III (ATIII)

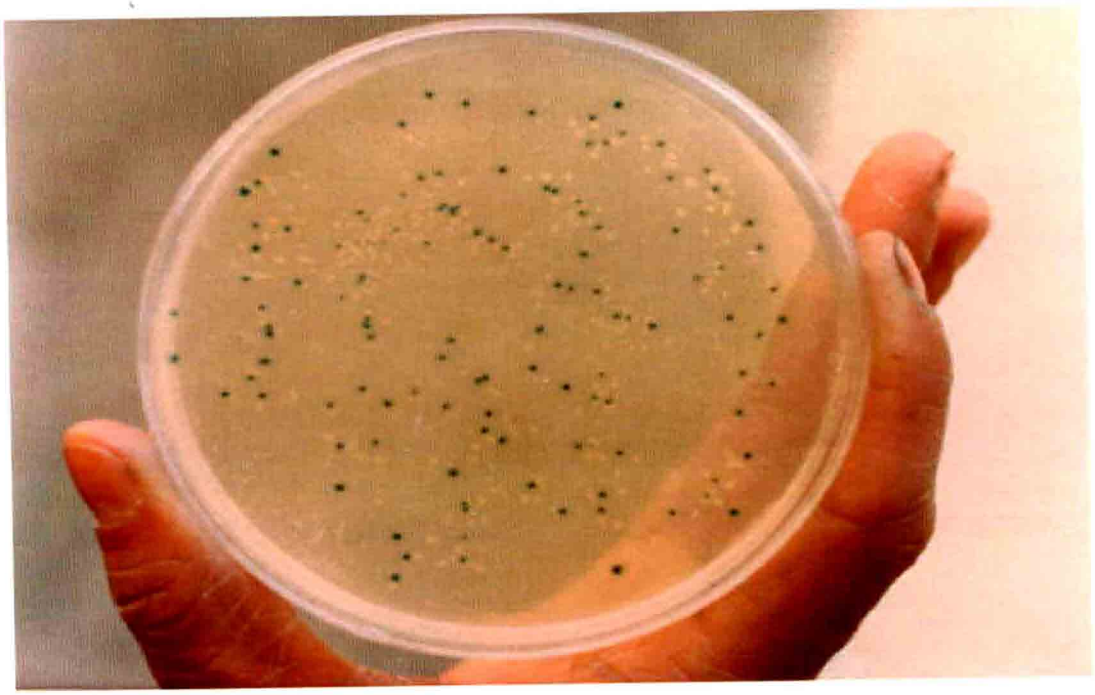

Fig. 10. Blue/white selection of recombinant clones on LB-ampicillin plates with IPTG/X Gal

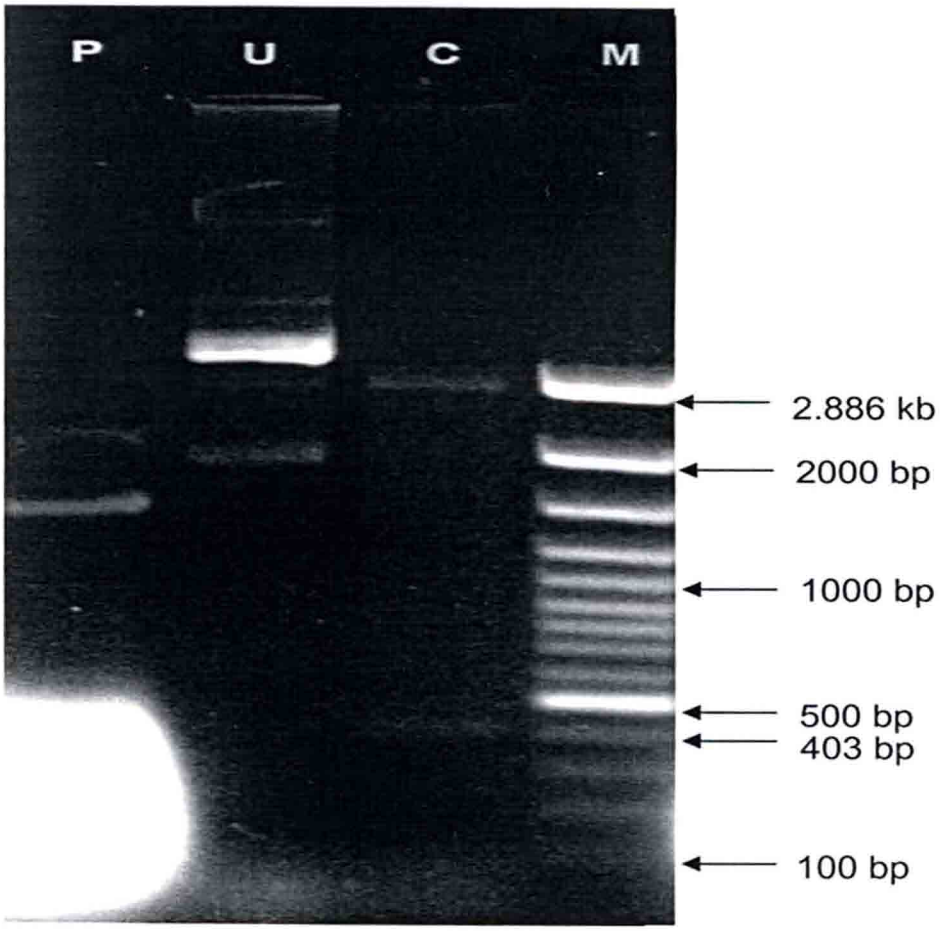

Fig. 11. Restriction endonuclease analysis of the recombinant plasmid DNA

P – Plasmid vector pTZ57R

U – Uncut recombinant plasmid

C – Linearised plasmid after double digestion with *Bam*HI and *Xba*I

M – 100 bp DNA ladder

4.6.3. Sequencing of PCR amplicon

Nucleotide sequencing of the purified PCR product as well as the cloned plasmid DNA was carried out using BigDye® Terminator v 3.1 cycle sequencing kit at Molecular Biology Unit, NCCS, Pune and 340 bp unambiguous sequence (Fig. 12) was obtained.

4.6.4. Analysis of the deduced sequence

The sequence producing significant alignment after random BLAST of the 340 bp nucleotide sequence in the GenBank has been presented in Table 22. As it is seen from the table, the nucleotide sequence has matched at higher score, i.e., 369 and above, only with invasive gene sequences of different *Salmonella* spp., and no other sequence in the GenBank database matched with similarity score above 58.

Salmonella spp. sequences that matched with the deduced sequence were retrieved and the particular segment of the subject sequences matching with the deduced sequence was traced (Table 23). The sequences were then submitted for multiple alignment in the GenBank database in ClustralW (1.82) multiple sequence alignment programme (Table 24). It is evident in the table that, a portion of the deduced sequence (i.e., from position 14 to 340) had near perfect matching (more than 97%) with the various *Salmonella* spp. invasive gene sequences.

4.7. Molecular Characterisation of *Salmonella* Strains by PCR Ribotyping

The results of PCR ribotyping have been given in Fig. 13, 14 and15 and the ribotype pattern has been analysed in Table 25. Out of the total 40 strains belonging to 28 serotypes that were used for PCR ribotyping, only 34 strains belonging to 23 serotyping produced the polymorphism. The polymorphism of 34 strains has been grouped into 29 PCR ribotypes (Table 25).

4.8. Evaluation of the *Salmonella* spp. Specific PCR Method in a Multiplex PCR Assay

The multiplex PCR assay involving four pairs of primers, each one specifically targeting *uid*A gene of *E. coli*, *ctx* gene of *V. cholerae*, *inv*A gene of *Salmonella* spp. and *tl* gene of *V. parahaemolyticus* resulted in 147 bp, 302 bp, 403 bp and 450 bp DNA fragments, respectively in a common protocol of PCR ingredient mixture and temperature schedule (Fig. 16). When four primers and four template

aggcatgttttcccacttctttaaaaaaatcaggaaatttcgcttccagttggtccagcatatgttttgtttcctgaataccga
aatattcattgacgttgcgcgccagcgtcaccgccagacagtggtaaagctcatcaagcgcgttccgcaacacatag
ccaagctcccggagtttctccccctcttcatgcgttacccagaaatactgactgctaccttgctgatggattgttggattaat
accaaaggacacgacttcatcggaataatttaccactcgcatcaaatcaaaatagaccgtaaattgttcaacacggat
ctcattaatcaacaatac

Fig. 12. Deduced nucleotide sequence of the PCR amplicon from atypical *Salmonella* isolate III (ATIII)

Table 22. Extract of the nucleotide-nucleotide BLAST of the deduced nucleotide sequence in the GenBank database

