BIOCHEMICAL AND MOLECULAR INVESTIGATIONS ON *SALMONELLA* SEROVARS FROM SEAFOOD

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DECLARATION

I hereby declare that the thesis entitled "Biochemical and molecular investigations on *Salmonella* serovars from seafood" is a record of bonafide research work done by me under the supervision and guidance of Dr. P.K. Surendran, and Dr. Nirmala Thampuran, and it has not previously formed the basis for award of any degree, diploma, associateship, fellowship or other similar title or recognition to me, from this or any other university or society.

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CERTIFICATE

This is to certify that this thesis entitled "Biochemical and molecular investigations on *Salmonella* serovars from seafood" embodies the result of original work conducted by Mr. Rakesh Kumar, under our supervision and guidance from November 2004 to March 2009. We further certify that no part of this thesis has previously formed the basis for the award to the candidate, of any degree, diploma, associateship, fellowship or other similar titles of this or any other University or Society. He has passed the Ph.D. qualifying examination of the Cochin University of Science and Technology, held in April 2006.

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Rakesh Kumar

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Abbreviations

AOAC	Association of analytical chemists (communities)
BGA	Brilliant green agar
BHI	Brain heart Infusion Agar
BPW	Buffer peptone water
BSA	Bismuth sulphite agar
CDC	Center for disease control and prevention
cs	Centisome
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylene diaminetetra acetic acid
ERIC	Enterobacterial repetitive intergenic consensus
g	Gram
h	Hour
HACCP	Hazard analysis critical control point
HEA	Hektoen enteric agar
l	Litre
LIA	Lysine iron agar
LPS	Lipopolysaccharide
MDR	Multi-drug resistance
mg	Milligram
min	Minutes
mM	Millimolar
MR	Methyl Red
NBT	Nitroblue tetrazolium
NCCLS	National committee for clinical laboratory standards
ng	Nanogram (10^{-9})
NMKL	Nordic committee on food analysis
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
pg	Picogram (10^{-12})
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
RV	Rappaport Vassiliadis
s	Second
SCB	Selenite cystine broth
SDS	Sodium dodecyl sulphate
SPI	*Salmonella* pathogenicity Island
spv	*Salmonella* plasmid virulence
stn	*Salmonella* enterotoxin

TAE	Tris acetate ethylene diamine tetra-acetic acid
TBE	Tris borate ethylene diamine tetra-acetic acid
TSI	Triple sugar iron agar
TT	Tetrathionate broth
UPGMA	Unweighted pair group with arithmetic averages
USFDA	United States food and drug administration
VP	Voges-Proskauer
XLD	Xylose lysine desoxycholate agar
µg	Microgram
µl	Microlitre
µM	Micromolar

INTRODUCTION

1. INTRODUCTION

Traditionally, seafood is a popular food diet in Indian sub-continent and other parts of the world. In India, particularly the coastal areas, seafood provides the main source of dietary animal protein and also generates income avenues for 14 million fisher folk and people associated with seafood industry. Seafood sector is playing an important role in the economy and nutritional security of the nation. The export earnings from seafood for India in the year 2007-08 were to the tune of over Rs. 7620 crores (Anon., 2008). Today, more people are turning towards fish as a healthy food due to low fat content and presence of n-3 polyunsaturated fatty acids in fish. However, consumption of fish and shellfish may also cause various diseases to the consumers due to infection or intoxication by food-borne pathogens. The presence of food borne pathogens also cause huge monetary loses to the fisherman and the exporters. The seafood exporters in the country have been facing tremendous challenges in meeting the food safety requirements from the European Union (EU) and United States. The EU commission has imposed border testing of frozen seafood products for *Salmonella* and *Vibrio* spp. which resulted in a decline in export to the EU countries.

Seafood being a relatively high risk perishable food, are subjected to a range of food safety requirements related to general biological and chemicals hazards. Among foodborne pathogens, *Salmonella* comes top in the rank for being responsible in foodborne outbreaks. Food borne pathogens are inherent in seafood from aquatic and terrestrial environments. In a 2-year period (1980–1981) 8.7% of disease outbreaks in the Netherlands were associated with seafood and 10.1% of outbreaks in the United States during a period of 1972-87 were connected with seafood (Huss et al., 2000). The

loss due to food borne outbreaks costs the United States, alone $1.1 billion to 1.5 billion annually. Food borne outbreaks are not properly documented in developing countries, unlike the western counterparts; hence, less number of reports are available in these countries. Presence of *Salmonella* in seafood is well documented. In numerous incidences, *Salmonella* serovars have been isolated from seafood in India and abroad.

Salmonella is a leading food borne pathogen; causes both typhoid fever and salmonellosis illnesses in humans. Till date, more than 2540 *Salmonella* serotypes have been identified, based on somatic (O), flagellar (H) and capsular (Vi) antigenic profile (Popoff et al., 2004). The natural habitat of *Salmonella* spp. is in the gastrointestinal tract of animals, birds, reptiles and even some serotypes have been isolated from marine sources. Outbreaks due to *Salmonella* have been associated with consumption of chilled boiled salmon, halibut, cooked cockles, fish and chip (Francis et al., 1989). The incidences of *Salmonella* in India associated with seafoods were reported in some of the earlier studies (Iyer and Shrivastava, 1989b; Nambiar and Iyer, 1991; Hatha and Lakshmanaperumalsamy, 1997; Shabarinath et al., 2007).

Most commonly, conventional culture method has been used for the isolation and identification of *Salmonella* serotypes in seafood. The basic principle behind the isolation and identification of *Salmonella* in culture method is the biochemical substrate utilization pattern, although, considerable variations observed in biotyping pattern. Majority of *Salmonella* are recognized as non-lactose fermenters (lac^-) and hydrogen sulfide (H_2S^+), although, majority of *Salmonella enterica* subsp. *arizonae* and *Salmonella enterica* subsp. *diarizonae* are lactose fermenters and certain H_2S negative *Salmonella* serovars are also available. The conventional approach requires confirmatory test of all typical and atypical colonies on selective plates and it becomes

very cumbersome to identify these suspected *Salmonella* isolates. Hence, alternative molecular approaches need to be incorporated in the detection assay. The process of isolation and identification of *Salmonella* in seafood by conventional method requires multiple steps of pre-enrichment, selective enrichment, followed by plating on selective media and finally biochemical confirmation with key reactions. The entire process takes 5-7 days to identify a *Salmonella* isolate. Thus, there are considerable interests in the development of more rapid techniques, particularly for detection of *Salmonella* in seafood. Different array of tests have been developed in the form of miniaturized biochemical kits, immunoassays and DNA-based tests for rapid screening of large number of food or seafood in a short duration. Rapid methods provide an alternative approach for screening large number of samples in a short duration. A large number of modern rapid methods have been approved by AOAC and other international agencies such as USDA and NMKL (Swaminathan and Feng, 1994; Fung, 1997). The main disadvantage of the commercial kits available in market is that they are expensive in nature. Thus, development of indigenous rapid, sensitive and competitive technique based on PCR and DNA probe assays for identification of *Salmonella* serovars in seafood would be an ideal step for rapid screening of seafood samples.

In epidemiological studies, biotyping, serotyping, and antimicrobial typing methods have been frequently used for characterization of *Salmonella* serotypes from different environments. Biotyping assay consists of the utilization pattern of various sugars, amino acids and other organic compounds and is most simple and commonly used typing technique. Disadvantage of this method is that it is less discriminating, in nature, between strains. Serotyping is another phenotypic method, which confirms the relatedness among the isolates from common and different environments based on antigenic property. This technique is quite specific and most commonly used for

characterization of *Salmonella* isolates but it is complex and laborious in nature. Indiscriminate use of antibiotics in humans and farm animals has led to development of antibiotics resistance in bacterial pathogens. Use of antibiotics in the aquaculture ponds also contributed to development of antibiotics resistance in bacteria. Antimicrobial resistance typing profile gives the impact of chemical hazards on environment, particularly in microbes. This technique has been successfully used for the detection of antimicrobial resistance profile of *Salmonella* serotypes. The microbial typing methods have been used in wide range of microorganisms, but none of these typing methods offers an ideal approach for the subtyping of microbial species. Thus, the combination of different methods may be the best approach to characterize the *Salmonella* isolates.

The dynamics of species variability arise from bacterial mutation and conjugative intra and inter generic exchange of transposons and plasmids encoding determinant traits. Different molecular typing methods based on the variation in genetic makeup have been now used in complement with traditional typing methods for fingerprinting of *Salmonella* serotypes. Nucleic acid, protein and lipoppolysaccharides are the only macromolecules that carry information in their sequences and compositions to allow the study of microbial diversity and the development of molecular typing methods that would be the more holistic approach for characterization of *Salmonella* isolates. Molecular typing of a *Salmonella* serotypes can be based on plasmid typing, enterobacterial repetitive intergenic consensus sequences (ERIC)-PCR, virulence gene characterization, and pulsed field gel electrophoresis (PFGE) analysis. These molecular fingerprinting methods will provide the genetic variation in *Salmonella* serovars associated with seafood in this part of the country.

Against this background, the main objectives of the proposed investigations are:

- Isolation and characterization of *Salmonella* serovars from fresh and unprocessed seafood from Cochin (India).
- Development of biotyping profile of different serovars based on utilization of various sugars and amino acids.
- Antibiotic resistance profile of *Salmonella* serovars isolated from seafood.
- Development of molecular typing patterns based on PCR-ribotyping, for *Salmonella* serovars associated with seafood.
- PFGE based fingerprinting profile of *Salmonella* serovars.
- Characterization of different *Salmonella* virulence genes.
- Development of rapid and sensitive detection assays for *Salmonella* in seafood.
- Quantitative detection of *Salmonella* in seafood by real-time PCR.

About this thesis

The present investigation was envisaged to determine the prevalence and identify the different *Salmonella* serovar in seafood from Cochin area. Though, the distribution of *Salmonella* serovars in different seafood samples of Cochin has been well documented, the present attempt was made to identify the different *Salmonella* serovars and determine its prevalence in various seafoods. First part of this investigation involved the isolation and identification of *Salmonella* strains with the help of different conventional culture methods. The identified isolates were used for the further investigation i.e. serotyping, this provides the information about the prevalent serovars in seafood. The prevalent *Salmonella* strains have been further characterized

based on the utilization of different sugars and amino acids, to identify the different biovar of a serovar.

A major research gap was observed in molecular characterization of *Salmonella* in seafood. Though, previous investigations reported the large number of *Salmonella* serovars from food sources in India, yet, very few work has been reported regarding genetic characterization of *Salmonella* serovars associated with food. Second part of this thesis deals with different molecular fingerprint profiles of the *Salmonella* serovars from seafood. Various molecular typing methods such as plasmid profiling, characterization of virulence genes, PFGE, PCR- ribotyping, and ERIC–PCR have been used for the genetic characterization of *Salmonella* serovars.

The conventional culture methods are mainly used for the identification of *Salmonella* in seafood and most of the investigations from India and abroad showed the usage of culture method for detection of *Salmonella* in seafood. Hence, development of indigenous, rapid molecular method is most desirable for screening of *Salmonella* in large number of seafood samples at a shorter time period. Final part of this study attempted to develop alternative, rapid molecular detection method for the detection of *Salmonell*a in seafood. Rapid eight–hour PCR assay has been developed for detection of *Salmonella* in seafood. The performance of three different methods viz., culture, ELISA and PCR assays were evaluated for detection of *Salmonella* in seafood and the results were statistically analyzed. Presence of *Salmonella* cells in food and environmental has been reported low in number, hence, more sensitive method for enumeration of *Salmonella* in food sample need to be developed. A quantitative real-time PCR has been developed for detection of *Salmonella* in seafood. This method would be useful for quantitative detection of *Salmonella* in seafood.