Sequences producing significant alignments:	Score (bits)	E Value
gi|16421434|gb|AE008832.1| Salmonella typhimurium LT2, sect...	632	e-178
gi|1236876|gb|U43273.1|SGU43273 Salmonella gallinarum invas...	617	e-174
gi|1236874|gb|U43272.1|SEU43272 Salmonella enterica invasio...	617	e-174
gi|1236804|gb|U43237.1|SEU43237 Salmonella enterica invasio...	617	e-174
gi|154154|gb|M90846.1|STYINVA Salmonella typhimurium invA g...	617	e-174
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	609	e-171
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	609	e-171
gi|1236872|gb|U43271.1|SEU43271 Salmonella enterica invasio...	601	e-169
gi|1236806|gb|U43238.1|SEU43238 Salmonella enterica invasio...	593	e-167
gi|1236826|gb|U43248.1|SEU43248 Salmonella enterica invasio...	543	e-152
gi|1236824|gb|U43247.1|SEU43247 Salmonella enterica invasio...	543	e-152
gi|1236822|gb|U43246.1|SEU43246 Salmonella enterica invasio...	486	e-134
gi|1236808|gb|U43239.1|SEU43239 Salmonella enterica invasio...	486	e-134
gi|1236834|gb|U43252.1|SEU43252 Salmonella enterica invasio...	482	e-133
gi|1236832|gb|U43251.1|SEU43251 Salmonella enterica invasio...	474	e-131
gi|1236830|gb|U43250.1|SEU43250 Salmonella enterica invasio...	468	e-129
gi|1236828|gb|U43249.1|SEU43249 Salmonella enterica invasio...	460	e-127
gi|1236814|gb|U43242.1|SEU43242 Salmonella enterica invasio...	404	e-110
gi|1236820|gb|U43245.1|SEU43245 Salmonella enterica invasio...	396	e-107
gi|1236818|gb|U43244.1|SEU43244 Salmonella enterica invasio...	396	e-107
gi|1236816|gb|U43243.1|SEU43243 Salmonella enterica invasio...	396	e-107
gi|1236812|gb|U43241.1|SEU43241 Salmonella enterica invasio...	369	2e-99
gi|1236810|gb|U43240.1|SEU43240 Salmonella enterica invasio...	369	2e-99
gi|34103846|gb|AE016919.1| Chromobacterium violaceum ATCC 1...	58	1e-05
gi|4760332|emb|AL023704.2|SPCC553 S.pombe chromosome III co...	54	2e-04
gi|728525|emb|Z48621.1|CER07B1 Caenorhabditis elegans cosmi...	42	0.65
gi|12191184|emb|AL359273.11| Human DNA sequence from clone ...	42	0.65
gi|20068563|emb|AL607127.12| Mouse DNA sequence from clone ...	42	0.65
gi|24817647|emb|AL928758.3|CNS08CBV Oryza sativa chromosome...	40	2.6
gi|24197313|gb|AE011497.1| Leptospira interrogans serovar 1...	40	2.6
gi|21206069|gb|AC092490.10| Homo sapiens 12 BAC RP11-20D14 ...	40	2.6
gi|1495276|emb|Z71928.1|BSYVEFGNS B.subtilis pnbA, sigL, yv...	40	2.6
gi|1945641|emb|Z94043.1|BSZ94043 B.subtilis genomic DNA fra...	40	2.6
gi|2125817|emb|X99452.1|LEDIF54 L.esculentum mRNA for exten...	40	2.6
gi|13539193|emb|AL139020.5|CNS01DX2 Human chromosome 14 DNA...	40	2.6
gi|33146037|emb|AL929211.18| Mouse DNA sequence from clone ...	40	2.6
gi|23379824|gb|AY102286.1| Mus musculus RTN4 (Rtn4) gene, c...	40	2.6
gi|21672116|gb|AC092758.3| Papio anubis clone RP41-22J16, c...	40	2.6
gi|32468825|emb|Z99121.2|BSUB0018 Bacillus subtilis complet...	40	2.6
gi|27497219|gb|AC091621.4| Papio anubis clone RP41-57J19, c...	40	2.6
gi|3281967|emb|Z99129.1|HS425C14 Human DNA sequence from cl...	40	2.6
gi|17975438|gb|AC079305.6| Homo sapiens BAC clone RP11-483K...	40	2.6
gi|27262769|emb|AL929371.6| Mouse DNA sequence from clone R...	40	2.6
gi|34494988|gb|AC124412.3| Mus musculus BAC clone RP24-274J...	38	10
gi|28855325|gb|AE016874.1| Pseudomonas syringae pv. tomato ...	38	10
gi|14010946|gb|AC012372.4| Homo sapiens BAC clone CTD-2568K...	38	10
gi|34915123|ref|NM_194020.1| Oryza sativa (japonica cultiva...	38	10
gi|21615440|emb|Z73424.2|CEC44B9 Caenorhabditis elegans cos...	38	10
gi|38490507|emb|BX005012.7| Zebrafish DNA sequence from clo...	38	10
gi|21108606|gb|AE011873.1| Xanthomonas axonopodis pv. citri...	38	10

Table 23. Particulars of the sequences used for multiple alignment

Seq. Code	Source sequence (web-link)	Total length (bp)	Matching region (bp)	
			From	To
TestSeq.	PCR amplicon of atypical *Salmonella* III (ATIII)	340	01	340
AE008832	gi|16421434|gb|AE008832.1| Salmonella typhimurium LT2, section 136 of 220 of the complete genome	23125	7459	7785
STYINVA	gi|154154|gb|M90846.1|STYINVA Salmonella typhimurium invA gene, complete cds	2176	1318	1644
SEU43237	gi|1236804|gb|U43237.1|SEU43237 Salmonella enterica invasion protein (invA) gene, partial cds.	1950	1216	1542
SGU43273	gi|1236876|gb|U43273.1|SGU43273 Salmonella gallinarum invasion protein (invA) gene, complete cds	1950	1216	1542
SGU43271	gi|1236872|gb|U43271.1|SEU43271 Salmonella enterica invasion protein (invA) gene, partial cds	1950	1216	1542
AE016843	gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enterica serovar Typhi Ty2, section 10 of 16 of the complete genome	301311	172857	173183
AL627276	gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typhi (Salmonella typhi) strain CT18, complete chromosome	274050	188898	189224

Table 24. Multiple alignment of test sequence with few *Salmonella* spp. sequences

clustalw-20040204-10470707.aln: CLUSTAL W (1.82) multiple sequence alignment

```
TestSeq.    AGGCATGTTTTCCCACTTCTTTAAAAAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 60
AE008832    -------------CACTTCTTTAAGTAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 47
STYINVA     -------------CACTTCTTTAAGTAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 47
SEU43237    -------------CACTTCTTTAAGTAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 47
SGU43273    -------------CACTTCTTTAAGTAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 47
SEU43271    ---------^----CACTTCTTTAAGTAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 47
AE016843    -------------CACTTCTTTAAGTAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 47
AL627276    -------------CACTTCTTTAAGTAAATCAGGAAATTTCGCTTCCAGTTGGTCCAGCA 47
                         ***********  ******************************** **

TestSeq.    TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCAGCGTCACCGCCA 120
AE008832    TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCAGCGTCACCGCCA 107
STYINVA     TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCACGGTCACCGCCA 107
SEU43237    TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCACGGTCACCGCCA 107
SGU43273    TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCACGGTCACCGCCA 107
SEU43271    TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCACAGTCACCGCCA 107
AE016843    TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCAGCGTCACCGCCA 107
AL627276    TATGTTTTGTTTCCTGAATACCGAAATATTCATTGACGTTGCGCGCCAGCGTCACCGCCA 107
            ************************************************  **********

TestSeq.    GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 180
AE008832    GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 167
STYINVA     GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 167
SEU43237    GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 167
SGU43273    GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 167
SEU43271    GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 167
AE016843    GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 167
AL627276    GACAGTGGTAAAGCTCATCAAGCGCGTTCCGCAACACATAGCCAAGCTCCCGGAGTTTCT 167
            ************************************************************

TestSeq.    CCCCCTCTTCATGCGTTACCCAGAAATACTGACTGCTACCTTGCTGATGGATTGTTGGAT 240
AE008832    CCCCCTCTTCATGCGTTACCCAGAAATACTGACTGCTACCTTGCTGATGGATTGTTGGAT 227
STYINVA     CCCCCTCTTCATGCGTTACCCAGAAATACTGACTGCTACCTTGCTGATGGATTGTTGGAT 227
SEU43237    CCCCCTCTTCATGCGTTACCCAGAAATACTGACTGCTACCTTGCTGATGGATTGTTGGAT 227
SGU43273    CCCCCTCTTCATGCGTTACCCAGAAATACTGACTGCTACCTTGCTGATGGATTGTTGGAT 227
SEU43271    CCCCCTCTTCATGCGTTACCCAGAAATACTGGCTGCTACCTTGCTGATGGATTGTTGGAT 227
AE016843    CCCCCTCTTCATGCGTTACCCAGAAATACTGACTGCTACCTTGCTGATGGATTGTTGGAT 227
AL627276    CCCCCTCTTCATGCGTTACCCAGAAATACTGACTGCTACCTTGCTGATGGATTGTTGGAT 227
            ******************************** ****************************

TestSeq.    TAATACCAAAGGACACGACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 300
AE008832    TAATACCAAAGGACACGACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 287
STYINVA     TAATACCAAAGGACACGACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 287
SEU43237    TAATACCAAAGGACACGACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 287
SGU43273    TAATACCAAAGGACACGACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 287
SEU43271    TAATACCAAAGGACACGACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 287
AE016843    TAATGCCAAAGGAAACAACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 287
AL627276    TAATGCCAAAGGAAACAACTTCATCGGAATAATTTACCACTCGCATCAAATCAAAATAGA 287
            **** ******** ** ********************************************

TestSeq.    CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 340
AE008832    CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 327
STYINVA     CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 327
SEU43237    CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 327
SGU43273    CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 327
SEU43271    CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 327
AE016843    CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 327
AL627276    CCGTAAATTGTTCAACACGGATCTCATTAATCAACAATAC 327
            ***************************************
```

* Represents total homology of the particular base

$$\text{Degree of homology} = \frac{\text{No. of bases with total homology}}{\text{Total No. of bases under matching}} \times 100 = \frac{318}{327} \times 100 = 97.2\%$$

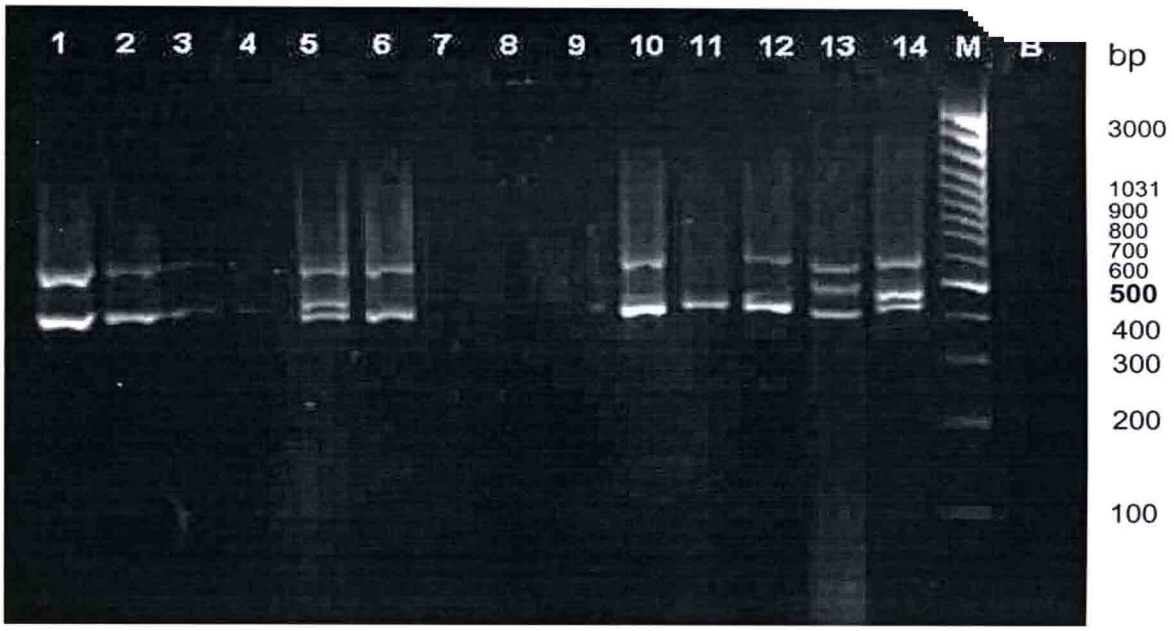

Fig 13. PCR ribotyping of standard *Salmonella* strains

1 - *S.* Typhi	6 - *S.* Enteritidis	11 - *S.* Infantis
2 - *S.* Paratyphi	7 - *S.* Kentuky	12 - *S.* Brunei
3 - *S.* Paratyphi A	8 - *S.* Abony	13 - *S.* Arizonae
4 - *S.* Paratyphi B	9 - Salmonella I.	14 - *S.* Weltevreden
5 - *S.* Typhimurium	10 - *S.* Virchow	B. Blank

M - DNA molecular weight marker

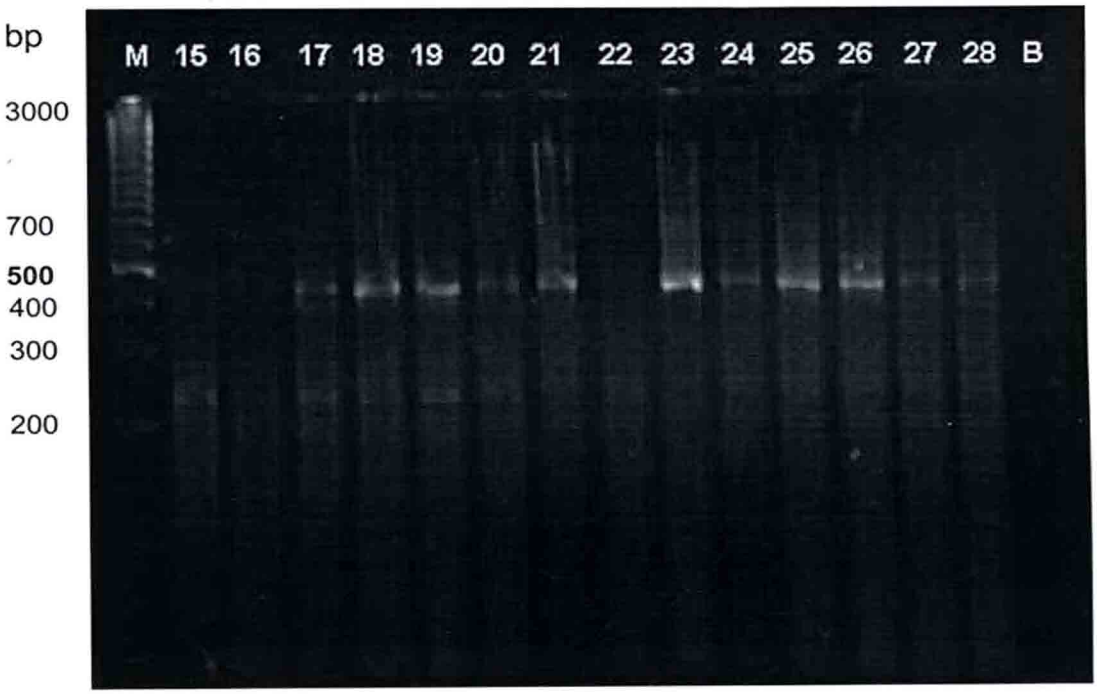

Fig 14. PCR ribotyping of *Salmonella* strains isolated from fish and fish handling environments

15 – *S*. Newport
16 – *S*. Senftenberg
17 – *S*. Saintpaul
18 – *S*. reading
19 – *S*. Saintpaul
20 – *S*. Reading
21 – *S*. Reading
22 – *S*. Senftenberg
23 – *S*. stanley
24 – *S*. Stanley
25 – *S*. Sarajane
26 – *S*. Saintpaul
27 – *S*. Sandiego
28 – *S*. Saintpaul
B – Blank

M – DNA molecular weight marker

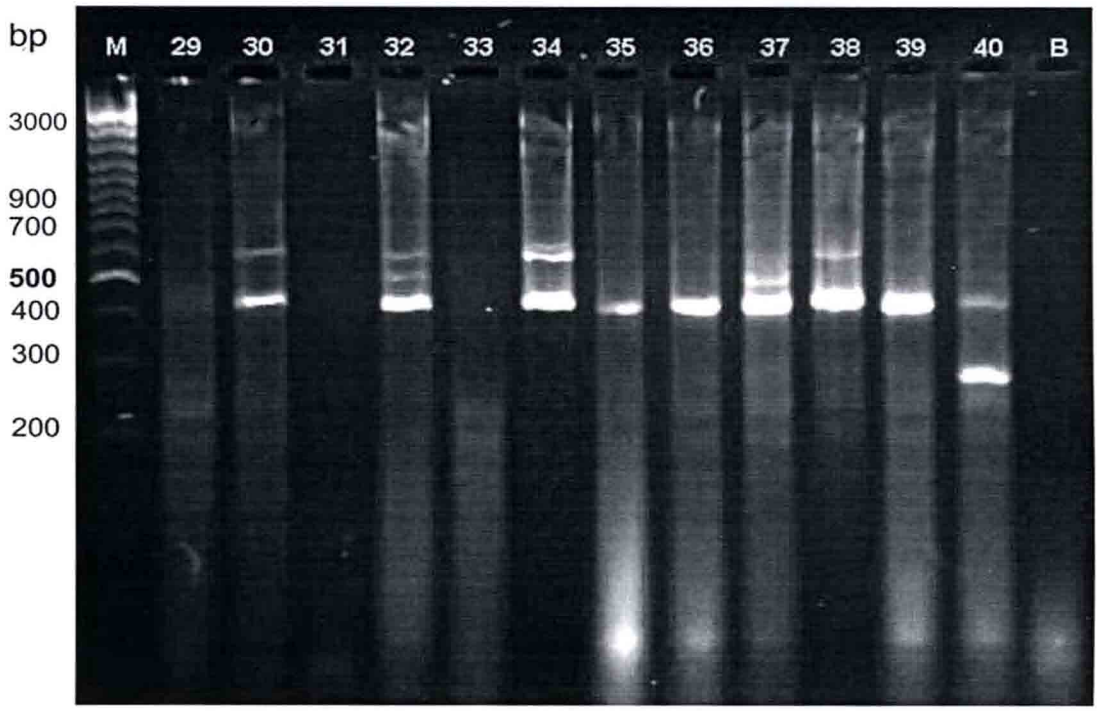

Fig. 15. PCR ribotyping of few reference and isolated *Salmonella* strains

29 – *S.* Gallinarum	33 – *S.* Typhimurium	37 – *S.* Reinckendorf
30 – S. Paratyphi	34 – *S.* Stanley	38 – *S.* Braford
31 – *S.* Typhimurium	35 – *S.* Stanley	39 – *S.* Typhimurium
32 – *S.* I. monophasic	36 – *S.* Heidelberg	40 – *S.* Ohio

M – DNA molecular weight marker

Table 25. PCR ribotype pattern of the reference as well as the isolated *Salmonella* strains

Well code *		**1**	**2**	**3**	**4**	**5**	**6**	**10**	**11**	**12**	**13**	**14**	**29**	**30**	**32**	**16**	**17**	**18**
Salmonella Strain		*Salmonella* Typhi	*S.* Paratyphi	*S.* Paratyphi A	*S.* Paratyphi B	*Salmonella* Typhimurium	*S.* Enteritidis	*S.* Virchow	*S.* Infantis	*S.* Brunei	*S. Ch.* Arizona	*Salmonella* Weltevreden	*S.* Gallinarum	*S.* Paratyphi	*Salmonella* I. monophasic	*Salmonella* Senftenberg	*S.* Saintpaul	*S.* Reading
Bands (bp)	I	768	568	600	600	574	577	593	441	600	562	574	230	602	592	225	422	413
	II	533	404	425	425	457	405	425		443	487	464	196	417	500		223	226
	III	387				405				425	400	425			413		188	190
PCR ribotype		1	2	3	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Well code *		**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
Salmonella Strain		*S.* Saintpaul	*S.* Reading	*S.* Reading	*Salmonella* Senftenberg	*S.* Stanley	*S.* Stanley	*S.* Sarajane	*S.* Saintpaul	*S.* Sandiego	*S.* Saintpaul	*S.* Stanley	*S.* Stanley	*S.* Heidelberg	*Salmonella* Reinickendorf	*S.* Bradford	*Salmonella* Typhimurium	*S.* Ohio
Bands (bp)	I	413	423	418	225	409	413	400	396	400	400	590	409	409	492	598	413	422
	II	223	228	234			226	221	217	217	217	422			412	426	201	250
	III											413			242	260		210
PCR ribotype		17	18	19	14	20	21	22	23	24	24	25	20	20	26	27	28	29

* - Please refer Fig. 15, 16 and 17 for the well code

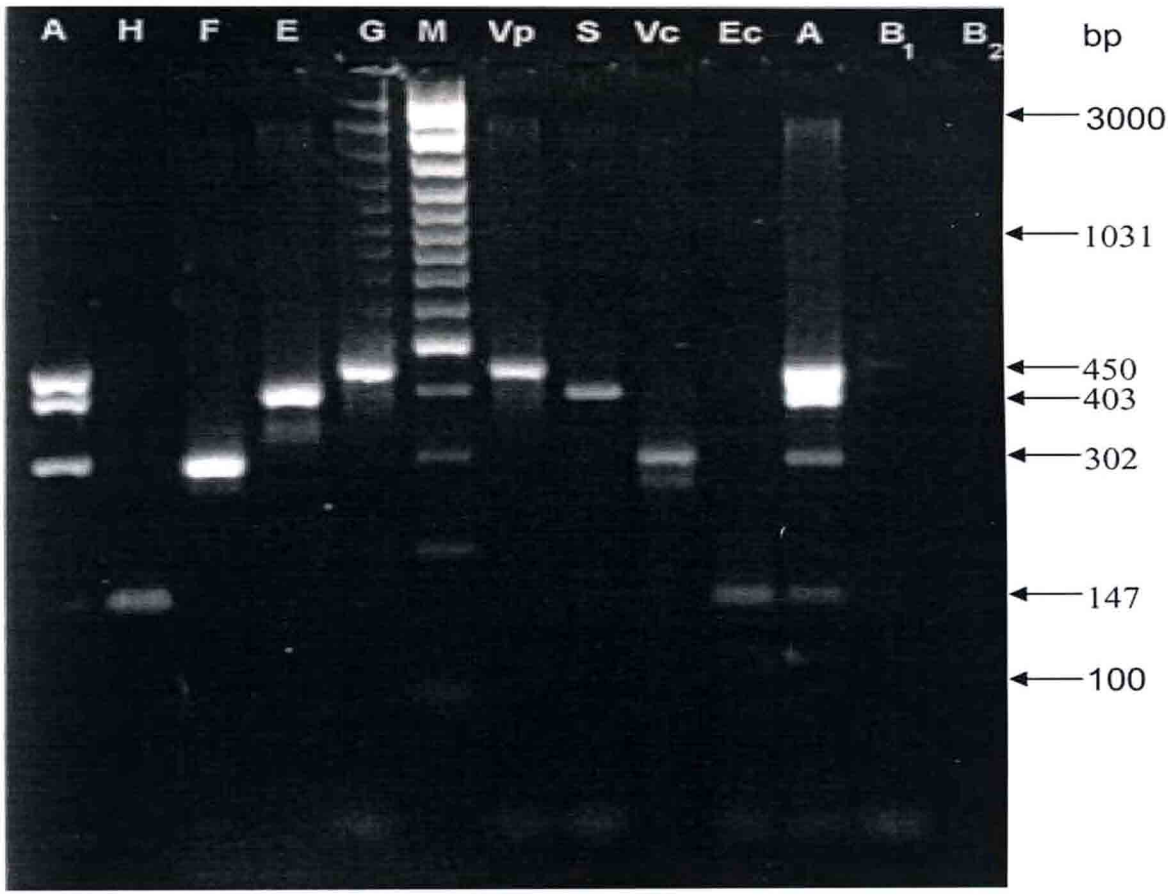

Fig. 16. Simultaneous detection of few common seafood – borne pathogens by multiplex PCR and two way cross-reaction among the participating primers and template DNAs

A – All primers + all strains

H – *EC* primers+ all strains

F – VC primers + all strains

E – S primers + all strains

G – VP primers + all strains

M – Mol. weight marker

Vp – VP strain + all primers

S – *Salmonella* strain + all primers

Vc – V. cholerae srrain + all primers

Ec – *E. coli* strain + all primers

B1 – No stain + all primers

B2 – All strains + no primer

DNAs were taken in a single tube PCR reaction, it resulted in four distinct bands. On the other hand, the two-way cross reaction involving one pair of the participating primers and the four template DNAs or vice versa resulted in a single band for which the pair of primers or the template DNA meant for (Fig. 16). This showed that all these four pairs of primers can be used for co-amplification and there was no cross reaction among the participating pairs of primers or with the genomic DNAs.