The thesis is divided into five major chapters and each chapter is further divided into subheads. The first chapter highlights the identification of problem and the theme of research work with suitable objectives. Second chapter deals about the review of literature. The review includes taxonomical status, morphology, isolation, growth and biochemical characteristics and antibiotics resistance of *Salmonella*. Different method of isolation and identification of *Salmonella* in food has been reviewed and more attention is given to rapid, immunological and molecular methods. Different typing methods such as biotyping and serotyping of *Salmonella* spp. are also reviewed. The epidemiology of salmonellosis and its public health significance and final part the review of literature covered the distribution of *Salmonella* in seafood, national and international perspectives. A brief review of statistical analysis is also included in the review of literature. Third chapter deals with material and methods. All method employed in the investigation are presented in detail. In chapter 4, results and discussion are presented. Results are mostly in tables and figures and also presented in dendrograms formats. The findings are discussed in detail. Finally, a summery of the entire work is presented in the chapter 5 and a detailed bibliography of the all citation made in the thesis is shown at the end of the thesis. A list of the publication from the study is also appended at the end of this thesis.

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REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Genus *Salmonella*

2.1.1 Background-historical

During early nineteenth century, the study of *Salmonella* began with Eberth's first recognition of organism in 1880, and subsequent isolation of the bacillus, responsible for human typhoid fever by Gaffky (Le Minor, 1991). Further investigations by European workers characterized the bacillus and developed a sero-diagnostic test for the detection of this human disease agent (D'Aoust, 1989; Le Minor, 1981). Thereafter, D.E. Salmon isolated the bacterium then thought to be etiological agent of hog cholera, but later disproved. The genus was named *Salmonella* by Lignieres in 1900 in honour of D.E. Salmon (Le Minor, 1991). Further investigations led to the isolation of other *Salmonellae*. It became a common practice to name each new isolate based on the disease it caused or the species of animal from which isolated. Early 20th century, great advances occurred in the serological detection of somatic and flagella antigens within *Salmonella* group. An antigenic scheme for the classification of *Salmonellae* was first proposed by White (1925) and subsequently expanded by Kauffmann (1941) into Kauffmann-White scheme, which currently includes more than 2540 serovars (Popoff and Le Minor, 2005).

2.1.2 Taxonomy and nomenclature

Salmonellae are facultative anaerobic, Gram-negative rod shaped bacteria belonging to the family *Enterobacteriaceae*. Although most members of this genus are motile by pertrichous flagella, a few non-flagellated variants such as *Salmonella enterica* subsp. *enterica* serovar Gallinarum and *Salmonella* Pullorum from poultry are non-motile. *Salmonellae* are chemoorganotrophic with ability to metabolize nutrients by both respiratory and fermentative pathways (D'Aoust et al., 2001). *Salmonella* nomenclature is very complex and Scientists use different system to refer to and communicate about this genus. Unfortunately, current usage often combines several nomenclature systems that divide the genus into species, subspecies, subgenera, groups, subgroups, and serotypes (serovars), and all these usages cause lots of confusion among researchers. *Salmonella* nomenclature has progressed through a succession of taxonomical and serological characteristics and on the principles of numerical taxonomy and DNA homology. The nomenclature for the genus *Salmonella* has evolved from the initial one serotype-one species concept proposed by Kauffmann (1966) on the basis of somatic (O), flagellar (H) and capsular (Vi) antigens. In the early development of taxonomic scheme, biochemical reactions were used to separate *Salmonella* into subgroups and the Kauffmann-White scheme was the first attempt to systematically classify *Salmonella* using scientific parameters. Thus, the effort culminated into development of five biochemically defined subgenera (I to V) where, individual serovars were designated status of a species (Kauffmann, 1966).

Table 2.1 Different Classification of *Salmonella*

Source; **Bergey's Manual of Systematic Bacteriology** (Brenner and Farmer III, 2005)

Classification used in Bergey's Manual of Systematic Bacteriology (1st Edition) and Bergey's Manual of Determinative Bacteriology (9th Edition	**Synonyms**	**Current classification in Bergey's Manual of Systematic Bacteriology** (Brenner and Farmer III, 2005)
Salmonella bongori[c] , *S. bongori*[d]	*Salmonella* subsp. *Bongori*, *Salmonella* subsp.V	*Salmonella bongori*
Salmonella choleraesuis[c], *Salmonella choleraesuis* subsp. *cholerasuis* Choleraesuis[d]	*Salmonella* subsp. I	*Salmonella enterica* subsp.*enterica* Cholreraesuis
Salmonella enteritidis[c],		*Salmonella enterica* subsp.*enterica* Enteritidis
Salmonella gallinarum[c], *Salmonella choleraesuis* subsp. Gallinarum[d]		*Salmonella enterica* subsp.*enterica* Gallinarum
Salmonella paratyphi-A[c], *Salmonella choleraesuis* subsp. *cholerasuis* Paratyphi A [d]		*Salmonella enterica* subsp.*enterica* Paratyphi A
NL[a]		*Salmonella enterica* subsp.*enterica* Paratyphi B
Salmonella typhi[c], *Salmonella choleraesuis* subsp. Typhi[d]		*Salmonella enterica* subsp.*enterica* Typhi
Salmonella typhimurium[c]		*Salmonella enterica* subsp.*enterica* Typhimurium
Salmonella salamae[c], *Salmonella choleraesuis* subsp. *salamae*	*Salmonella* subsp. II	*Salmonella enterica* subsp. *salamae*
Salmonella arizonae[c], *Salmonella choleraesuis* subsp. *arizonae*[d]	*Salmonella* subsp.IIIa	*Salmonella enterica* subsp. *arizaone*
Salmonella choleraesuis subsp. *diarizonae*[d]	*Salmonella* subsp.IIIb	*Salmonella enterica* subsp. *diarizonae*
Salmonella houtenae[c], *Salmonella choleraesuis* subsp. *houtenae*[d]	*Salmonella* subsp. IV	*Salmonella enterica* subsp. *houtenae*
Salmonella choleraesuis subsp. *indica*[d]	*Salmonella* subsp. VI	*Salmonella enterica* subsp. *indica*

NL[a], not listed, [c] Name used in Manual of Systematic Bacteriology , 1st Edition, 1984,
[d] Name used in Manual of Determinative Bacteriology , 9th Edition, 1994.

Note: The complete classification of *Salmonella* serovar is genus, species, subspecies and serovar e.g. *Salmonella enterica* subsp. *enterica* Typhimurium, but for convenience in this thesis used *Salmonella* Typhimurium.

Subsequently, three species nomenclature system was proposed using 16 discriminating tests to identify *Salmonella* Typhi, *Salmonella* Choleraesuis, and *Salmonella* Enteritidis and later scheme recognized member of *Arizona* group as a distinct genus (Ewing, 1972).

The scientific development in *Salmonella* taxonomy occurred in 1973 when Crosa et al. (1973) demonstrated, using DNA-DNA hybridization, that all serotypes and sub-genera I, II, and IV of *Salmonella* and all serotypes of "Arizona" were related at the species level. Thus, they belonged to a single species and an exception, described later was *Salmonella bongori*, previously know as subspecies-V. Further studies by DNA-DNA hybridization however, identified it as distinct species. Based on the multilocus enzyme electrophoretic pattern, *Salmonella enterica* susp. bongori was designated into a new species called *Salmonella bongori* (Reeves et al., 1989). Thereafter, *Salmonella choleraesuis* was designated as species name. Since, *Salmonella* Choleraesuis, causative agent of swine salmonellosis, appeared on the "Approved List of Bacterial Names" as the type species of *Salmonella*, it had priority as the species name. The name "choleraesuis", however, refers to both a species and a serotype, which caused more confusion for bacteriologist (Brenner et al., 2000). In addition, the serovar Choleraesuis is not representative of the majority of serotypes because it is biochemically distinct, being arabinose and trehalose negative. Other taxonomic proposals have been proposed based on the clinical role of a strain and biochemical characteristics that divided the serovars into subgenera and ultimately, on genomic relatedness (Brenner et al., 2000).

The antigenic formulae of *Salmonella* serovars are defined and maintained by the World Health Organization (WHO) Collaborating Centre for Reference and

Research on *Salmonella* at the Pasteur Institute, Paris. The new serovars are listed in annual updates of the Kauffmann-White scheme and the latest supplement no. 46 reported in year 2002, the identification and characterization of 18 new *Salmonella* serovars recognized by the WHO Collaborating Centre for Reference and Research on *Salmonella* (Popoff et al., 2004). Presently, *Salmonella* genus consists of two species: (1) *Salmonella enterica* and (2) *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies; S. *enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI) (Popoff and Le Minor, 2005). As per the recommendation of Popoff and Le Minor (1997) laboratories have to report the names of *Salmonella* serovars under the different subspecies of *enterica*. The names of the serovars are no longer italicized and first letter of the serovar should be written in a capital letter.

Table 2.2 Present number of serovars in each species and subspecies

***Salmonella* species and subspecies**	**No. of serovars** (Source Popoff et al., 2004)
Salmonella enterica	
subsp. *enteria* (I)	1504
susbsp.*salmae* (II)	502
subsp. *arizoane* (IIIa)	95
subsp. *diarizonae* (IIIb)	333
subsp. *houtene* (IV)	72
subsp. *indica* (VI)	13
Salmonella bongori	22
Total	2541

2.2 Characteristics of *Salmonella*

2.2.1 Morphology and isolation

Salmonella are 0.2 -1.5 x 2-5 μm in size, Gram negative, facultative anaerobic, rod shaped bacteria belonging to family *Enterobacteriaceae*. Members of this genus are motile by peritrichous flagella, except, *Salmonella* Pullorum and *Salmonella* Gallinarum. *Salmonella* are chemoorganotrophic, with an ability to metabobolize nutrients by both respiratory and fermentative pathways (Popoff and Le Minor, 2005). Hydrogen sulphide is produced by most *Salmonellae* but a few serovars like *Salmonella* Paratyphi A and *Salmonella* Choleraesuis do not produce H_2S. Most *Salmonellae* are aerogenic, however, *Salmonella* Typhi does not produce gas (Ziprin, 1994).

Most of the *Salmonellae* do not ferment lactose and this property has been the basis for the development of numerous selective and differential media for the culture and presumptive identification of *Salmonella* sp. (Rambach, 1990). Such media includes xylose lysine decarboxycholate agar, *Salmonella-Shigella* agar, brilliant green agar, Hektoen enteric agar, MacConkey's agar, lysine iron agar and triple sugar iron agar (Andrews and Hammack, 2001; Anderson and Ziprin, 2001). Isolation of *Salmonella* from food and environmental samples with culture method utilizes the multiple steps of pre-enrichment and enrichment on the selective and differential media in order to increase the sensitivity of the detection assay (Andrews and Hammack, 2001). Pre-enrichment is a process in which the sample is first cultured in a non-selective growth medium such as buffered peptone water or lactose broth with the intent of allowing the growth of any viable bacteria, and also useful in

allowing recovery of injured cells. In the case of *Salmonella*, the nest step of enrichment is usually achieved by culturing the pre-enriched samples in media containing inhibitors to restrict the growth of undesirable bacteria. Enrichment media commonly used to enrich *Salmonella* include the tetrathionate broth (Muller, 1923) and selenite cystine broth (Leifson, 1936).

More recently, selenite cystine broth has been replaced with Rappaport-Vassiliadis broth (Andrews and Hammack, 2001). The advantage of the Rappaport-Vassiliadis medium is that it can be used as broth or semisolid medium. Following the enrichment period, the enriched cultures are spread onto selective and differential agar plate, and then typical colonies for *Salmonella* has to be identified. Final confirmation of typical colonies is determined by series of biochemical and serological tests. A total of 18 key biochemical reactions have been used in the identification and confirmation of *Salmonella* isolate from food or seafood (Andrews and Hammack, 2001). A few *Salmonella* serovars do not exhibit the typical biochemical characteristics of the genus and these strains pose problem diagnostically because they may not easily be recovered on the commonly used differential media. About 1% of the *Salmonella* serovars submitted to Centres for Disease Control (CDC) ferment lactose; hydrogen sulphide production too was quite variable (Ziprin, 1994). Most recently developed *Salmonella* chrom agar medium has been described very promising for detection of both lactose positive and lactose negative *Salmonella* isolates from food samples (Dick et al., 2005).