4.8.1. Evaluation of specificity of the participating primers

Results of the test of evaluation for specificity of the primers targeting to *uid*A gene of *E. coli*, *inv*A gene of *Salmonella* spp., *ctx* gene of *V. cholerae* and *tl* gene of *V. parahaemolyticus* has been given in figures 17, 4, 18 and 19, respectively. The figures demonstrates that the primers based on invA and *ctx* gene are highly specific for the detection of the target pathogen, where as the primers targeting *uid*A and *tl* gene showed some cross-reactions.

The PCR protocol developed in this study has high potential to use for routine analysis to rapidly detect these four common seafood-borne human pathogens. Besides, this method can be conclusiveely used for fast, accurate and specific detection of *V. cholerae* and *Salmonella* spp. hazard in fish processing units.

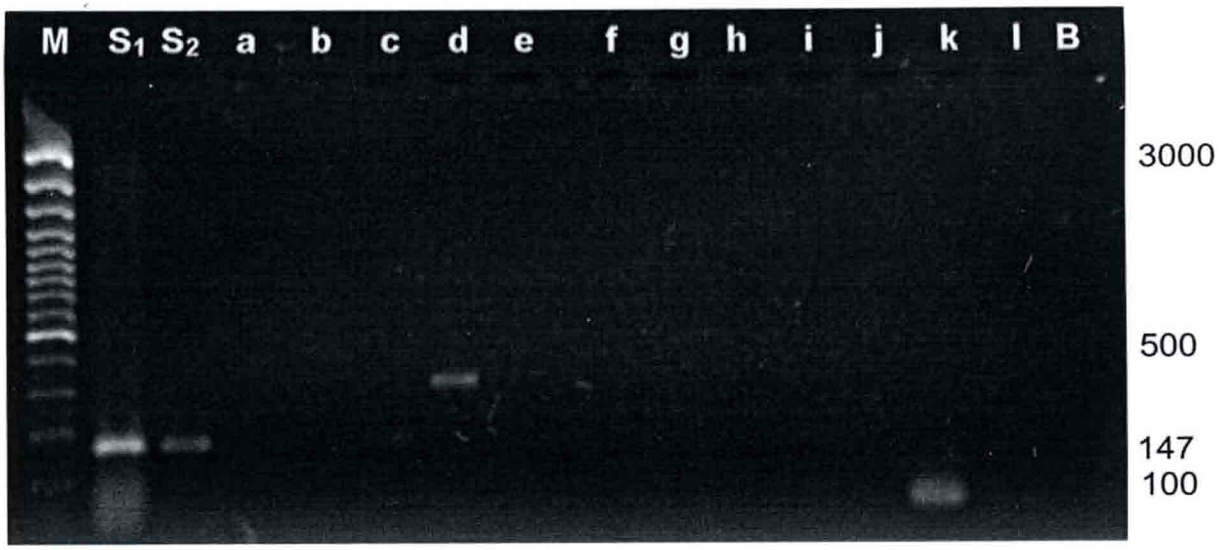

Fig. 17. Evaluation of specificity of primer set H targeting *uid*A gene of *E. coli*

M – Mol. weight marker
S_1 – EC (MTCC 1687)
S_2 – EHEC O157:H7
a – *Vibrio cholerae* O1
b – *V. parahemolyticus*
c – *V. vulnificus*
d – *S.* Typhi
e – *A. hydrophila*
f – *P. mirabilis*
g – *K. pneumonae*
h – *M. morganii*
i – *H. alvei*
j – *Staphylococcus aureus*
k – *V. cholerae* (non-toxigenic)
l – *V. parahaemolyticus*
B – Blank

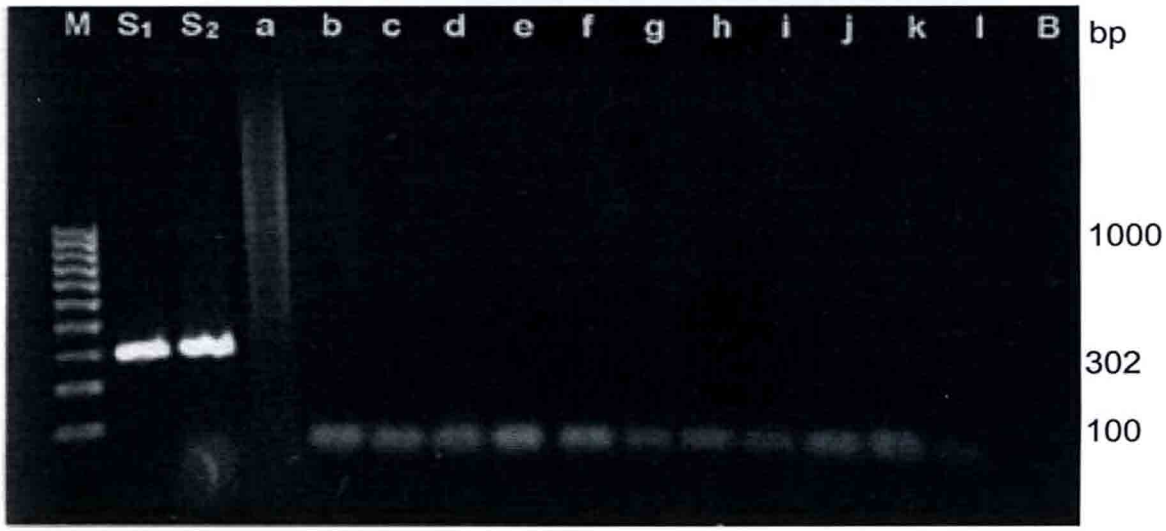

Fig. 18. Evaluation of specificity of Primer set F targeting *ctx* gene of V. *cholerae*

M – 100 bp DNA ladder
S_1 – *V. cholerae* O1
S_2 – *V. cholerae* O1
a – *V. cholerae* (non-toxigenic)
b – *E. coli*
c – *V. parahaemolyticus*
d – *V. vulnificus*
e – S. Typhi
f – *A. hydrophila*
g – *K. pneumonae*
h – *M. morganii*
i – *H. alvei*
j – *Staph. aureus*
k – EC O157:H7
l – *P. mirabilis*
B – Blank

Fig. 19. Evaluation of specificity of Primer set G targeting *tl* gene of *V. parahaemolyticus*

M – 100 bp DNA ladder

S_1 – *V. parahaemolyticus*

S_2 – *V. parahaemolyt*icus

a – *Vibrio cholerae* O1

b – *E. coli*

c – *S.* Typhi

d – *V. vulnificus*

e – *A. hydrophila*

f – *P. mirabilis*

g – *K. pneumonae*

h – *M. morganii*

i – *H. alvei*

j – *Staph. aureus*

k – *Vibrio cholerae* (non-toxigenic)

l – EC O157:H7

B – Blank

DISCUSSION

5. DISCUSSION

5.1. Hygienic Status of Fish and Fish Handling Environments of Mumbai

The increasing rate of incidence of food-borne diseases around the world has made HACCP based quality assurance system mandatory for the seafood processing facilities involved in international trading. This systematic approach of assuring quality relies on managing total quality of a production system right from the harvest of raw material through the stages of processing, including its packaging, storage, transport and the environment involved in the whole process to produce a zero defect product.

However, the basic objectives of HACCP framework have not been achieved in many fish processing facilities in India and there were several instances of rejection of seafood consignments in previous years. One of the principal factors responsible for this could be the lack of awareness among the fish processors on the conditions of the post-harvest handling and transportation. Hygiene in landing centre and handling and transportation of raw material, which are the important points of entry of human pathogens into the seafood, has been overlooked in many occasions. Venugopal (1999) has rightly pointed out these facts in his review of seafood quality assurance in India. According to Mortjemi and Kaferstein (1999), HACCP system is neither the penacea of all food safety problems nor is a magic wand that can turn unsafe food into a safe one. The earlier in the food chain that pathogens are controlled, the lesser are the chances that the consumers will be exposed to death (DeWaal, 2000). Hence, the hygienic handling of raw material and the environment in the landing centre have a major role in ensuring the quality of the final product. It is in this context, a brief surveillance study of landing centres and fish market of Mumbai, was undertaken to assess the prevailing hygienic condition of fish and fish handling environments.

Bacterial load in any food system serves as a general indicator of hygiene and the risks associated with consumption (Gram, 1992). In fish, this is more pronounced because of the apparent distinction from other muscle food in terms of extreme perishable nature and shortened shelf life (FAO, 1973). In the present study, the APC of fish and fish handling environments in all the sampling stations fell within 6.2×10^2 to 6.9×10^7cfu/g. Among the sampling stations,

considering all the samples, APC level was highest in the Sasoon Dock fish landing centre with an average level of 9.8 x 10^6 cfu/g (Table 16). Within a sampling station, the APC levels were usually in the higher side in fish and/or shrimp samples, with the only exception of one water sample collected from the fish auction/dressing surface at Sasoon Dock fish landing centre. This may be due to the fact that washing, dressing and/or auction of fish in Sasoon Dock fish landing centre is done on the same concrete surface that is used for movement of fishermen and traders. There is no elevated platform used separately for these purposes, which would have reduced the bacterial load. The overall hygiene of Versova landing centre and Four Bungalows fish market were found to be comparatively better. This may be due to the absence of concrete surface in either of the stations, which forces the fishermen to keep their catch in containers instead of heaping them on the surface. APC level of swab samples revealed that there were substantial level of bacterial load on the fish contact surfaces. The average bacterial load on the Individually Quick Frozen (IQF) shrimp samples was 1.9 x 10^3 cfu/g. In most of the samples the bacteriological load of fish and shrimp exceeded the prescribed level of 5 x 10^5cfu/g for fresh/chilled fish or shrimp (Iyer, 2000a). However, this should not be a reason for concern, since all the samples were taken for bacteriological study without giving even single washing in potable water, whereas multiple washing steps precede the final processing in usual process cycle. There are examples of seafood with a TPC load of 10^6 - 10^8 cfu/g without objectionable quality changes (Nickelson and Finne, 1992). But high initial bacterial load would mean faster spoilage due to improper handling and time-temperature abuse during transportation of raw material to the processing plants. Akhtar (2002) conducted a surveillance study on the bacterial load of fish at various stages of its processing in 10 different fish processing plants in and around Mumbai, and reported that the TPC load was of the range 10^6-10^7 cfu/g in the raw material, which decreased to 10^5-10^4 cfu/g during processing. Panda (2002) has reported APC load of 10^4 to 10^6 cfu/g in seafood from different processing plants sampled at various stages of processing.

5.2. Isolation and identification of *Salmonella* spp.

Fish and fishery products have the potential to cause a wide spectrum of public health problems through contamination with human pathogens from the point of harvest to the final preparation. The major possibility of contamination with

enteric pathogens is the improper sanitary practices during product handling and subsequent amplification of these pathogens because of time-temperature abuse in post-harvest condition (Kvenberg, 1991). *Salmonella* is regarded as the zero-tolerance pathogen in any food commodity. In the present study, presence of *Salmonella* spp. was considered as a representative assessment of pathogenic contamination of the raw material, particularly that during post-harvest handling and transport. There are several culture based methods available for the detection of food-borne *Salmonella* (Anon, 1984a; APHA, 1985; ISO, 1991; Anon,1991; FDA, 2001). However, the methods described by FDA (2001) was used in the present case with slight modification based on ISO 6579 (ISO, 1991), as it was more logical in its approach to isolate very small number of *Salmonella* from food. Twenty six *Salmonella* spp. were isolated (Table 17) from 84 different samplings made in this study. This indicates very high rate of incidence of *Salmonella* spp. in fish and fishery environment of Mumbai. There are over 2600 serovars of *Salmonella* spp. and almost all are considered pathogenic to human or animal (Jay, 1986; Aabo *et al.*, 1993; Kwang *et al.*, 1996) and presence of even a single *Salmonella* bacterium in food is considered significant (Tetjen and Fung, 1995). Generally *Salmonella* spp. is not a part of normal microflora in the aquatic environment, therefore their presence in fish and fish handling environments is regarded as poor sign of hygiene and sanitation (Dalsgaard, 1998).

Out of the total 26 *Salmonella* isolates, eight were from Versova fish landing centre, mainly found in water and swab samples. Interestingly, none of the fish and shrimp samples of Versova was found contaminated with *Salmonella* spp. Similarly, *Salmonella* spp. were not found in any of the samples of Sasoon Dock fish landing centre. The maximum number of *Salmonella* isolate i.e., 18, were from the Four Bungalows fish market (Table 17). Freshwater fish samples were frequently found to be contaminated with *Salmonella* spp. and as high as 7 isolates were obtained. *Salmonella* contamination was found in all categories of samples from Four Bungalows fish market. Frequent Isolation of *Salmonella* spp. from different samples in Four Bungalows fish market reflects that the hygienic condition of the fish market is very poor.

There are several instances of incidence of *Salmonella* spp. in fish and fishery products of India as well as in other part of the world (Iyer and Srivastav, 1989; Nambiar and Iyer, 1991; Reilly *et al.*, 1992; Bandekar *et al.*, 1995). Recently

Panda (2002) has reported the incidence of *Salmonella* spp. in one of the processed shrimp samples collected from fish processing plants in Mumbai. There are several reports on the incidence of *Salmonella* spp. in poultry, meat and other flesh-food worldwide (Bachhil and Jaiswal, 1988; Thorberg and Engvall, 2001; Stock and Stolle, 2001; Bailey *et al.*, 2002; Tavechio *et al.*, 2002). When the fish and fishery environment that is the source of raw material is contaminated, and if not properly handled, there is a great probability that the products processed out of it may contain *Salmonella* spp. out of cross contamination, re-contamination or inadequate processing. This would be the most probable reason for the repeated rejection of shrimp from India and other Southeast Asian countries due to the contamination with *Salmonella* spp. (Karunasagar and Karunasager, 1999).

5.3. Biochemical and Serological Characterisation of Salmonella Isolates

The *Salmonella* spp. isolated and identified following FDA (2001) were subjected to detailed biochemical characterisation as described by Ewing (1986). All the isolates produced acid and gas from dulcitol, glucose, maltose and manitol. All of them reduced nitrogen, utilised citrate and lysine, but could not utilise urea, phenylalanine, gelatin and malonate. All the isolates were positive to oxidase test and H and O serum agglutination test. These are the typical reactions for most of the members of *Salmonella* genus (Andrews and Hammack, 2001).

The reactions of some of the isolates to few biochemical tests were atypical (Table 18). Isolate BSCC18 and BSCC19 could utilise lactose and sucrose and produced acid and gas, which are not the typical reaction for *Salmonella* (FDA, 2001). Further, the isolate BSCC18 was VP +ve and MR –ve and isolate BSCC19 was Indole +ve, which are not the usual reactions for *Salmonella* spp. However, these two isolates were considered as presumptive *Salmonella* spp. and subjected to serological classification because of their positive reaction to both the Poly H and O serum agglutination tests. There are reports that up to 4 % of all *Salmonella* cultures isolated by FDA analysts from certain foods, especially seafoods, have been atypical (FDA, 2001).

All the 26 isolates were subjected to detailed serological characterisation leading to their sero-grouping using definitive antisera at NSC, IVRI, Izatnagar. The isolates were identified as belonging to 11 serovars (Tables 17). The

isolates BSCC18 and BSCC19, showing atypical biochemical reactions (Tables 18), were identified as *Salmonella* Bradford and *S.* Typhimurium respectively. It was found that, the 8 *Salmonella* isolates obtained from Versova fish landing centre were belong to only 2 serovars. An interesting feature observed in the samples from Versova fish landing centre is that, all the isolates were obtained only from environmental samples i.e., water and swabs taken from fish contact surfaces, and one particular serovar *Salmonella* Ohio, was frequently isolated. This may be due to the fact that, the fish and prawns are not expected to harbour *Salmonella* in their natural environment (Dalsgaard, 1998) and the bacteria might not had established in the fish or prawn by the time of sampling. In Four Bungalows fish market all the 7 *Salmonella* isolates from freshwater fish were identified as belong to only two serovars. However, it was observed in the study that the Four Bungalows fish market was contaminated with maximum number of *Salmonella* serovars, as 9 different serovars (18 isolates) were isolated from 39 samples. The vast swampy area on the back of Four Bungalows fish market and the water from an unprotected well present in the swampy area might be playing a major role in contaminating fish in that market. However, detailed study is required to establish this fact.

The pattern of *Salmonella* serotypes in India has been reported by National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh (Nath *et al.*, 1966 and 1970; Saxena *et al.*, 1983). Nambiar and Iyer (1991) have reported on the isolation of 16 different *Salmonella* serotypes from 156 fresh and 127 frozen fish samples from the retail trade in Kochi. Iyer and Shrivastava (1989) have worked out on the incidence of *Salmonella* spp. in different aqua-products and had isolated 34 different serotypes, with the highest number of serotypes isolated from frozen frog legs. Bandekar *et al.* (1995) have reported on the isolation of five major strains of *Salmonella* from fish and chicken in markets of Mumbai. There are many reports on incidence of specific serotypes from various sources (Saxena *et al.*, 1983; Anon 1984b, c, 1985 and 1986; Iyer and Varma 1987).

The salmonellae may be divided into three groups based on host predilections (COS, 1969). None of the serovars isolated in the present study belong to typhoid or paratyphoid group that has human as the specific host and causing systemic typhoid fever. Only one serovar, *S.* Typhimurium causes disease in human

and many other animals (Baumler *et al.*, 1998). Except this, all the other serovars isolated in the present study do not have well-known host specificity. These un-adapted serovars of *Salmonella* are responsible for food-borne gastroenteritis in human (Jay, 1986). Therefore, the isolation of these serovars from fish and fish handling environments is of great significance from the point of health hazard. Since majority of *Salmonella* serovars was isolated from Four Bungalows fish market, which caters fresh fish to the local inhabitants, a great deal of care is required while preparation of such contaminated fish. It is well known that, *Salmonella* spp. are sensitive to heat (Nambiar and Iyer, 1991) and certainly cannot survive the Indian way of preparing food. However, cross-contamination of prepared food items with the infected fish might cause infection.

5.4. Antibiotic Susceptibility Profile of *Salmonella* Isolates

Antimicrobial drug resistance is a major problem in salmonellosis, which mainly develops through drug resistant plasmid "R" plasmid, by transposons or by adoption mechanism (Hirsh *et al.*, 1983). R plasmid is considered to be conjugative in nature and has been implicated in inter-species and intergenic spread of multiple drug resistance (Stone, 1975). All the 26 *Salmonella* isolates were tested for their antibiotic resistance pattern using 22 antibiotics representing different families (Table 8). Antibiotic susceptibility profile of the isolates revealed that majority of the isolates were resistant to five or more antibiotics tested (Table 19). MAR Index of many of the isolates indicated their origin form high risk sources of contamination. The environmental isolates of Versova fish landing centre, *S.* Ohio, were found to be resistant to minimum 11 antibiotics. These isolates were referred as multiple-antibiotic resistant strains. However, detailed study of these isolates leading to the establishment of minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC) is required to find out the probable threat of these multiple antibiotic resistant isolates. WHO (1997) has reported a notable increase in cases related to a multiple-drug resistant *S. enterica* serovar Typhimurium, DT 104, in several European countries. Saxena (2000) has reported four strains of *Salmonella* that were resistant to all the 14 antibiotics tested. Schuman *et al.* (1989) have also isolated a *S.* Typhimurium *Hf* strain from milk that was resistant to as high as 14 antibiotics. Further, they have established that the resistance of the *S. typhimurium*

Hf was mediated through the four plasmids of different sizes it harboured. Similarly, incidence of antibiotic resistant *S.* Typhimurium (Bhatacharya, 1997), *S.* Enteritidis (Bakshi, 1998) and *S.* Gallinarum (Joseph, 1994; Gopinath, 1996) have also been reported.