2.2.2 Physiology and biochemical characteristics

The biochemical properties of *Salmonella* spp. show that almost all *Salmonella* serovars do not produce indole, hydrolyze urea, and de-aminate phenylalanine or tryptophan. Most of the serovars readily reduce nitrate to nitrite and most ferment a variety of carbohydrates with the production of acid, and reported to be negative for Voges-Proskauer (VP) reaction (Popoff and Le Minor, 2005). The other prominent characteristics of *Salmonella* are that most serovars produce hydrogen sulfide (H_2S) and decarboxylate lysine, arginine and orinithine with few exceptions (e.g. *Salmonella enterica* subsp. *arizonae* and *Salmonella enterica* subsp *diarizonae*). Most of *Salmonellae* utilize citrate with a few exceptions such as *Salmonella* Typhi , *Salmonella* Paratyphi A and a few *Salmonella* Choleraesuis serovars. Dulcitol is generally utilized by all serovars except *Salmonella enterica* subsp. *arizonae* (IIIa) and *Salmonella enterica* subsp. *diarizonae* (IIIb), whereas, lactose will not be utilized by most of the *Salmonella* serovars (Popoff and Le Minor, 2005).

Though, lactose may not be utilized by most of the *Salmonella* serovars, it has been reported that less than 1 % of all *Salmonellae* ferment lactose (Ewing, 1986). Most commonly, lactose negative (lac^-) *Salmonella* serovars are isolated and identified from food including seafood, which are more prevalent in nature. Several factors are responsible for lower detection of lactose positive (lac^+) *Salmonella* serovars in food or seafood. Lac^+ *Salmonella* serovars, which are sporadic in presence and also tricky to identify as many of the *Enterobacteriaceae* look similar with Lac^+ *Salmonellae* on selective media plates, hence escaped detection during

analysis. Further, *Salmonella* isolation from different sources with routine selective and differential media utilizes non-lactose fermentation as a key biochemical property and most commonly used differential plating media for isolation of *Salmonella* contains lactose. Littell (1977) has demonstrated that routine selective and differential media for *Salmonella* was not efficient enough to identify *Salmonella arizonae* (IIIa) group.

The natural habitat of the *Salmonella* subspecies; *Salmonella enterica* subsp. *salamae* (II), subsp. *arizonae* (IIIa), subsp. *diarizonae* (IIIb), subsp. *houtenae* and subsp. *indica* (VI) are considered to be the cold-blooded animals and environments (Popoff and Le Minor, 2005) and large number of *Salmonella* serovars in these subspecies are lactose fermenting in nature. Thus, it is suspected that seafood being cold blooded animals may harbour naturally lac^+ *Salmonella* serovars and actual incidence of lac^+ *Salmonella* in seafood may be much higher than the reported incidences. Outbreaks of disease from lac^+ *Salmonella* have been reported (Camara et al., 1989; Ruiz et al., 1995). In India, *Salmonella arizonae* (IIIa) infection in infants and children has been reported by Mahajan et al. (2003). *Salmonellae* are considered resilient microorganisms that readily adapt to extreme environmental conditions. *Salmonella* grow best at moderate temperature (35 -37°C), they can grow over a much wider temperature range, as low as 4 °C (D'Aoust, 1991) and as high as 48 °C (Baird-Parker, 1991). Thermal stress mutants of *Salmonella* Typhimurium has been reported to grow at elevated temperature of 54°C (Droffner and Yamamoto, 1992) and some other serovars exhibited psychrotrophic properties by their ability to grow in foods stored at 2 to 4°C (D'Aoust, 1991). The physiological adaptability of *Salmonella* spp. was demonstrated by their ability to proliferate at pH values ranging

from 4.5 to 9.5 (Chung and Goepfert, 1970). Leyer and Johnson (1992) demonstrated the increased survival of acid-adapted *Salmonella* in fermented milk and refrigerated temperature. Further studies showed that brief exposure of *Salmonella* Typhimurium to mild acid environment of pH 5.5 to 6.0 followed by exposure of the adapted cells to pH 4.5 (acid shock) triggers a complex acid tolerance response (ATR) that potentiates the survival of the microorganism under extreme acid environments (Foster and Hall, 1991; Hickey and Hirshfield, 1990). Another factor such as high salt concentration have long been recognized for their ability to extend the self life of foods by inhibiting the growth of inherent microflora (Pivnick, 1980). Although, *Salmonella* spp. are generally inhibited in the presence of 3 to 4 % NaCl, bacterial salt tolerance increases with increasing temperature in the range of 10 to 30 °C. D'Aoust (1989) suggested that the magnitude of this adaptive response was food and serovar specific. A recent report on anaerobiosis and its potentiation of greater salt tolerance in *Salmonella* raises concerns regarding the safety of modified -atmosphere and vacuum-packed foods that contain high levels of salts (Anon., 1986). A mathematical model has been developed that predict the survival of *Salmonella* spp. in food based on the interactive forces generated by temperature, pH and salt and other environmental forces (Gibson et al., 1988).

2.3 Antibiotic resistance

During early sixties, *Salmonella* resistance to single antibiotic was reported and since then multiple drug resistance (MDR) has been reported worldwide (Bulling et al., 1973; Threlfall et al., 1997). Current global scenario has showed that an increased number of antibiotic resistant *Salmonella* spp. from humans and farm

animals (Murray, 1986; Pacer et al., 1989; Lee et al., 1994). This resulted into major public health concern that *Salmonella* spp. could become resistant to antibiotics used in human medicine, thus, reducing therapeutic options and threatening the lives of infective individuals. The uncontrolled use of antibiotics in farm animals and aquaculture system has contributed tremendously to the emergence and persistence of resistant strains (Institute of Medicine, 1988; Novick, 1981; World Health Organization, 1988; Young, 1994). A study carried out for antibiotic resistance pattern in *Salmonella* isolated from swine by Gebreyes et al. (2000) demonstrated that a total of 625 out of 1257 *Salmonella* strains exhibited MDR pattern. Antimicrobial resistance in *Salmonella* serovars isolated from imported food was reported by Zhao et al. (2003) and results highlighted nalidixic acid resistance in *Salmonella* isolated from catfish and tilapia from Taiwan and Thailand, respectively. Multidrug-resistant phenotypes have been increasingly described among *Salmonella* species worldwide according to the World Health Organization (WHO) report on infectious disease (WHO, 2000).

The widespread use of fluoroquinolone is in practice due to broad spectrum of activity, high efficiency, and various applications in human and veterinary medicine (WHO, 1998). The increased resistance of *Salmonella* strains to fluoroquinolones was recently documented in England and Wales (Threlfall et al., 1997). The incidence of quinolone resistance over the period 1986 to 1998 in veterinary *Salmonella* isolates from Germany was reported by Malorny et al. (1999). As a result, several European countries have banned the non-human use of fluoroquinolones and USFDA has banned use of fluoroquinolones in poultry (D'Aoust et al., 2001). Plasmid based gentamicin resistance were detected in

Salmonella spp. isolated from treated livestock (Threlfall, 1992; Pohl et al., 1993). The genetic basis of quinolone and fluoroquinolone resistance in *Campylobactor* and *Salmonella enterica* was due to single point mutation in *gyr*A which encoded A subunit of DNA gyrase and rarely in *gyrB* (Griggs et al., 1996; Piddock et al., 1998). The other mechanism was proposed based on mutations in *parC* gene characterized the multiple-antimicrobial resistant gene in *Salmonella* serovars isolated from retail meat, thus, highlighted the role of genes in antimicrobial resistance (Heisig, 1996; Chen et al., 2004).

2.4 Rapid detection methods for *Salmonella*

As already discussed earlier (2.2.1) the process of isolation and identification of *Salmonella* in food involves multiple steps of pre-enrichment, selective enrichment, followed by plating on selective media and finally biochemical confirmation with key reactions. This entire process takes 5-7 days to identify *Salmonella* isolate (Rose 1998; Andrews and Hammack, 2001; ISO, 2000). There are considerable interests in the development of more rapid techniques without compromising the sensitivity, particularly for diagnostic purposes. These new lines of diagnostic methods are often called "rapid methods". A vast array of tests has been developed for detection of *Salmonella* and other pathogenic bacteria in the form of miniaturized biochemical kits, immunoassays and DNA-based rapid tests (Dziezak, 1987; Feng, 1996; Zhu et al., 1996; Kalamaki et al., 1997). Rapid detection of *Salmonella* is important in quality control of seafood and several factors are involved for reliable detection of *Salmonella* in food, in general, most important being type of method involved for the assay.

2.4.1 Biochemical property based methods

Bacterial pathogens from food are generally identified by biochemical characteristics and often required several days to weeks for identification. Most of these biochemical profiles for identification are labour intensive, time-consuming, and media-consuming process. The efforts to reduce or miniaturization of testing process began in the late 1940s (Cox et al. (1987a). The use of smaller chambers or vessels greatly economized the use of media and concentrated inocula considerably reduced the incubation times (Hartman et al., 1992). Over the years, various forms of miniature biochemical test system were introduced and steadily gained the popularity, especially in clinical microbiology. As the benefits of using such minisystems to identify food-borne bacteria became apparent, many studies confirmed the utility of these systems in food microbiology (Fung et al., 1981; Cox et al., 1984). These kits include specialized media combination to simple modifications of conventional assays, for rapid detection of *Salmonella* as result in saving labour, time, and materials. In most of the cases disposable cardboards containing dehydrated media, which eliminates the need for agar plates, constituting savings in storage, incubation and disposal procedures (Cox et al., 1987b; Fung, 1991). Others incorporate specialized chromogenic and fluorogenic substrates in media to rapidly detect trait enzymatic activity (Manafi et al., 1991; Hartman et al., 1992; Gaillot et al., 1999). There are also tests that measure bacterial adenosine riphosphate (ATP), which (although not identifying specific species), can be used to rapidly enumerate the presence of total bacteria.

Hartman et al. (1992) investigated many kits for enteric bacteria and evaluated their performance. Most of kits consist of multichamber disposable strips containing 15 to 20 dehydrated media especially designed to identify a target bacteria or species. With the exception of a few systems in which results can be interpreted in 4 h, most tests required 18 to 24 incubation (Swaminathan and Feng, 1994). The performances of most of the miniaturized biochemical tests appeared to be comparable and showed 90- 99% accuracy when compared standard methods for the identification of *Enterobacteriaceae* (Hartman et al., 1992; Fung, 1997). O'Hara et al. (1993) compared the API 20E (bioMerieux, France) system with conventional biochemical tests for identification of biochemical typical and atypical members of family *Enterobacateriaceae* and demonstrated 92.1% of the *Enterobacteriaceae* were correct to genus and species by API 20E test. Several miniaturized biochemical systems have been developed for the identification of non-*Enterobacteriaceae*. The enterotube II system and Oxi-ferm tube (Roche, Switzerland), and API 20NE (bioMerieux, France) are commonly used for the detection of non-enteric bacteria in food. Hanai et al. (1997) compared the six commercial bacterial identification kits with USFDA and Japanese standard method for identification of *Salmonella* in food and reported that xylose-lysine-brilliant green agar method was most efficient technique among the commercial kits for detection of *Salmonella* in food.

2.4.2 Immuno assays

The first use of immunological methods for diagnostic purpose occurred in the early 1900s, when researchers discovered that the serum and urine of the patients

with typhoid contained a soluble substance that would precipitate when mixed with rabbit anti-*Salmonella* antiserum (Marcon, 1995). Infact, most of the early methodology utilizing antigen-antibody reactions was available in the clinical laboratories long before such methods came into use by food microbiologists. As a result of growing interest in detecting infectious agents more rapidly and precisely, the technology has undergone tremendous changes, particularly in the development and usage of monoclonal antibodies. Use of monoclonal antibodies in the technology has improved the sensitivity and specificity of enzyme immunoassays (Robison, 1997). Now a days, large number of immuno assay formats are available that employ antibodies to specifically detect food borne pathogens, but, enzyme linked immunosorbent assay (ELISA) is most commonly used (Candish, 1991; Ramsay, 1998).