All the *Salmonella* isolates were found to be resistant to penicillin G (benzylpenicillin) and erythromycin and more than 80% of the isolates were resistant to nitrofurantoin, vancomycin and furazolidon (Table 20). On the other hand, almost all the isolates were sensitive to norfloxacin, ciprofloxacin, levofloxacin, co-trimoxazole and chloramphenicol. Norfloxacin and ciprofloxacin are newer broad-spectrum quinolones (Black, 2002). Chloramphenicol, a broad spectrum antibiotic, has been the drug of choice to treat typhoid fever, until 1970, when its fatal side effects were understood (Greenwood, 1997). Co-trimoxazole and ciprofloxacin are now being used as the second line of treatment for typhoid fever. Therefore, above 90% of the isolates susceptible to chloramphenicol and all of them susceptible to co-trimoxazole, norfloxacin and ciprofloxacin is a sign of relief. On the other hand, vancomycin, a glycopeptide, is a large molecule that is unable to penetrate the outer membrane of Gram-negative bacteria (Greenwood, 1997). Therefore, it is expected that *Salmonella* should exhibit inherent resistance to vancomycin. Similarly penicillins, which target peptidoglycan strand and erythromycin, which interferes with translocation of mRNA on the bacterial genome, have no useful activity against enteric Gram-negative bacilli (Greenwood, 1997). The results obtained validate the method adopted for antibiotics resistance study. Mode of action of nitrofurans, nitrofurantoin and furazolidon has not yet been fully elucidated. Therefore, the resistance of more than 80% of the isolates to these two antibiotics and at a different rate to other antibiotics has to be viewed with caution. To sum up, it has been observed that, though few *Salmonella* isolates were found to be resistant to many antibiotics, they have not acquired resistance against the drugs being used today to treat infections due to *Salmonella.*

5.5. Development of *Salmonella* spp. Specific PCR

Salmonella spp. are considered as a major food-borne pathogens of humans. The genus comprises of more than 2600 serovars and almost all are considered pathogenic to human and animals (Jay, 1986; Portillo, 2000). In many countries, *Salmonella* is the leading cause of food-borne outbreaks and infections

(Schmidt, 1995; Wallace *et al.*, 2000; Tirado and Schmidt, 2001). For the last two decades, incidence of salmonellosis has been constantly increasing (CDC, 1987; Tauxe, 1997; Waage *et al.*, 1999). Presence of *Salmonella* in fish and fishery products in India and its consequences has been widely reported (Iyer and Shrivastava, 1989; Nambiar and Iyer, 1991; Bandekar *et al.*, 1995; Karunasagar and Karunasagar, 1999; Panda, 2002). To minimise the risk of infection, microbiological control of the food process line is being increasingly applied. The traditional end-point evaluation has been replaced by HACCP based systematic approach of quality assurance. In such a system, for the in-process monitoring of CCPs the number of samples to be tested for the presence of *Salmonella*, in a batch of product, right from the harvest of raw material, through the stages of processing, till the food is finally consumed has increased several fold when compared to the traditional testing of only the end product. Thus, for the effective implementation of HACCP, the availability of rapid, reliable and sensitive test system that can provide quick results having scope of production control has become increasingly important. However, the conventional culture based methods of isolation and identification fail to meet the requirement, as they require minimum 5-7 days for the determination of presence or absence of *Salmonella* in the test sample (Andrews, 1985; Meiscier *et al.*, 1992).

The need for timely results has led to the development of many rapid methods of detection with high specificity and sensitivity among which, the *in-vitro* amplification of DNA by PCR has been reported as a powerful technique that offers rapid, sensitive and specific detection of food-borne pathogens (Olsen, 2000). A number of PCR methods have been developed targeting specific gene sequence for the detection of *Salmonella* (Malorny *et al.*, 2003). However, the development of PCR primers specific for the detection of salmonellae with all the serotypes possesses some difficulties (Tsen *et al.*, 1994). In *Salmonella*, neither virulence factor nor toxin gene specific for all serovers have been reported (Fitts *et al.*, 1983), making the search for a genus specific DNA sequence complicated. Eventhough, the recent nomenclature based on DNA homology has classified more than 2600 serovars of the *Salmonella* genus into two species, *S. enterica* and *S. bongori* (Le Minor and Popoff, 1987; Boyd *et al.*, 1996), the data available is insufficient to establish a specific gene or nucleotide sequence that is universal in the *Salmonella* genus. Only the *inv* genes, which enable the bacteria to invade cells, have been

reported to have uniform distribution among various serovars of *Salmonella enterica* (Galan and Curtis, 1991; Swamy *et al.*, 1996). The *inv*A gene is located on pathogenecity island 1 of *Salmonella* spp., encoding proteins of a type III secretion system, appears to be present in all serovers of *S. enterica* (Chiu and OU, 1996; Galan, 1996; Malorny *et al.*, 2003).

In the present study on the development of a *Salmonella* specific PCR, out of three pairs of primers used, two pairs of primers were based on *inv*A gene and the third one on the published sequence of *S. typhi* specific DNA probe (GenBank Accession No. U78640). PCR screening of the primers was carried out one by one with the pre-standardised PCR protocol. Seventeen standard *Salmonella* strains and ten non-*Salmonella* strains were taken in the first phase of screening.

Evaluation of primer set A (L-INVA, R-INVA), which was taken from the published literature (Brasher *et al.*, 1998), has been given in Fig. 2. The reaction produced the desired 571 bp DNA band in almost all the *Salmonella* strains with the only exception of *Salmonella* I. monophasis (well No. 26). There were false positive bands with few non-*Salmonella* strains, *V. parahaemolyticus* (well No16), *V. vulnificus* (well No. 17), *M. morganii* (well No. 21) *and Staphylococcus aureus* (well No. 23) in repeated trials and the primer set was rejected.

Evaluation of specificity of the set B primers (BS1-F, BS1-R), which was designed for this study based on the nucleotide sequence U78640 of the GenBank, gave positive reaction for many *Salmonella* strains (Fig. 3). However, the primers failed to detect *S.* Paratyphi A (well No. 2), *S.* Kentuky (well No.6), *S.* Arizona (well No. 11) and *Salmonella* I. monophasis (well No. 26) in the primary screening, and thus this set was rejected.

The primer set C (BS2-F, BS2-R) gave positive reaction (Fig. 4) for all the standard *Salmonella* strains tested in the preliminary screening (well No. 1 to 13 and 24 to 26). At the same time there was no false positive reaction in any of the non-*Salmonella* strains tested (well No 14 to 23). The result obtained was reproducible and free from any ambiguity. Therefore, primer set C was short-listed as the best among the three set of primers screened so far.

5.6. Evaluation of Bacterial Cell Lysate for PCR

One of the pre-requisite of a successful PCR assay for food-borne pathogens is the use of an effective DNA extraction and purification procedure to remove the contaminating substances from the food that may inhibit the activity of DNA polymerase (Lantz *et al.*, 1994; Romero and Lopez-Goni, 1999). Partial or total inhibition of PCR reaction may also be encountered due to compounds in selective media or chemicals from DNA extraction procedures (Wernars *et al.*, 1991; Rossen *et al.*,1992). Several workers have investigated on the DNA extraction methods and came out with varying results (Rossen *et al.*, 1992; Fluit *et al.*, 1993a, 1993b; Widjojoatmodjo *et al.*, 1995).

The rapid DNA preparation method in the present study was based on the findings of Medici *et al.* (2003) in their attempt to develop a rapid, reproducible and robust method of DNA extraction and purification for use in real-time PCR. They compared four different methods and selected the rapid boiling lysis method of DNA preparation because of its simplicity and rapidity. Similar method has been adopted as the faster, economical and sensitive method of DNA preparation by Mukhopadhyay and Mukhopadhyay (2002) and Malorny *et al.* (2003). Lin *et al.* (1996) used DNA prepared by boiling the bacterial culture and found that the RAPD fingerprint pattern as consistent as those obtained from phenol-chloroform extracted DNA. In the present study of evaluating bacterial cell lysate as the template DNA (Fig. 5) using primer set C resulted in specific DNA bands of 403 bp from all the 13 standard *Salmonella* strains reproducibly. The evaluation of bacterial cell lysate was further carried out using all the 26 microbiologically confirmed *Salmonella* strains isolated in the present study (Fig. 6). As can be seen in the figure, all the 26 isolates (well No. 1 to 26) yielded the specific 403 bp PCR product with primer set C. The effectiveness of the bacterial cell lysate was also evaluated by artificially seeding the shrimp and kamaboko homogenate with one representative from each of the *Salmonella* serovars isolated in the present study (Fig. 7).

These evaluations conclude two different things. The first, that there was total agreement between the conventional culture based method of isolation and identification of pathogen and the PCR based method of detection of bacterial pathogen. The second was that, the bacterial cell lysate prepared by rapid boiling

lysis method is sufficient enough for routine analysis by PCR. Besides, this method of template DNA preparation is very fast, takes less than an hour to prepare the DNA directly from the test tissue sample. Whereas, several hours is required for preparing DNA from purified broth culture using the standard phenol-chloroform extraction method. Further, this method did not incorporate any chemical and thus reduced the chances of PCR inhibition. While preparing lysate from seeded shrimp homogenate, which may be applicable to any processed fish sample, the tissue was diluted ten times of its original volume and this would help in diluting the inhibiting substances present in processed shrimp (Rossen *et al.*, 1992).

The primer set C (BS2-F, BS2-R) that gave encouraging results in the evaluations carried out till this stage as well as in the second phase screening using bacterial cell lysate of four standard *Salmonella* strains and two non-*Salmonella* strains (Fig. 7). The performance of the PCR method was consistent and all the results are reproducible even after the change of thermocycler. Thus it can be stated that, the newly developed PCR method based on the primer set C is an ideal PCR method for rapid and specific detection of *Salmonella* spp. contamination in fish and fishery products. This PCR method has lot of potential in the fish processing industry of India to keep track of *Salmonella* contamination, determination of CCPs and effectiveness of the corrective action taken in the fish processing line and will be certainly useful for quality management of fish and fishery products meant for export.

5.7. Analysis of Atypical *Salmonella* Isolates

The reactions of three presumptive *Salmonella* isolates (BSCC25, BSCC28 and BSCC29) to the biochemical tests were atypical (Table 21). The strain BSCC25 could utilise lactose and sucrose to produce acid and gas, and was VP +ve and MR –ve, which are not typical reactions of *Salmonella* spp. (FDA, 2001). However, this isolate showed very faint agglutination with *Salmonella* Poly H antiserum and clear agglutination with Poly O antiserum and was retained as presumptive *Salmonella.* Similarly, the isolate BSCC28 was VP +ve, MR –ve and malonate +ve, but the strain gave positive result in *Salmonella* Poly H and Poly O agglutination test (Table 21). On the other hand, the reaction of BSCC29 to all the biochemical tests and Poly H and O agglutination test was in accordance to the typical reaction of *Salmonella*. These three strains when submitted to NSC, IVRI for

confirmation and serotyping along with others were identified as not *Salmonella* (Table 21). At the same time, two other isolates (BSCC18 and BSCC19) showing similar atypical reactions (Table 18) were identified as members of two different serovars. Based on these results, it may be stated that the conventional biochemical identification and serotyping are sometimes not dependable and are inadequate to determine the *Salmonella* hazard in seafood.