ELISA technique was first described by Engvall and Perlman (1971) in Sweden and van Weemen and Schuurs (1971) in Holland. The assay was based on antigen and antibody reaction and a 'lebel' attached to the antibody allow the reaction to be visualized. Depending upon the substrates used, enzyme assay either can be colorimetric or fluorogenic. The technique most commonly used to detect the bacterial antigens in foods is a version of noncompetitive ELISA called the sandwich ELISA (Robison, 1997). Usually designed as a sandwich assay, an antibody bound to a solid matrix is used to capture the antigen from enrichment cultures and a second antibody conjugated to an enzyme is used for detection. Antibodies coupled to magnetic particles or beads are also used in immunomagnetic separation (IMS) technology to capture pathogens from pre-enrichment media. IMS is analogous to selective enrichment, but instead of using antibiotics or harsh reagents that can cause

stress-injury, an antibody is used to capture the antigen, which is a much milder alternative and captured antigens can be plated or further tested using other assays (Oggel, 1990). Immunoprecipitation or immunochromatography, another antibody assay in a sandwich format but, instead of enzyme conjugates, the detection antibody is coupled to colored latex beads or to colloidal gold. Using only a 0.1 ml aliquot, the enrichment sample is wicked across a series of chambers to obtain results (Olsvik et al., 1994; Feldsine et al., 1997). These assays are extremely simple, require no washing or manipulation and are completed within 10 minutes after cultural enrichment. Enzyme based immunoassays has been successfully used for detection of *Salmonella* in meat and poultry (Emswiler-rose et al.,1984; Croci et al., 2004; Schneid et al., 2006) and an automatic Vidas system has been compared with conventional culture method for detection of *Salmonella* in food (Uyttendaele et al., 2003). The culture method and two commercial enzyme immunoassays for detection of *Salmonella* in porcine fecal and cecal contents were compared by Wegener and Baggesen (1997).

2.4.3 Nucleic acid based methods

2.4.3.1 Polymerase chain reaction (PCR)

Nucleic acid (DNA or RNA) based methods has become very popular for rapid detection of foodborne pathogens. The first *in vtro* amplification of mammalian genes using the Klenow fragment of *Escherichia coli* DNA polymerase was carried out by Kary Mullis (Saiki et al., 1985; Mullis and Faloona 1987). This assay is now popularly known as polymerase chain reaction (PCR). PCR assay has proven to be a most powerful molecular tool and revolutionized the entire molecular

biology. PCR assay require the target template DNA, primers, dNTPs and *Taq* polymerase, and based on the repeated cycles of enzymatic amplification of small quantities of target DNA in a thermocycler provide more than billion copies (Tenover et al., 1997). Role of PCR is applied in various field of food microbiology such as detection of microorganisms, detection of virulence genes and detection of genes responsible for antimicrobials (Cohen et al., 1996; Malorny et al., 2003a; del Cerro et al., 2002). More recently, PCR methods are used in the typing of bacterial isolates in epidemiological investigation. PCR based methods are more promising and found to be very sensitive for detection of foodborne pathogens including *Salmonella* in food (Hill, 1996). Different PCR validation studies showed that PCR method is one of the most promising techniques for the rapid detection of *Salmonella* spp. in food (Makino et al., 1999, Ferretti et al., 2001; Kumar et al., 2005). Several PCR based detection assays for rapid and specific detection of *Salmonella* in seafood has been developed and assays were compared with conventional method and reported PCR method was comparable to the culture method (Fach et al., 1999; Kumar et al. 2003). Vazquez-Novelle et al. (2005) demonstrated the samples positive by eight-hour PCR assay were also positive by standard microbiological method. However, PCR assay was reported to be far superior than of the conventional culture methods for detection of *Salmonella* in meat samples (Fratamico, 2003). Oliveira et al. (2003) showed the 15 meat samples positive for *Salmonella* by culture method and 33 samples were found positive by PCR method, when a total of 87 field meat samples were analyzed for the presence of *Salmonella* by culture and PCR assay. The main disadvantage for the adoption of *Salmonella* PCR in naturally contaminated foods is difficulties in terms of

amplification of dead cells DNA and the occasional inhibition for PCR assay by food matrix, thus, presenting a few false results in terms of sensitivity and specificity. More recently, RNA based techniques have been used in the detection of viable and non-culturable (VBNC) and live and dead cells. The amplification of mRNA by reverse transcription-PCR showed the ability to distinguish between living and dead *Escherichia coli* cells (Sheridan et al., 1998). Detection of *Salmonella* Entertidis by RT-PCR was reported by Szabo and Mackey (1999).

2.4.3.2 Real-time PCR

Quantitative microbial risk assessment (QMRA) is an important step for food safety in which risk factor that influence food safety are identified. This approach is very important when low numbers of foodborne bacterial cells are present in a food sample. Currently, nearly all quantitative data generated for *Salmonellas* were obtained from traditional bacteriological methods (Jensen et al., 2003; Blodgett, 2006). Quantitative culture based method are both cumbersome and time consuming, thus limiting the usage in routine analysis. PCR based method has been standardized by ISO and now being used for food testing (Malorny et al., 2003c). More recently, a second generation PCR called real-time PCR is developed and it offered the possibility of estimating the number of bacteria in different samples. The quantitation in real-time PCR is not based on the end point signal but rather based on the exponential increase in the initial target DNA amount with the number of PCR cycles performed. In real-time PCR, serial dilution of known number of target copies are used to set up a standard curve which is used to determine an unknown amount of DNA in a sample, hence, provides an absolute quantitative data of target sample (Fey et al., 2004). The specificity of the real-time PCR is confirmed by the melting

temperature (T_m) analysis of the amplicon obtained, which shows the temperature at which 50% of DNA amplicon is denatured (Ririe et al., 1997).

The automation of DNA sample preparation method and availability of large real-time PCR formats are undoubtly useful for generating a large amount of quantitative data at a high speed and low cost. Real–time PCR has been successfully used to detect *Salmonella* in clinical, food, and environmental samples (Levin, 2004; Josefsen et al., 2007). Apart from the quantitative detection, there are several advantages of real-time PCR over conventional PCR. Conventional PCR requires post-PCR gel electrophoresis analysis to confirm the presence of the target in the sample. In contrast, the real-time method is based on the increase in fluorescence, which indicates the presence of the target and is monitored during PCR assay, thus, no post PCR handling of the samples and reducing the risk of the false positive due to contamination in the laboratory. Ellingson et al. (2004) developed a rapid and quantitative real-time PCR for detection of *Salmonella* in raw and ready-to eat meat products and reported to detect 1cfu/ml of food homogenate. More recently, several real-time PCR based assays have been developed and perfected for quantitative detection of *Salmonella* in meat or food (Hein et al., 2006; Josefsen et al., 2007).

2.4.3.3 Probe based methods

The identification of bacteria by DNA probe hybridization methods is an important DNA method used for rapid detection of bacterial pathogens. This assay is in contrast to most other biochemical and immunological test that are based on the detection of gene products. Gene probes are a set of specific oligonucleotide sequence, which are labeled suitably, so that it can be detected in order to determine when hybridized with complementary DNA strand to form a double stranded DNA.

Probe based molecular method has been used as rapid and specific detection method for food borne pathogens including *Salmonella* (Riley and Caffrey, 1990; Knight et al., 1990; Hanes et al., 1995). Probe based assay is an important molecular screening technique for recombinant library of specific DNA sequences or target a specific gene with the help of labelled probe. The technique provides a sensitive and rapid approach for detection of positive colonies in a heterologous background. The process involves detection of the target strands of the DNA molecules or a bacterial colony with many copies of a single-stranded DNA or RNA molecule, called a probe. The entire process involved in several steps and finally the hybridized strands are visualized with chemilumiscent and colorimetric process (Lampel et al., 1992; Sambrook and Russel, 2001). Several restriction endonuclease fragments selected randomly from the *Salmonella* chromosome were used as probes to identify members of the genus (Holmes, 1989). More recently, non- radiolabeled probes are becoming popular among researchers, because of less hazardous in nature. Ribosomal gene based *Salmonellae* specific probes was designed for detection of *Salmonella* (Curiale et al., 1990) and *Salmonella* plasmid virulence (*spv*) gene based probe was developed for specific detection of *Salmonella* Enteritidis in food (Hanes et al., 1995).

2.5 *Salmonella* typing methods

2.5.1 Biotyping

Salmonella strains in a particular serovar may be differentiated into biotypes by their utilization pattern of selected substrates such as carbohydrates and amino acids. In many serovars there are few biochemical tests in which significant

numbers of strains behave differently and so the number of identifiable biotypes with in a serovar can be obtained. The organisms expressing different phenotypes of a given serotype are considered a different biotype, and that differences can be associated with differences in virulence properties (Anderson and Ziprin, 2001). Duguid et al. (1975) developed a scheme for biotyping to study the epidemiology of infections with *Salmonella* Typhimurium. This scheme was based on the use of 15 biochemical characters. Thirty-two potential primary biotypes were defined by the combinations of positive and negative reactions shown in the 5 tests (d-xylose, m-inositol, l-rhamnose, d- tartrate and m-tartrate) most discriminating in *Salmonella* Typhimurium. These primary biotypes were designated by numbers (1-32) and the full biotypes was developed by additional 10 secondary tests and finally, a total 24 primary and 184 full biotypes have been identified. Most recently, de la Torre (2005) used the biochemical kinetic data to determine strain relatedness among *Salmonella enterica* subsp. *enterica* isolates. *Salmonella* Mbandaka isolates in animals and their feed in Poland has been biotyped based on utilization of glucose, mannitol, sorbitol, rhamnose, sucrose, melibiose, amygdaline and arabinose, however, limitation of *Salmonella* Mbandaka biotyping in epidemiological investigation was reported (Hoszowski and Wasyl, 2001). In India, *Salmonella* Paratyphi B isolated from animals and their products were biotyped by Agarwal et al. (2003).

The mutants of *Salmonella* Typhimurium were found to be defective in utilization of the branched chain amino acids (Kiritani, 1974). Modified versions of the *Salmonella* Typhimurium biotyping scheme have been successfully applied to the epidemiology of *Salmonella* Agona. An international collection of 419 isolates of *Salmonella* Agona was biotyped and demonstrated that 92.6% of the isolates

belonged to biotype 1a, a rhamnose non-fermenting variant. A maltose late fermenting group was also established among the isolates (Barker and Old, 1982). *Salmonella* Montevideo isolates were grouped into different biotyped groups (Old et al., 1985). The ability of *Salmonella* Mbandaka isolates to utilise glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdaline and arabinose were determined by Hoszowski et al. (1999). *Salmonella* Typhi isolated from the hospitalized children in Kolkata, India were biotyped based on xylose fermenting property (Saha et al., 2003). Different biochemical tests results were used in the determination of strain relatedness among different serovars of *Salmonella enterica* subsp. *enterica* (59 *Salmonella* Typhimurium strains, 25 *Salmonella* Typhimurium monophasic variant strains, 25 *Salmonella* Anatum strains, 12 *Salmonella* Tilburg strains, 7 *Salmonella* Virchow strains, 6 *Salmonella* Choleraesuis strains, and 1 *Salmonella enterica* (4,5,12::) (de la Torre et al., 2005).

2.5.2 Serotyping

The basis of *Salmonella* serotyping depend upon the complete determination of different antigens i.e. somatic (O), flagellar (H) and capsular (Vi) antigens.

2.5.2.1 Somatic (O) antigens

These are heat stable antigens which are composed of phospholipid-polysaccharide complexes. Analysis of O antigens revealed polysaccharide (60%), lipid (20 to 30%), and hesomione (3.5 to 4.5%) (Edwards and Ewing, 1972). The nature of terminal groups and the order in which they occur in the repeating units of the polysaccharide chain provide the specificity to the numerous kinds of O antigens. Somatic antigens are resistant to alcohol and dilute acid and different form variants

(smooth, rough and form variant) are prevalent in *Salmonella* (Edwards and Ewing, 1972) and these variational phenomenon affect the serological typing of *Salmonella*. Smoooth (S) to rough (R) variations occur in *Salmonellae* and White (1925) showed the successful agglutination from the rough strains. The O antigens groups were first designated by letters of the alphabet (A-Z) and additional antigens were later delineated. The O antigens group factors were denoted by Arabic numerals. Since each letter of the alphabet was already used to describe an O antigen group, numbers (51-67) were too used to describe the latest O antigen groups. (Popoff and LeMinor, 2005). Kaufffmann and Petersen (1956) described somatic antigens which were named as T antigens. The first T antigen (T_1) was found in *Salmonella* Paratyphi B and *Salmonella* Typhimurium and second T antigen (T2) was reported to be present in *Salmonella* Bareilly (Edwards and Ewing, 1972). The T_1 was found in certain serotypes within serogroups B, E_1, E_4 and G. The change in O antigens due to bacteriophages and form variation has been discussed by Edwards and Ewing (1972).

2.5.2.2 Flagellar (H) antigens

These are heat-labile antigens that present in the flagella of *Salmonella* and proteineous in nature, which was called flagellin. The fllagelin was a keratin-myosin-epiderm-fibrinogen group protein of 40 Kda in molecular weight (McDonough, 1965). The amino acid content and the order in which these acids present in the flagellins determine the specificity of the different H antigens (Edwards and Ewing, 1972). The flagellar agglutination occurs very rapidly and the aggregates formed are loosely knit and floccular forms. The phase variations in H antigens were reported in *Salmonella arizonae* by Edwards et al. (1947). *Salmonella*

strains may produce one (monophasic) or two (diphasic) sets of flagellar antigens. These homologous surface antigens are chromosomally encoded by the H1 (phase 1) and H2 (phase 2) of the vh2 locus (Le Minor, 1981). By convention each serotype has been denoted by an antigenic formula with the major O antigen listed first, followed by H phase 1 antigen (s), and then H phase 2 antigen (s). The antigen H phase 1 are designated by lowercase letters and then phase 2 H antigens by Arabic numerals or some instances, by components of e or z series (Ewing, 1986) (See Table 2.3).

2.5.2.3 Capsular (Vi) antigens

The capsular antigens are present in *Salmonella* Typhi, *Salmonella* Dublin and *Salmonella* Paratyphi A. The Vi antigen in *Salmonella* Typhi was reviewed by Webster et al. (1951) and Martin et al. (1969), and demonstrated that Vi antigen could be purified by chemical method. The thermal solubilization of capsular antigen (Vi) antigen is necessary for the immunological detection of serotypes containing capsular antigens. There were reports of some other mucoid (M) and nonmucoid (N) antigens of *Salmonellae* (Kauffmann, 1936). Certain other antigen i.e. X was also reported to be present in *Salmonella* cultures and was first showed by Topley and Ayrton (1924). They reported the X antigen was formed in cultures incubated at 34°C, and to be present in both smooth and rough form of *Salmonellae*.

The biochemical identification of food borne and clinical *Salmonella* isolates are coupled to serological confirmation based on somatic (O) flagellar (H) and the capsular (Vi) antigen. (Le Minor, 1981). Officially recognised by the World Health Organization (WHO), the Kauffmann-White diagnostic scheme involves the

primarily subdivision of *Salmonella* into serogroups and further delineated into serotypes based on the O, H and Vi antigenic formula (Ewing, 1986).

Table 2.3 Antigenic formulae of a few important serovars of the genus *Salmonella*: The Kauffmann-White scheme.

Salmonella serotype	Serogroup	Somatic (O) antigen	Flagella (H) antigen	
			Phase 1	Phase 2
S. Paratyphi A	A	1, 2, 12	a	[1,5]
S. Typhimurium	B	1, 4, [5], 12	i	1,2
S. Braenderup	C1	6,7, 14	e, h	e, n, z_{15}
S. Cochin	D2	9,46	k	1,5
S. Weltevreden	E1	3,10, [15]	r	Z_6
S. Luciana	F	11	a	e,n,z_{15}
S. Poona	G	1,13,22	z	1,6
Salmonella IV	Z	50	b	Z_6
S. Utrecht	O:52	52	d	1,5

The heat stable O antigen consist of lipopolysaccharide-protein chain exposed on the cell surface and are classified as major and minor antigens. The major category consists of antigens such as such as somatic factors O;4 and O;3, which are specific determinants of serogroups like B and E, respectively. In contrast minor somatic antigenic components, such as O;12 are nondiscriminatory, as evidenced by their presence in serogroups (D'Aoust et al ., 2001). These are heterogeneous structures and the antigenic specificity is determined by the composition and the lineage of the O group sugars and sometimes mutation affect the sugars leading to new O antigen (Kreig and Holt, 1984).

The aim of the serological testing procedure is to determine the complete antigenic formula of individual *Salmonella* isolate. Commercially available polyvalent somatic antisera kits consist of mixtures of antibodies specific for major antibodies. Following a positive agglutination with polyvalent antisera, single factor group would be used to define the serogroup of the isolate. Flagellar (H) antigens would then be determined by broth agglutination reaction using polyvalent H antisera or the Spicer-Edwards series of antisera. In the former assay, a positive agglutination reaction with one of the five polyvalent antisera (poly A-E; Difco laboratories, USA) would lead to testing with single factor antisera to specifically adentify the phase 1 and phase 2 flagellar antigens present. Agglutination in poly flagellar antiserum and subsequent reaction of the isolate with single grouping H antisera would confirm the presence of the phase 1 antigen. Phase reversal in a semisolid media of phase 1 antiserum would immobilize phase 1 *Salmonellae* at or near the point of inoculation, therefore, facilitating the recovery of phase 2 cells from the edge of the zone of migration (D'Aoust et al., 2001). The serological typing of *Salmonellae* has led to identification of large number of *Salmonella* serovars. In the Kauffmann-White scheme, there are currently 2541 *Salmonella* serotypes from different sources (Popoff et al., 2004). The antigenic formulas of some of the important *Salmonella* serovars are shown in Table 2.3.

2.5.3 Molecular Typing of *Salmonella*

Traditionally, food and clinical laboratories are using the conventional typing system, usually based on specific phenotypic characterizations. Unfortunately, as a result of inconsistently expressed phenotypic traits, these classical typing approaches

are often unable to discriminate between related outbreak strains. The ability to characterize and determine the genetic relatedness among bacterial isolates involved in a food borne outbreak is a prerequisite for epidemiological investigations. Detailed strain identification is essential for the successful epidemiological investigation of *Salmonella* outbreaks. Investigations have relied traditionally on serological and antibiogram techniques. In contrast, modern typing methods are based on characterization of the genotype of the organism. Hence, molecular typing or fingerprinting of *Salmonella* isolates is an invaluable epidemiological tool that can be used to track the source of infection and to determine the epidemiological link between isolates from different sources.

The ability of molecular typing systems is to distinguish among epidemiologically unrelated isolates based on genetic variation in chromosomal DNA of a bacterial species (Swaminathan and Matar, 1993). Usually this variability is high, and differentiation of unrelated strains can be accomplished using a variety of fingerprinting techniques. The genotyping methods are those methods, which are based on the genetic structure of an organism and include polymorphisms in DNA restriction patterns based on cleavage of the chromosome. The digestion of the chromosomal DNA provides variable number of the DNA fragments, thus, reveals genetic variations. Genotyping methods are less subject to natural variation, though various factors may be responsible for genetic variants such as insertions or deletions of DNA into the chromosome, the gain or loss of the extra chromosomal DNA, and random mutations that may create or eliminate restriction sites. (Tenover et al., 1997). There is currently no gold standard typing system available for *Salmonella* fingerprinting, however, pulsed field gel electrophoresis (PFGE) has been

considered most suitable molecular typing method. The combination of different genotyping methods such as plasmid profile analysis, ribotyping, enterobacterial repetitive intergenic consensus sequence analysis (ERIC-PCR), random amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis methods have been evaluated for more precise subtyping of *Salmonella* serovars.

2.5.3.1 Plasmid Profile

Plasmids of *Salmonella* are varying in size from 2 to 200 kb. Virulence plasmids are the most described group of plasmids in *Salmonella.* Different serovars viz.; *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Enteritidis, *Salmonella* Choleraesuis, *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Abortusovis reported to possess serovar specific plasmids which shared considerable homologies (Montenegro et al., 1991). Large plasmids were found to be absent in strains of the following serotypes; *Salmonella* Agona, *Salmonella* Bovismorbificans, *Salmonella* Heidelberg, *Salmonella* Infantis, *Salmonella* Panama, *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella* Saintpaul, *Salmonella* Seftenberg and *Salmonella* Typhi (Popoff et al., 1984). Besides the serovar specific virulence plasmids, *Salmonella* also harboured additional high molecular weight plasmids which can transfer resistance to antibiotics or low molecular weight plasmids with sizes below than 20 kb of unknown functions (Rychlik et al., 2006).

The virulence plasmid can be experimentally exchanged without affecting the virulence property of a new host (Barrow and Lovell, 1989). Plasmid profile analysis has been used as a rapid molecular typing method and has shown some success in the discrimination of *Salmonella* serotypes (Crichton et al., 1996; Holmberg et al., 1984). Plasmid analysis has been used in the epidemiological

investigation of an instance of salmonellosis outbreak in England and found that a single strain was responsible for recurrent infection of immuno compromised patients (Mayer and Hanson, 1986). Plasmids are not always useful for epidemiological tools since all strains do not contain plasmid. In a study of *Salmonella* Typhi isolates obtained during a typhoid outbreak in Chile and Peru, only 17 of 141 isolates contained plasmid and the plasmid profile were not useful for dividing the major Vi-phage groups as absence of plasmid was observed among the other 124 *Salmonella* isolates (Maher et al., 1986). Helmuth et al. (1985) demonstrated that 90 % of 337 *Salmonella* isolates originating from 29 different countries contained serotype specific plasmids and further proved that four serovars; *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Enteritidis and *Salmonella* Choleraesuis contained virulence plasmids, whereas, three serotypes (Infantis, Panama, and Heidelberg) harboured plasmids unrelated to virulence. Beninger et al. (1988) showed that plasmids of different size and endonuclease restriction patterns are found in *Salmonella* Enteritidis and *Salmonella* Choleraesuis and share a common 4 kb Eco-R 1 restriction fragment with the 80 kbp virulence plasmid of *Salmonella* Dublin (pSDL2). Plasmid pSDL2 is required for the development of a lethal systemic infection in the mouse virulence test and portions of pSDL2 were homologous to a virulence plasmid of *Yersinia* species (Krause et al., 1991). Naturally occurring strains of *Salmonella* Dublin, *Salmonella* Enteritidis and *Salmonella* Choleraesuis most often isolated from non-typhoid human systemic salmonellosis, typically carry the virulence plasmids. It was presumed that 4 kbp region of the virulence plasmid was partly responsible for the ability of these serotypes to cause the human systemic infections (Roudier et al., 1990).

2.5.3.2 PCR-ribotyping

All strains of *Salmonella* appear to be at least 70 % related by DNA hybridization, but intra-serovar differentiation is essentially designed for epidemiological investigations during outbreaks and for long time it was carried out with conventional methods (Crosa et al., 1973). The fingerprinting of rRNA coding sequence i.e. ribotyping has been used for detection of genetic variation among *Salmonella* serovars (Altwegg et al., 1989; Esteban et al.,1993; Jensen et al., 1993).

The main disadvantage of ribotyping was involvement of lengthy and cumbersome processes of restriction digestion, probe development, hybridization and followed by detection. This has led to development of more promising and simple PCR-ribotyping technique, which is based on the amplification of the spacer sequences between the 16S and 23S genes in the rRNA transcriptional units (Lagatolla et al., 1996). Ribosomal RNA loci are present in 2 to 11 copies on the chromosome of most bacterial species. A high degree of sequence homology exit for rRNA genes and the intergenic spacer regions showed extensive sequence and length variations that can be used for characterization of bacteria at the genus (Jensen et al., 1993) species, and subspecies levels (Dolzani et al., 1995; Soto et al., 2001). PCR-ribotyping technique detected the polymorphism in 16S-23S regions of *Salmonella* isolates, either by direct analysis of the amplification products or after digestion by restriction enzymes (Nastasi and Mammina, 1995; Baudart et al 2000). *Salmonella* contain seven '*rrn*' operons with a different degree of sequence divergence, which is larger in the 16S-23S regions than in rRNA genes. The amplification of intergenic 16S-23S regions of the '*rrn*' ribosomal operons in

Salmonella was carried out by Kostman et al. (1992). *Salmonella* from animal origin were fingerprinted based on PCR-ribotying method (del Cerro et al., 2002).