In contrast to results of biochemical reactions, these three isolates produced *Salmonella*' spp. specific 403 bp DNA band in the PCR reaction developed by us (Fig. 9). Similarly, up to 4 % of all *Salmonella* cultures isolated by FDA analysts from certain foods, especially seafoods, have been atypical (FDA, 2001) and Baudart *et al.* (2000b) failed to serotype approximately 1% of the environmental strains, since O and H antigens are not always expressed. It may be stated that, the isolates declared as "not *Salmonella*" by NSA, IVRI, Izatnagar were turned out to be *Salmonella* positive by PCR. Therefore, the isolates BSCC25, BSCC28 and BSCC29 were designated as atypical *Salmonella*, ATI, ATII and ATIII, respectively.

There are several ways in which PCR fragments can be characterized, among which sequencing the PCR amplicon is reported to provide the strongest confirmatory data (Karunasagar and Karunasagar, 1999). Therefore, the PCR amplicon of one of the atypical *Salmonella* strain (AT III) was sequenced for its characterization. The sequencing reaction was attempted in two approaches. The PCR product was successfully cloned to the T/A cloning vector (pTZ57R/T) and conveniently screened using blue/white selection on LB-ampicillin agar plate containing X Gal and IPTG (Fig. 10). The cloned plasmid was sequenced using universal sequencing primer. Secondly, the PCR product was used directly for sequencing. The unambiguous sequence of 340 bp (Fig. 12) deduced after sequencing was used for analysis by random BLAST (Table 22). The deduced nucleotide sequence matched at higher score, i.e., 369 and above, only with invasive gene sequence of various *Salmonella* spp. and none of the non-*Salmonella* sequences had similarity score above 58. This clearly shows that the nucleotide sequence generated in the present study is a fragment of the invasive gene of *Salmonella* spp., and hence, it may be conclusively stated that, the atypical *Salmonella* strain AT III (i.e., BSCC29) is a member of *Salmonella* genus. This

proves the superiority of the PCR method we have developed over the conventional culture based methods to detect the biochemically and serologically atypical *Salmonella* isolates. Similarly, importance of PCR method for identifying biochemically atypical *V. parahaemolyticus* has been reported by Dileep *et al.* (2003). Dalsgaard and Olsen (1995) reported the incidence of *Salmonella* spp. from manure samples of shrimp farm, which had been declared as free from *Salmonella* by conventional culture based methods.

Multiple alignment of the deduced sequence with the matching portion of the sequences from GenBank database revealed that a portion of the deduced sequence, i.e., starting from position 14 to 340, has a near perfect matching (i.e., more than 97%) with the different *Salmonella* spp. invasive gene sequences (Table 24). Such a high degree of homology certainly reflects the high potential of the deduced sequence to be used as *Salmonella* genus specific hybridization probe. Identification of bacteria by DNA probe hybridization method is based on the presence or absence of particular genes. This is in contrast to most biochemical and immunological tests that are based on the detection of gene products such as antigens or chemical end products of a metabolic pathway (Hill *et al.*, 2001). A number of reports are available for the *Salmonella* genus specific detection by hybridization probes (Fitts *et al.*, 1983; Flowers *et al.*, 1987a; Tsen *et al.*, 1991; Olsen *et al.*, 1991, 1995; Bandekar, *et al.*, 1995; Gopo *et al.*, 1988). All these probes differ in their detection limit and accuracy. The nucleotide sequence deduced in the present study is based on *inv*A gene sequence that is strongly conserved but unique to *Salmonella* spp. as already reported by Galan and Curtiss (1991) and Galan *et al.* (1992). Therefore, further use of the deduced sequence as a highly specific hybridization probe for genus specific detection of *Salmonella* is highly recommended.

5.8. Molecular Characterisation of *Salmonella* Isolates by PCR Ribotyping

Salmonella spp. are ubiquitous enteric bacteria comprising of more than 2600 serovars. The conventional serotyping of *Salmonella* is based on the antigenic structure of the cell surface lipopolysaccharide (O antigen) and flagellar protein (H antigen) (Popoff and Le Minor, 1997). Other classic techniques like phase typing, biotyping, antibiogram and plasmid profiling are generally carried out to

establish intra-serovar differentiation particularly during epidemiological investigations (Threlfall and Frost, 1990). These traditional techniques are being replaced by modern molecular typing methods, i.e., ribotyping, IS200 fingerprinting etc. to type different *Salmonella* isolates. However, these are complex techniques and require skills that are beyond the reach of many laboratories (Lagatolla *et al.*, 1996). PCR ribotyping that is based on the amplification of the spacer sequences between the 16S and 23S genes in the rRNA transcriptional units (Kostman *et al.*, 1992) seems to be more promising and simpler in this regard. rRNA loci are present in 2 to 11 copies on the chromosomes of most bacterial species. While a high degree of sequence homology exists for rRNA genes, the intergenic spacer regions show extensive sequence and length variations which have been used to characterise at the genus (Jensen *et al.*, 1993), species (Jensen *et al.*, 1993; Dolzani *et al.*, 1995) and subspecies level (Kostman *et al.*, 1992; Dolzani *et al.*, 1994).

The results of the PCR ribotyping carried out for forty strains of *Salmonella* belonging to 28 serotypes have been given in fig. 13, 14 and 15. A nelson's eye on the figures have failed to produce any differential pattern among the serovars. However, the PCR ribotyping pattern (Table 25) worked out based on number and size of DNA bands could classify only 34 strains (belonging to 23 serotypes) into 29 ribotypes. Seventeen of the 34 strains were found to have serovar specific banding pattern. Two strains of *S.* Paratyphi were different in their banding pattern, whereas the banding pattern of S. Paratyphi A and B were similar. Similarly, three isolates of *S.* Reading had three different patterns and three *S.* Stanley strains had two types of banding pattern, one of which matches with that of *S.* Heidelberg. Nevertheless, PCR ribotyping can be considered a good technique for sub-typing strains of *Salmonella*, the protocol followed in this study did not provide a greater discriminatory power among the serotypes and within the serotypes. Therefore, further study is required to substantiate the procedure for efficient molecular subtyping of the isolates based on ribosomal primers.

5.8. Evaluation of Multiplex PCR for Detection of Seafood Pathogens

Fish and fishery products are implicated with several pathogenic bacteria comprising the indigenous flora of the natural environment, contaminants of sewage or faecal waste during handling, processing, storage and distribution (NAS,

1991). Among all, *Salmonella* spp., the indicator of safety of food and water supply of a country; pathogenic *V. cholerae*, the causative agent of Cholera epidemic; and *V. parahaemolyticus*, one of the principal cause of seafood related gastroenteritis, are the important ones affecting quality of fish and fishery products. Molecular detection using PCR has been reported as the rapid procedure with high sensitivity and specificity for the immediate detection and identification of specific pathogenic bacterial contamination. Several PCR methods are available for the specific detection of *Salmonella* spp. (Rahn *et al.*, 1992; Jones *et al.*, 1993; Aabo *et al.*, 1993; Hasimoto *et al.*, 1995; Woodward and Kirwan, 1996; Soumet *et al.*, 1999); *V. cholerae* (Fields *et al.*, 1992; Karunasagar *et al.*, 1995, 1997b) and *V. parahaemolyticus* (Tada *et al.*, 1992; Lee and Pan, 1993; Karunasagar *et al.*, 1996, 1997a; Dileep *et al.*, 2003). However, identification of individual pathogens by PCR becomes time-consuming and costly. When search for newer methods of rapid diagnosis of pathogenic contamination having greater scope of production control is in vogue, simultaneous detection of all the important pathogens of fish and fishery products in a multiplex PCR format is a valued proposition. Such a method would be relatively rapid and also cost effective (Brasher *et al.*, 1998). A number of multiplex PCR methods have been reported for different food borne pathogens (Trost *et al.*, 1993; Wang *et al.*, 1997; Franck *et al.*, 1998; Brasher *et al.*, 1998; Soumet *et al.*, 1999; Wesley *et al.*, 2002). However, not a single multiplex PCR protocol for these pathogens from fish and fishery products has been published from Indian laboratories.

The performance of the *Salmonella* spp. specific PCR developed in this study has been evaluated in a multiplex PCR methodology for the simultaneous detection few common seafood-borne human pathogens (Fig. 16). It is evident that, the primers targeting *uid*A, *ctx*, *inv*A and *tl* gene of *E. coli*, *V. cholerae*, *Salmonella* spp. and *V. parahaemolyticus* specifically produced the 147 bp, 302 bp. 403 bp and 450 bp bands, respectively in the two way cross-reactions performed as shown in the figure. Again, all the primers when taken together with DNA from all the participating bacteria produced the four specific bands in a single tube PCR reaction (Fig. 16). This shows that each of the four target DNAs could be co-amplified with other and there was no cross-reaction of any primer with any DNA or vice versa. The result obtained in this multiplex PCR is highly encouraging and it has high potential in the management of quality of fish and fishery products.

Specificity of the participating pair of primers have also been evaluated in the presence of DNA from both target and non-target pathogens. The evaluation of primer based on *inv*A gene targeting *Salmonella* spp. (Fig. 4) has already been discussed else where in this chapter. Fig. 17 depicts the reaction of primer set based on *uid*A gene of *E. coli* with two *E. coli* strains and 12 non *E. coli* strains. The pair of primers was specific to *E. coli*, which produced precise 147 bp PCR product with both the non-toxigenic (MTCC 1687) and toxigenic (EHEC O157:H7) *E. coli*. There were no false positives with any of the non-E. *coli* strains tried in the experiment. However, there was a non-specific PCR product of about 300 bp by *Salmonella* Typhi. This non-specific band of higher size is of little significance under situations when one is detecting the *E. coli* contamination only in any fishery products. However, in multiplex PCR, this band might interfere with the 302 bp *V. cholerae* specific band using *ctx* gene-based primers. Therefore, in such a situation, a separate PCR reaction targeting specifically *V. cholerae* can be set up along with the multiplex PCR for further confirmation.

The pair of primers targeting *ctx* gene of *V. cholerae* was highly specific towards the toxigenic *V. cholerae* and did not produce any false positives with the other strains including non-toxigenic *V. cholerae* (Fig. 18). Cholera toxin is the only major virulence factor of *V. cholerae*, encoded by *ctx* gene (Mekalanoes *et al.*, 1983; Finkelstein, 1988; Shimada *et al.*, 1994) and the objective of seafood quality control laboratory is to differentiate these pathogenic strains from non-pathogenic ones. The conventional biochemical and serological methods are reported to be labour intensive, time consuming and inadequate to address many intriguing aspects of *V. cholerae*, its virulence and the associated public health hazards (Sakazaki and Dovovan, 1984; Shimada *et al.*, 1987; Colwell and Huq, 1994). However, the PCR method targeting the *ctx* gene developed in our study has lot of potential for rapid and specific monitoring of pathogenic *V. cholerae* contamination in fish and fishery products.

Results of the test for specificity of primers targeting *tl* gene of *V. parahaemolyticus* has been given in Fig. 19. The thermolabile hemolysin (*tl*) gene is reported to be present in all the *V. parahaemolyticus* strains irrespective of its virulence (Taniguchi *et al.*, 1986). Therefore, the PCR method targeting *tl* gene can

serve as the species specific detection of *V. parahaemolyticus*. In the present investigation, both the standard *V. parahaemolyticus* strains produced the specific 450 bp bands. However, there were few false positive DNA bands developed with some of the non-target bacteria.

The overall performance of the multiplex PCR has been satisfactorily good for routine and rapid detection of the four pathogenic bacterial contaminations in fish and fishery products. However, the performance of *ctx* and *inv*A gene based primers was found to be highly encouraging for the specific detection of toxigenic *V. cholerae* and *Salmonella* spp., respectively. As members of the *Salmonella* genus and toxigenic *V. cholerae* are highly objectionable in fish and fishery products and their presence should be nil in the products meant for export, the multiplex PCR method developed by us for these two pathogens has promising role for effective and conclusive quality management of fish and fishery products.

SUMMARY

SUMMARY

Salmonella is one of the leading causes of food-borne diseases in the world. It is associated with the systemic typhoid fever and gastroenteritis in many countries. *Salmonella* is regarded as the zero-tolerance pathogen in food and being used as an indicator of public hygiene of food and water supply of a country. The alarming rate of increase of incidence of *Salmonella* in food and thereby causing salmonellosis has become a matter of great concern among consumers, industry and regulatory agencies. To minimise such menace, HACCP based quality assurance system has been made mandatory for the seafood processing plants involved in international trading. In such a systematic approach of total quality management, the quality control laboratories has to be equipped for handling large number of samples and produce reliable results at the shortest possible time.

Conventional culture based methods for the detection of *Salmonella* contamination in fish and fishery products are laborious and requires minimum 5-7 days to produce a diagnosis and thus are inadequate for the in-process monitoring of critical control points (CCPs). Therefore, a rapid detection method that is specific, sensitive and internationally accepted is the need of the hour.

The need for timely results has led to the development of many rapid methods, among which polymerase chain reaction (PCR) is a simple technique for specific and sensitive detection of food-borne pathogens. Therefore, a PCR based method for the specific detection of *Salmonella* contamination has been developed in the present study. Besides this, a representative survey on the prevailing hygienic status of fish and fish handling environments of Mumbai was made with the special emphasis on *Salmonella* contamination. The salient findings of the study are as follows:

- Eighty four fish, shrimp, water and swab samples were obtained from two fish landing centres, one fish market and one fish processing plant of Mumbai to assess the prevailing hygienic status of fish and fish handling environments.
- APC *of fish and shrimp sample ranged from* 3.7×10^4 *to* 1.7×10^7 *cfu/g; while that for water, swab and IQF shrimp samples were between* 1.3×10^4 *and* 6.9×10^7 cfu/ml, 4.8×10^3 and 2.0×10^6 cfu/cm^2 and 6.2×10^2 and 3.1×10^3 cfu/g,

respectively. Among the sampling stations, APC level was the highest in the Sasoon Dock fish landing centre with an average load of 9.8 x 10^6 cfu/g. Within a sampling station, the APC levels were usually in the higher side in fish and/or shrimp samples.

- Twenty six *Salmonella* spp. were isolated in the present study, which were confirmed and sero-grouped into 11 serovars. Eight *Salmonella* strains, were isolated from Versova fish landing centre, whereas there were no *Salmonella* spp. isolated from Sasoon Dock fish landing centre and the processed shrimp obtained from the fish processing plant. The maximum number of *Salmonella* isolates i.e., 18 belonging to 9 serovars, were from the Four Bungalows fish market, which shows that the hygienic condition of the fish market is very poor.
- Antibiogram study of the *Salmonella* isolates involving 22 commonly used antibiotics revealed that majority of the isolates were resistant to five or more antibiotics tested. The multiple antibiotic resistance (MAR) Index for many of the isolates indicated their origin form high risk sources of contamination. Resistance of few environmental isolates of *Salmonella* serovar *S.* Ohio to as high as 11 antibiotics is a matter of concern.
- A new pair of PCR primers, Primer set C (BS2-F, BS2-R), developed in this study based on the *inv*A gene sequence of *Salmonella* spp., was found to be highly specific to all the 21 standard *Salmonella* strains, while there was no false positives with any of the 12 non-*Salmonella* strains used in the study.
- A simple template DNA preparation method based on rapid boiling lysis was found to be rapid, reproducible and robust method of DNA extraction for use in PCR detection of *Salmonella* spp. from fish and fishery products. The method takes less than an hour to prepare DNA from the test tissue sample in contrast to several hours required for preparing DNA from purified broth culture using the standard phenol-chloroform extraction method. By this the rapidity of PCR detection could be increased to a great extent.
- There was total agreement between the conventional culture based method of isolation and identification and the newly developed PCR-based method of detection of *Salmonella* spp., as all the 26 *Salmonella* isolates of the present

study were detected reproducibly from the cell lysate of bacterial pure culture and seeded tissue homogenate.

- There were three isolates whose reactions to few biochemical tests were not typical of *Salmonella* spp. Serotyping using definitive antiserum at National Salmonella Centre, IVRI, Izatnagar, identified them as 'not *Salmonella*'. However, *Salmonella* spp. specific 403 bp band was obtained with the PCR method developed in this study, which were later confirmed as the members of *Salmonella* genus by nucleotide sequencing of the PCR product of one of these isolates and analysis of the deduced sequence with GenBank database. This finding reveals the superiority of the PCR-based method developed by us to the conventional culture based methods of isolation and identification *Salmonella* from fish and fishery products.

- Based on the above evaluations, it can be stated that the PCR method developed based on the newly designed primers is the ideal procedure for rapid and specific detection of *Salmonella* spp. contamination in fish and fishery products. This method has lot of potential in the fish processing industry of India to keep track of *Salmonella* contamination, determination of CCPs and effectiveness of the corrective action taken in the fish processing line.

- Random BLAST followed by the multiple alignment of the deduced sequence from one atypical *Salmonella* isolate with the NCBI GenBank database revealed that a portion of the deduced sequence has a near perfect matching with the different *Salmonella* spp. invasive gene sequences. Such a high degree of homology, i.e., more than 97%, reflects the potential of the deduced sequence to be used as *Salmonella* genus specific hybridization probe.

- The PCR ribotyping carried out for 40 strains of *Salmonella*, comprising both standard reference strains and isolates of the present study, could not differentiate the serovars successfully. It could classify only 34 strains of *Salmonella* into 29 ribotypes.

- Performance of the *Salmonella* spp. specific PCR was also evaluated in a multiplex PCR along with few common seafood-borne pathogens. Four pairs of primers produced 147 bp, 302 bp, 403 bp and 450 bp bands specifically targeting *E. coli*, toxigenic *V. cholerae*, *Salmonella* spp. and *V.*

parahaemolyticus, respectively in a single tube reaction. Performance of the *ctx* and *inv*A gene based primers was highly encouraging for the specific detection of toxigenic *V. cholerae* and *Salmonella* spp., respectively in comparison to that of *E. coli* and *V. parahaemolyticus* . Thus this PCR method has a lot of potential in fish processing industry of India for effective quality management with respect to toxigenic *V. cholerae* and *Salmonella* spp. that are highly objectionable in fish and fishery products.

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APPENDIX

APPENDIX : ABBREVIATIONS

APC	:	Aerobic Plate Count
AOAC	:	Association of Official Analytical Chemists
BLAST	:	Basic Local Alignment Search Tool
bp	:	base pair(s)
CCPs	:	Critical Control Points
cfu	:	Colony Forming Units
Conc.	:	Concentration
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTPs	:	Deoxynucleoside triphosphates
DAEC	:	Diffusely Adhering *E. coli*
EAggEC	:	Enteroaggregative *E. coli*
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EEC	:	European Economic Commission
EIA	:	Export Inspection Agency
EIC	:	Export Inspection Council
EIEC	:	Enteroinvasive *E. coli*
EHEC	:	Enterohaemorrhagic *E. coli*
ELISA	:	Enzyme Linked Immunosorbent Assay
EPEC	:	Enteropathogenic *E. coli*
ERIC	:	Enterobacterial Repetitive Intergenic Consensus Sequences
ETEC	:	Enterotoxigenic *E. coli*
EC	:	European Commission
EU	:	European Union
Fig.	:	Figure
h	:	Hour (s)
HACCP	:	Hazard Analysis Critical Control Point System
ICMSF	:	International Council of Microbiological Specification of Foods
IPTG	:	β-D-isopropyl-thiogalactopyranoside
IPQC	:	In-Process Quality Control
kb	:	Kilo base pair
l	:	Litre
LB	:	Luria Bertani
M	:	Molar
MAR	:	Multiple Antibiotic Resistant
mg	:	Milligramme (10^{-3}g)
min	:	Minute(s)
ml	:	Millilitre (10^{-3} l)
MLEE	:	Multilocus Enzyme Electrophoresis
mm	:	Millimetre
mM	:	Millimolar

MW	:	Molecular Weight
N	:	Normal
ng	:	Nanogramme(s) (10^{-9}g)
NSC, IVRI	:	National Salmonella Centre (Vet.), Indian Veterinary Research Institute, Izatnagar, Bareilly
°C	:	Degree Celsius
OD	:	Optical Density
PCR	:	Polymerase Chain Reaction
PFGE	:	Pulse-Field Gel Electrophoresis
pmol	:	Pico mole(s)
QAMS	:	Quality Assurance and Monitoring System
RAPD	:	Randomly Amplified Polymorphic DNA
REA	:	Restriction Endonuclease Analysis
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic Acid
RNase	:	Ribonuclease
rpm	:	Revolutions per minute
SDS	:	Sodium dodecyle sulphate
sec	:	Second(s)
SLT	:	Shiga-like toxins
SPS	:	Sanitary and Phytosanitary Measures
TE	:	Tris EDTA
TDH	:	Thermostable direct haemolysin
TRH	:	TDH-related haemolysin
U	:	Unit
USFDA	:	United States Food and Drug Administration
UV	:	Ultraviolet
V		Volt
VBNC	:	Viable but non-culturable
v/v	:	Volume/Volume
WHO	:	World Health Organisation
WTO	:	World Trade Organisation
w/v	:	Weight/Volume
X-Gal	:	5-bromo-4-chloro-3-indolyl-β-galactopyranoside
μg	:	Microgramme (10^{-6} g)
μl	:	Microlitre (10^{-6} l)