2.5.3.3 Enterobacterial repetitive intergenic consensus (ERIC) -PCR

In bacterial genomes, numerous families of short (30–150 bp) interspersed repetitive sequences have been identified (Lupski and Weinstock, 1992; Bachellier et al. 1996; Tobes and Ramos, 2005) and most families are restricted to single species or very closely related species. This suggested that if these repeats have any functions they have been acquired recently, may not apply to all members of the family, and not found to be responsible in fundamental aspects of bacterial growth, survival, and replication. However, few repetitive sequences have been reported to act as binding sites for a variety of proteins, including DNA polymerase and DNA gyrase (Gilson et al., 1990). There are considerable variations among strains with respect to the presence of an element in any particular intergenic region, but some copies appear to have been conserved since before the divergence of *Escherichia coli* and *Salmonella enterica*. In comparisons of orthologous copies between the species, ERIC sequences are surprisingly conserved and signified that they have acquired some function related to mRNA stability (Newbury et al., 1987).

Enterobacterial repetitive intergenic consensus (ERIC) sequences, also described as intergenic repetitive units, differ from most other bacterial repeats in being distributed across a wider range of species. ERIC sequences were first described in *Escherichia coli*, *Salmonella* Typhimurium, and other members of the *Enterobacteriaceae*, as well as *Vibrio cholerae* (Sharples and Lloyd, 1990; Hulton, et al., 1991). ERIC sequences are an imperfect palindrome of 127 bp. In addition, shorter sequences produced by internal deletions have also been described (Sharp

and Leach, 1996), as well as longer sequences due to insertions of about 70 bp at specific internal sites (Cromie et al., 1997). ERIC sequences have been found only in intergenic regions, apparently only within transcribed regions (Hulton et al., 1991). The number of copies of the ERIC sequence varies among species; it was initially estimated by extrapolation that there may be about 30 copies in *E. coli* K-12 and 150 in *Salmonella* Typhimurium LT2 (Hulton et al., 1991). To date, the most extensively analyzed family of bacterial short repetitive sequences is that of the 30- to 40-bp REP/PU sequences found in *E. coli*, *Salmonella enterica*, and their close relatives (Stern et al., 1984; Gilson et al., 1991). ERIC-PCR based method has been utilized for the genotyping of different bacterial pathogens (Alam et al., 1999; Marshall et al., 1999).

The strain differentiation in Indian isolates of *Salmonella* Abortusqui, *Salmonella* Choleraesuis, *Salmonella* Dublin, and *Salmonella* Bareilly were carried out with ERIC-PCR (Saxena et al., 2002). ERIC-PCR technique has been extensively used in the fingerprinting of *Salmonella* serovars isolated from different sources and molecular epidemiology of different *Salmonella* serovars involved in disease outbreaks (Burr et al., 1998; Ling et al., 2001; Lim et al., 2005). *Salmonella* Typhimurium bovine isolates identified from the farms and meat sources were evaluated with ERIC-PCR and results were compared with other molecular markers (Millemann et al., 2000). Fourteen isolates of *Salmonella* Weltevreden of seafood origin were fingerprinted with ERIC-PCR technique and found the multiple clones of *Salmonella* Weltevreden in seafood (Shabarinath et al., 2007).

2.5.3.4 Pulsed field gel electrophoresis (PFGE) analysis

The advancement in molecular fingerprinting has helped the food and health laboratories to determine the source of contamination and understand the epidemiology of infection. Pulsed field gel electrophoresis (PFGE) is one such molecular fingerprinting approach that identifies the organism based on their genotype pattern (Bohm and Karch, 1992; Birren and Lai, 1993; Tenover et al., 1995). PFGE involves the use of rare cutter restriction enzyme to generate a limited number of high molecular weight restriction fragments. These fragments are then separated by alternative directional agarose gel electrophoresis. The resulting eletrophoretic patterns are highly specific for strains from a variety of organisms and also provide an opportunity to analyze multiple variations to the entire genome of the organisms so as to identify specific strains and accurately link them with disease outbreaks. PFGE technique has been considered most powerful technique for molecular characterization of foodborne bacterial pathogens and extensively used for epidemiological study of *Salmonella* serovars from different environments including food (Schlichting et al.,1993; Barrett et al., 1994; Ribot et al., 2001; Berge et al., 2004).

The high discriminatory power and reproducibility are the main advantages of PFGE technique over other molecular typing methods and found to be the method of choice for molecular characterization of *Salmonella* serovars. Genetic diversity of clinical and environmental strains of *Salmonella* Weltevreden isolated in Malaysia has been analyzed by PFGE technique (Thong et al., 2002). PFGE was found to be very useful to identify the *Salmonella* intraserovar clonal variations and PFGE technique was used for characterization of heterogeneity and clonality in *Salmonella*

isolates obtained from the carcasses and faeces of swine samples at slaughters (Wonderling et al., 2003). Multidrug -resistant *Salmonella* Newport was fingerprinted based on PFGE analysis to identify the genetic variation among different isolates (Berge et al., 2004). Different *Salmonella* serovars, such as *Salmonella* Typhimurium, *Salmonella* Mbandaka and *Salmonella* Enteritidis were characterized by PFGE assay (Thong et al., 1995; Hoszowski and Wasyl, 2001; Tamada et al., 2001). Most recently, a report on *Salmonella* Enteritidis isolated from food, animals and humans showed that certain clones were prevalent worldwide (Pang et al., 2007).

2.5.3.5 *Salmonella* Pathogenicity and virulence genes

The nature of pathogenicity of an organism lies in virulence genes or virulence factors. However, these terms are still not strictly defined (Wassenaar and Gastraa, 2001). The possible virulence factors of *Salmonella* have been understood with the gain of knowledge on the molecular mechanism behind the pathogenicity of *Salmonella.* Very recently, involvement of effector protein in survival and replication of *Salmonella* in host cells has been elucidated. The majority of virulence genes of *Salmonella* are clustered in a region distributed over the chromosome, called *Salmonella* pathogenicity islands (SPI) (Groisman and Ochman, 1996; Marcus et al., 2000). Until recently, five SPIs (SPI-1 to SPI-5) have been identified on the *Salmonella* chromosome at centisome 63, 31, 82, 92 and 25 cs, respectively (Blanc-Portard and Groisman, 1997; Hayward and Koronakis, 2002). Each SPI was responsible in various cellular activities towards the virulence factor of the organism (Wong et al., 1998; Wood et al., 1998). On completion of genome sequence of *Salmonella* Typhi strain CT 18 another five regions were identified and

designated as SPI-6, SPI-7, SPI-8, SPI-9 and SPI-10. The 6.8 kb large SPI-8 encodes for genes conferring resistance to bacteriocin, SPI-9 for type I secretion system whereas SPI-10 encode for *sef* fimbrial operon (Galan et al., 1992; Parkhill et al., 2001). SPI-6 encode for *saf* and *tcf* fimbrial operon and SPI-7 encode for Vi biosyntheis genes and also for IV fimbrial operon (Parkhill et al., 2001; Pickard et al., 2003). The flagella mediated bacterial motility acceleates but not required for *Salmonella* Enteritidis invasion in differentiated Caco-cells (van Asten et al., 2004).

The *Salmonella* virulence factor were also detected in virulence plasmids in certain *Salmonella* serovars namely *Salmonella* Abortusovis, *Salmonella* Cholerasuis, *Salmonella* Dublin, *Salmonella* Enteritidis, *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Typhimurium, although not all isolates of these serotypes carry the virulence plasmid. (Rotgar and Casadesus, 1999). All plasmids contain the 7.8 kb *Salmonella* plasmid virulence (*spv*) locus. This locus harbored five genes, designated spv RABCD and expressions of *spv* genes might play a role in the multiplication of intracellular *Salmonellae* (Chu et al., 2001). The results showed that *spv*B together with *spv*C conferred virulence to *Salmonella* Typhimurium when administered subcutaneous to mice (Matsui et al., 2001). More recently in *Salmonella* Typhi CT 18 exhibited a 106 kb large cryptic plasmid with some homology to a virulence plasmid of *Yersinia pestis*. However, the majority of *Salmonella* Typhi tested did not harbour this plasmid. Cryptic plasmid has also been reported for *Salmonella* Paratyphi C, *Salmonella* Derby, and *Salmonella* Copenhagan, *Salmonella* Durban, *Salmonella* Give and *Salmonella* Infantis (Ou et al., 1990; Rotgar and Casadesus, 1999). Hybridization analysis has shown a few

other serotypes such as *Salmonella* Johannesburg, *Salmonella* Kottbus and *Salmonella* Newport found to bear the virulence plasmids.

Salmonella produce both endotoxin and exotoxin and virulence due to these toxins were well understood. The endotoxin, lipid portion (lipid A) of the outer lipopolysaccharide (LPS) membrane of *Salmonella* elicited a variety of *in vitro* and *in vivo* biological responses (Hitchcock et al., 1986). The best studied exotoxins of *Salmonella* was the heat labile *Salmonella* enterotoxin (*stn*) of approximately 29 kDa and encoded by *stn* gene (Chary et al., 1993; Prager et al., 1995). A study on 90 kDa heat labile enterotoxins of *Salmonella* Typhimurium was also reported by Rahman and Sharma (1995). The role of fimbriae and flagella of *Salmonella* have been well identified in the attachment and movement of the organism but role in pathogenesis is still not understood (Folkesson et al., 1999; Edwards et al., 2000).

Characterization of different virulence factors in *Salmonella* serotypes have been carried out by amplifying different gene sequences responsible for specific phenotypic properties. The amplification of *inv*A gene by PCR indicates presence of invasion gene in *Salmonella* serovars, thus, highlighting the presence virulence factor in *Salmonella*. A PCR based study demonstrated that *stn* gene was present in all *Salmonella enterica* serovars, whereas, it was absent in *Salmonella bongori* (Prager et al., 1995). The cumulative effect of virulence by these genes were found to be responsible for invasion to the epithelial cells of intestine and thereafter, leading to gastrointestinal disorder. PCR assays for several virulence (*inv*, *him*), and functional (*iro*B, *fim*Y) genes were developed for detection of *Salmonella* in natural environmental or in food or faeces samples (Bej et al., 1994; Baumler et al., 1997; Yeh et al., 2002; Malorny et al., 2003a). The *fli*C gene also has been successfully

used for molecular typing studies on *Salmonella*, based on high variability of the central region (Kilger and Grimont, 1993; Dauga et al., 1998).

2.6 Salmonellosis

2.6.1 Reservoirs and epidemiology

The primary reservoir of *Salmonellae* is the intestinal tract of birds and animals, particularly of poultry and swine. The organisms are excreted in faeces from which they may be transmitted by insects and other creatures to a large number of places such as water, soils and kitchen surfaces. There are host adaptation patterns among serovars, namely highly host adaptive, less host adaptive and non-host adaptive (Ecuyer et al., 1996). Human host adaptive serovars like *Salmonella* Typhi cause of typhoid fever; in contrast, the highly host adaptive chicken pathogens viz., *Salmonella* Pullorum and *Salmonella* Gallinarum are not human pathogen. There is no report of *Salmonella* Typhi host range extending beyond human beings (Ziprin and Hume, 2001). Hence, isolation of *Salmonella* Typhi from food or water must be indication of contamination from human beings. Other *Salmonella* serovars are found to be host adapted animal pathogens and sources of zoonotic infections i.e. an etiological agents of disease in animals that are secondary transmitted to human beings (Davis et al., 1968). *Salmonella* Choleraesuis is a pathogen of swine but sometime causes severe systemic infections in humans (Ziprin, 1994; Wang et al., 1996). Similarly, *Salmonella* Dublin may cause septicemia in cattle and be transmitted to human from milk and milk products (Fang and Fierer, 1991; Reher et al., 1995). *Salmonella* Enteritidis and *Salmonella* Senftenberg are host adapted to chicken and turkey, respectively. The typhoid serovars are not host adapted and are found to be present in wide array of animal products including seafood, fruits,

vegetables, water and processed foods (Ziprin, 1994). Most other *Salmonella* serovars are not host adapted and also tend to be less virulent than the host-adapted serotypes, but they are found to be responsible for overwhelming number (90 %) of incidents of human salmonellosis (Webber, 1996; Hunter, 1997). In recent years, certain sea animals such as sea turtles, sea lions and elephant seals have been found positive for *Salmonella* (Fenwick et al., 2004; Stoddard et al., 2005).

The widespread occurrence of *Salmonella* spp. in the natural environment have been attributed to the intensive animal husbandry practices used in the meat, fish, and shellfish industries. The recycled raw material of offal and inedible raw materials into the animal and aquaculture feeds have favoured the widespread transmission of this human pathogen in the food chain (D'Aoust, 1991). Many sectors within the meat, poultry and eggs industries remained a prominent reservoir of *Salmonella* spp. in many countries. Rapid depletion of capture fisheries in recent years has greatly increased the importance of the aquaculture industry as an alternative source of fish and shellfish. The high-density farming conditions are required to maximize the yield. The growing demand play an important role in the dissemination of various human pathogens including *Salmonella*. The feeding of poultry waste and raw meat scrap and offal potentially contaminated with different *Salmonella* serovars is practiced in many part of the world (D'Aoust et al., 2001). In an effort to actively control the problem of *Salmonella* in meat, the Food Safety Inspection Service (FSIS) of the U.S. Department of Agriculture implemented the HACCP (Hazard Analysis Critical Control Point) system in 1996. After the implementation of HACCP system the level of *Salmonella* contamination in chicken was reduced to half (D'Aoust, 1994).

The information about incidence and serovars distribution of *Salmonellae* in domestic animal populations is essential for understanding the relationships within and among reservoirs of *Salmonellae* in animals and humans that are ultimately responsible for zoonotic disease transmission (Gast, 1997). *Salmonella* infection is usually acquired by the oral route, mainly by ingesting contaminated food or drink. *Salmonella* can be transmitted directly from human to human or from animal to human without the presence of contaminated food or water, but this is not a common mode of transmission.

2.6.2 Foodborne outbreaks and Public health impact

Salmonella is one of the microorganisms most frequently associated with food-borne outbreaks based illnesses. Despite the recent improvement in procedures for the epidemiologic investigation of foodborne outbreaks and quality standards, the global increase in foodborne salmonellosis is reported (Todd, 1994). The true incidence of *Salmonella* infection is difficult to determine as most the salmonellosis cases in poor countries are not documented properly. The reported cases represent only a small proportion of the actual number because it is only large outbreaks that are investigated and documented. The actual food borne diseases has been presumed to be 350 times more frequent than actually reported (Anon. 1997). Different food items such as meat, eggs, fruit juice and vegetable have been found to be the vehicle of salmonellosis outbreaks (Kapperud et al., 1990; Ponka et al., 1995; Beers, 1997). Most recent, outbreaks showed that *Salmonella* Enteritidis, *Salmonella* Paratyphi B, *Salmonella* Oranienburg and *Salmonella* Muenchen were responsible for foodborne salmonellosis (Gordenker, 1999).

Table 2.4 Worldwide major foodborne salmonellosis outbreaks

Sl No.	Country	Source	*Salmonella* serovar	No. of cases	References
1.	Holland (1981)	Salad	Indiana	600	Beckers et al., 1985
2.	Scotland (1981)	Raw milk	Typhimurium PT204	654	Cohen et al., 1983
3.	Canada (1984)	Cheese	Typhimurium PT10	2700	D'Aoust et al., 1985
4.	USA (1985)	Pasteurized milk	Typhimurium	16284	Lecos, 1986
5.	China (1987)	Egg drink	Typhimurium	1113	Ye et al., 1990
6.	Japan (1988)	Cuttlefish	Champaign	330	Ogawa et al., 1991
7.	USA (1991)	Cantaloupes	Poona	400	Francis et al., 1991
8.	France (1993)	Mayonnaise	Enteritidis	600	Geiss et al., 1993
9.	Germany (1993)	Paprika chips	Saint-paul, Javiana	670	Lehmacher et al., 1995
10.	Finland (1994)	Alfalfa sprouts	Bovismorbificans	492	Ponka et al, 1995.
11.	USA (1995)	Orange Juice	Hartford	62	Parish, 1998
12.	Canada (1998)	Cheddar cheese	Enteritidis PT8	700	Ratman et al.1999
13.	Japan (1999)	Cuttlefish chips	Oranienburg, Chester	1500	Tsujii and Hamada, 1999
14.	Australia (1999)	Orange Juice	Typhimurium	427	Anon. 1999
15.	USA (1999)	Orange Juice	Muenchen	220	Boase et al., 1999
16.	USA (2000)	Orange Juice	Enteritidis	74	Butler, 2000
17.	Germany (2004)	Fermented sousage	Goldcoast	24	Bremer et al., 2004

The major world wide incidences of foodborne salmonellosis are given in Table 2.4. Typhoid and non-typhoid salmonellosis remain major public health problems and are clearly the most economically important food-borne disease. The incidence of typhoid salmonellosis is stable, with very low numbers of cases in developed countries, but cases of non-typhoid salmonellosis are increasing worldwide. Non-

typhoid cases account for 1.3 billion cases of acute gastroenteritis/ diarrhoea with 3 million deaths and for 16 million cases of typhoid fever with nearly 600,000 deaths (Pang et al., 1995).

In the US 1997, the estimated annual incidence of salmonellosis was 13.8 cases per 100,000 people. However, most cases are unreported, and the true incidence may be much higher. Although the incidence is greatest among children, outbreaks are common among individuals who are institutionalized and residents of nursing homes. Far fewer cases of typhoid fever occur each year (0.2 per 100,000 people), and these are increasingly associated with travel to developing countries (currently 72% of cases) (Zapor and Moran, 2005). The Center for Disease Control and Prevention (CDC) in Atlanta, GA in 1999 estimated that there were about 1.5 million cases with 500 deaths associated with the consumption of food contaminated with *Salmonella*. The United States Department of Agriculture Economic Research Service (USDA ERS) estimated that for the six major bacterial pathogens, the costs associated with human illness were $9.3 to $12.9 billion annually (Busby et al., 1996).

In many countries, the incidence of salmonellosis has markedly increased; however, a paucity of good surveillance data exists. In the Netherlands, which has a population of 15.8 million, 50,000 cases of salmonellosis are reported each year (incidence, 3 per 1,000 person-years) (Van Pelt and Valkenburgh, 2001). An estimated 12-33 million cases of typhoid fever occur globally each year, and the disease is endemic in many developing countries of the Indian subcontinent, South and Central America, and Africa (Zapor and Moran, 2005). Bean et al. (1997) have carried out the surveillance of foodborne outbreaks in USA during the period, 1988-

92. In their report they showed that among the bacterial outbreaks *Salmonella* Enteritidis contributed the maximum (79%) in the outbreaks. The Center for Disease Control and Prevention (CDC) estimates 76 million people suffer food-borne illnesses each year in the United States, accounting for 325,000 hospitalizations and more than 5,000 deaths. Food-borne disease is extremely costly and health experts estimate that the yearly cost of all food-borne diseases in the US is five to six billion dollars in direct medical expenses and lost productivity. Infections with *Salmonella* alone account for one billion dollars yearly, in direct and indirect medical costs (Mead et al., 1999).

2.6.3 *Salmonella* serovars in food

2.6.3.1 Prevalence and distribution of *Salmonella* serovars in meat, poultry and eggs

2.6.3.1.1 National scenario

Presence of the *Salmonella* serovars in food animals are well studied in the country. The quality of the meat (goat, sheep and buffalo) from retail outlets of Bareilly and Haldwani (India), were investigated with the 446 meat samples. They showed, out of 446, 57 samples (12.78%) yielded *Salmonella* serotypes viz. *Salmonella* Bareilly, *Salmonella* Stanley, *Salmonella* Weltevreden, *Salmonella* Newport, *Salmonella* Saintpaul, *Salmonella* Typhimurium, *Salmonella* Agona, *Salmonella* Anantum, *Salmonella* Chester and *Salmonella* Senftenberq (Sharma et al., 1989). Similarly, prevalence of *Salmonella* among goat meat in Bareilly (Northern India) was determined with indirect ELISA and results highlighted the presence of *Salmonella* in 46% of the goat meat (Chandra et al., 2006). Presence of avian *Salmonella enterica* serovars infections in poultry in geographical locations of

India was investigated (Prakash et al., 2005). The study revealed the presence of 23 *Salmonella* isolates from different disease outbreaks.

Chandra et al (2007) detected *Salmonella* in 35 out of 206 slaughtered goat meat samples in Bareilly (India). *Salmonella* was isolated either from mesenteric lymph nodes (11) or from gall bladder (15) or from both of the organ (9) of the goats examined. 60 *Salmonella* isolates included 34 from gall bladder (of 24 goats) and 26 from mesenteric lymph node (of 20 goats) samples. The serotyping revealed 17 serovars and majority of serovars were identified as *Salmonella* Czernyring, *Salmonella* Louga, *Salmonella* Rovaniemi, *Salmonella* Kirkee, *Salmonella* Sarajane, *Salmonella* Altona. Sharma et al. (1987) investigated a total of 343 pork and pork products in north Indian cities for the presence of *Salmonella*. Out of these, 42 (12.24%) samples yielded different *Salmonella* serotypes viz. *Salmonella* Oranienburg, *Salmonella* Senftenberg, *Salmonella* Heidelberg, *Salmonella* Weltevreden, *Salmonella* Infantis, *Salmonella* Indiana, *Salmonella* Newport, *Salmonella* Bareilly, *Salmonella* London, *Salmonella* Stanley, *Salmonella* Saintpaul, *Salmonella* Derby and *Salmonella* Bovismorbificans. The level of contamination in different pork products ranged from 7.7% in hot dog to 41.7% in pork sausage.

Suresh et al. (2006) conducted a study to determine the incidence of *Salmonella* Enteritidis and other *Salmonella* serovars on eggshell, egg contents and on egg-storing trays from retails markets of Coimbatore, South India. A total of 492 egg samples and 82 egg-storing trays were examined over a period of one year and study highlighted the *Salmonella* contamination in 38 of 492 (7.7 %) eggs, out of which 29 was in eggshell (5.9 %) and 9 in egg contents (1.8 %). A 7.5 % of the egg-storing trays were also found to be contaminated with *Salmonella*. *Salmonella*

Enteritidis was the main serovars identified in egg shell and egg content, however, a few other serovars encountered were *Salmonella* Cerro, *Salmonella* Molade and *Salmonella* Mbandaka from eggshell and *Salmonella* Cerro from egg-storing trays.

2.6.3.1.2 International scenario

The incidence of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 was determined in 100 Turkish sausage *(Soudjouck)* samples collected from shops and markets in the Afyon province (Turkey). *Salmonella* spp. were detected in 7 % of the samples and all of the isolates were *Salmonella* Paratyphi B (Siriken et al., 2006). Distribution of *Salmonella* in swine herds in Québec (Canada) was studied by Letellier et al. (1999) with a 208 farm environmental samples and 87 samples (42 %) were found contaminated by *Salmonella* spp. Ten serotypes of *Salmonella* (n = 132) were identified in the production pyramid with a predominance of *Salmonella* Derby (37.1%) and *Salmonella* Typhimurium (34.1%). Duffy et al. (1999) investigated the Irish retail meat (n=74) and poultry samples (n=106) for the presence of naturally occurring *Salmonella* spp. The pathogen was detected in 28 poultry (n=106), two pork (n=22) and one cooked meat samples (n=20) examined. *Salmonella* was not isolated from minced beef or lamb samples tested. The most commonly isolated serotype was *Salmonella* Bredeney accounting for 48·4%, followed by *Salmonella* Kentucky (35·5%) and *Salmonella* Enteritidis (6·5%). Burkhalter et al. (1995) analyzed *Salmonella* spp. in eggs and compared the three different methods. *Salmonella* serovars prevalence at the population and herd level in pigs in the Netherlands was studied by ELISA method (van der Wolf et al., 2001) and results showed the variable titer that out of 406 finishing herds, 69.7 % had

Salmonella-status I (low prevalence), 21.7 % status II (moderate prevalence) and 8.6 % status III (high prevalence, cutoff OD%>40). During 2002 and 2003, *Salmonella* isolates (380) were recovered from animal diagnostic samples obtained from four state veterinary diagnostic laboratories in USA. Forty-seven serovars were identified, the most common being *Salmonella* Typhimurium (26%), *Salmonella* Heidelberg (9%), *Salmonella* Dublin (8%), *Salmonella* Newport (8%), *Salmonella* Derby (7%), and *Salmonella* Choleraesuis (7%) (Zhao et al., 2007). *Salmonella enterica* serovars viz., *Salmonella* Typhi, *Salmonella* Enteritidis, *Salmonella* Anatum, *Salmonella* Reading, *Salmonella* Melagridis, *Salmonella* Typhimurium, *Salmonella* Paratyphi B, *Salmonella* Newport, *Salmonella* Bovis-morbificans, *Salmonella* Braenderup, *Salmonella* Infantis, *Salmonella* Tennessee, *Salmonella* Montevideo and *Salmonella* Typhi were detected in raw minced meat, raw fresh sausages and raw burger patties from retail outlets in Botswana (Mrema et al., 2006).

Pork is a major cause of foodborne salmonellosis throughout the world. A recent study of pork in U.S. retail stores found 9.6% of samples were contaminated by *Salmonella* (Duffy et al., 2000). Studies on 90 Alberta (Canada) swine farms showed that sixty (66.7%) farms had at least one *Salmonella*-positive sample. *Salmonella* were detected in 14.3% of faecal and 20.1% of environmental samples. Among environmental samples, *Salmonella* were most frequently recovered from boots (38.6%) and the main drain (31.8%). Twenty-two serovars were detected on the 60 *Salmonella*-positive farms. Serovars Typhimurium (78 isolates), Derby (71 isolates) and Infantis (47 isolates) were the most common (Rajic et al., 2005). *Salmonella* Goldcoast, was identified in 25 isolates in Germany and further revealed

that consumption of a raw fermented sausage manufactured by a local company remained significant was responsible for salmonellosis (Bremer et al., 2004). A pre-harvest surveillance of *Campylobacter* and *Salmonella* in Danish broiler flocks for a two year period was investigated with a total of 44,550 samples from the same flocks in the broiler houses at the farms level. 5.5 % of the flocks were positive for *Salmonella* (Wedderkopp et al., 2001). Poultry products and eggs were considered the vehicle for transmission of *Salmonella* Enteritidis and during 1998-2003, prevalence of *Salmonella* Enteritidis in meat, poultry, and pasteurized egg products regulated by the U.S. Food Safety and Inspection Service, studied with 293,938 samples. Of these samples, 12,699 (4.3%) were positive for *Salmonella*, and 167 (1.3%) of the positive samples (0.06% of all samples) contained *Salmonella* Enteritidis (White et al., 2007).

The prevalence of *Salmonella* in food in tropical Asian and African countries was found to be at high level as compared to the rest of the world. Aissa et al. (2007) showed the trend in *Salmonella enterica* serotypes isolated from human, food, animal, and environment in Tunisia from 1994–2004. The top three frequently isolated serotypes during the 11-years were *Salmonella* Enteritidis (25.5%), *Salmonella* Anatum (14%), and *Salmonella* Corvallis (13.2%). Among human isolates, *Salmonella* Enteritidis was the most common serotype, accounting for 24% of all isolates and non-human isolates, *Salmonella* Anatum (28%), *Salmonella* Enteritidis (69%), and *Salmonella* Corvallis (17.3%) were reported as the first common serotypes for food, animal and environmental samples, respectively. Survey of *Salmonella* contamination of poultry droppings used as manure, retail fresh beef,

fresh beef retailers' aprons and fresh beef retail tables, was carried out in Awka, Nigeria (Orji et al., 2005) and reported *Salmonella* Paratyphi A had an isolation rate of 12.5% from poultry droppings, 4.2% from fresh beef, and 2.1% and 4.2% from meat retailers' aprons and tables, respectively. Other serotypes isolated from the sources included *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Gallinarum, *Salmonella* Pullorum, *Salmonella* Typhi and *Salmonella* Agama. Prevalence of foodborne microorganisms (*Salmonella* spp., *Campylobacter* spp., *Arcobacter* spp., and *Enterococcus* spp.) in retail foods i.e. raw chicken, beef, pork, and chicken eggs from fresh markets and supermarkets in Thailand was studied and showed alarmingly high rate of *Salmonella* (121/200), a total of 61% samples were positive for at least one *Salmonella* serogroup and 175 *Salmonella* spp. were isolated. Study further showed that *Salmonella* Anatum was most common isolated serotype, followed by *Salmonella* Corvallis and *Salmonella* Derby (Vindigni et al., 2007). Prevalence of different foodborne bacterial pathogens in food items including raw milk, meat and poultry were reported from China (Chao et al., 2007). Their study showed the presence of *Salmonella* (3.46%), *Listeria monocytogenes* (5.79%), *Staphylococcus aureus* (7%), *Vibrio parahaemolyticus* (0.24%) and *Escherichia coli* O157:H7 (0%), in Chinese food products.

2.6.3.2 Prevalence and distribution of *Salmonella* in milk, dairy farms and milk products

2.6.3.2.1 National scenario

There are very few reports on microbial quality of milk, diary products and dairy farms in India. Varadaraj and Nambudripad (1986) studied the production of enterotoxins and thermostable deoxyribonuclease by *Staphylococcus aureus* in raw

cow milk and their carryover to Khoa, a heat-concentrated (98 degrees C for 15 to 20 min) Indian milk product and demonstrated the enterotoxins and thermostable deoxyribonuclease carried over to Khoa from raw milk, hence, highlighted the use of good quality raw milk free from pathogenic organisms for preparation of milk products such as Khoa.

The microbiological quality of the ice-cream sold in Hisar (India) was evaluated by Prasad et al. (1986). Similarly, microbiological examination of milk in Tarakeswar, India was studied with special reference to coliforms and found pasteurized milk was good in quality (Chatterjee et al., 2006). Presence of *Staphylococcus aureus, Salmonella sp.,* Coliforms, *Listeria monocytogenes, Yersinia enterocolitica* and *Bacillus cereus* in dairy product 'Pedha' in Munbai (India) was reported and microbial quality improvement using gamma radiation was recommend by Bandeker et al. (1998).

2.6.3.2.2 International scenario

Salmonella serovars and *Listeria monocytogenes* were isolated from 250 Tulum cheese from various markets located in Istanbul Turkey, during a period from 2004 to 2005. Study showed 12 (4.8%) and 6 (2.4%) samples positive for *Listeria monocytogenes* and *Salmonella* spp, respectively (Colak et al., 2007). Chocolate-associated *Salmonella* outbreak originating from Germany, was epidemiologically investigated and molecular subtyping by PFGE analysis revealed that two brands from the same company, one exclusively produced for that chain, tested positive for *Salmonella* Oranienburg (Werber et al., 2005). Microbiological quality of retail cheeses made from raw, thermized or pasteurized milk in the UK was studied by Little et al. (2008). Raw or thermized milk cheeses were of unsatisfactory quality

due to levels of *Staphylococcus aureus* at $>10^4$ cfu g^{-1}, *Escherichia coli* at $>10^5$ cfu g^{-1}, and/or *Listeria monocytogenes* at $>10^2$ cfu g^{-1}, whereas pasteurized milk cheeses were of unsatisfactory quality due to *S. aureus* at $>10^3$ cfu g^{-1} and/or *E. coli* at $>10^3$ cfu g^{-1}. *Salmonella* was not detected in any samples. The occurrence of *Salmonella* and *Shigella* in infant formula from Indonesia and Malaysia were determined with 74 packages (5 different manufacturers) of dehydrated powdered infant formula. All the samples were detected neagative for *Salmonella* and *Shigella*, however, other *Enterobacteriaceae* (*Enterobacter sakazakii*, *Pantoea spp.*, *Escherichia hermanii*, *Enterobacter cloacae*, *Klebsiella pneumoniae* subsp. *pneumoniae*, *Citrobacter* spp., *Serratia* spp. and *Escherichia coli*) were detected in the infant formula (Estuningsih et al., 2006).

During 2000-2001, *Salmonella* isolated from dairy herds in New York, Minnesota, Michigan, and Wisconsin in USA. Serogroup and antimicrobial susceptibility characteristics were determined for *Salmonella* from cattle and environmental samples. At least 1 *Salmonella* isolate resistant to 5 or more antimicrobial agents was found on 23.6% of herds. This resistance phenotype was most common among serogroups B and E1 and among samples from calves and farmer-designated sick cows (Ray et al., 2007). Similarly, persistence of multi-drug-resistant (MDR) *Salmonella* Newport (USA) on 2 dairy farms was investigated. The prevalence (32.4% and 33.3% on farms A and B, respectively) of isolating *Salmonella* from samples from joint hospital-maternity pens was significantly higher than the prevalence in samples from pens housing preparturient cows (0.8%, both farms) and postparturient cows on Farm B (8.8%). Multi-drug-resistant *Salmonella* Newport was isolated in high numbers from bedding material, feed refusals, lagoon

slurry, and milk filters. Most isolates were of the C2 serogroup and were resistant to third-generation cephalosporins (Cobbold et al., 2006). Prevalence of *Salmonella* in Dutch dairy farms was studied in a matched case–control study with 47 case farms and 47 control farms. The study included a total of 47 case farms experienced a clinical outbreak of salmonellosis which was confirmed with a positive bacteriologic culture for serovar Typhimurium in one or more samples. Serovar Typhimurium phage type 401 and 506 (definitive type 104, DT104) were the most frequently isolated phage types (13 isolates). On most farms (66%), clinical signs were seen only among adult cows (Veling et al., 2002). Between October 1999 and February 2001, Davison et al. (2006) *Salmonella* status of 449 dairy farms in England and Wales determined by environmental sampling on up to four occasions in a year. Study highlighted the region, herd size, month of visit and the lack of a clean visitor parking area were significantly associated with the prevalence of *Salmonella* species, and there was a significant trend towards an increased risk in late summer and autumn.

2.7 Prevalence and distribution *Salmonella* serovars in seafood

2.7.1 Indian scenario

The microbial quality and presence of foodborne bacterial pathogens in fish and fishery products of the Cochin area has been investigated by many authors (Varma et al., 1988; Nambiar and Iyer, 1991; Thampuran and Surendran, 1998; Surendran et al., 2002; Lalitha and Surendran, 2002). Nambiar and Iyer, (1990)

the coastal area of the Mangalore was investigated by Srikantaiah et al. (1985). They reported 7-11 % of estuary sediments samples were contaminated with *Salmonella*. Quality of the fish in retail market of Bombay reported the presence of high count of the indicator bacteria and 7.7 % of the samples were contaminated either with *Salmonella* or *Clostridium perfringens* (Iyer et al., 1986). *Salmonella* isolated from the shrimp processing units were identified as *Salmonella* Farmsen, *Salmonella* Newport, *Salmonella* Havana, *Salmonella* Bareilly, *Salmonella* Weltevreden, and *Salmonella* Typhimurium. Study also included the culture pond water, coastal seawater, shrimp processing water, ice and processing table and floors (Iyer and Varma, 1990).

The prevalence of *Salmonella* in 500 market prawn samples from local markets of the west coast region of India was found to be reasonably very low (1 %) and *Salmonella* Infantis and *Salmonella* Newport were isolated from the prawn samples (Prasad and Pandurangarao, 1995). Iyer and Joseph (1980) showed the presence of *Salmonella* Roan in seafood. *Salmonella* serovars have also been isolated from the shrimp sold in the Mumbai (Valsan et al., 1985), and Mangalore market and fish landing centers (Shabarinath et al., 2007). The quality of the frozen cephalopod (*Loligo* spp. and *Sepia* spp.) products from India was investigated. Based on the biochemical indices, 85% of the samples maintained good quality and the bacterial quality of the products was reported to be good, albeit, only 3.2 % were infected by *Salmonella*. (Lakshamanan et al., 1993). Although, the wide variety of *Salmonella* serovars were prevalent in seafood in India but, *Salmonella* Weltevreden was found to be the most common serovar in seafood. A study on *Salmonella* in

imported seafood reported that *Salmonella* Weltevreden was the most frequently isolated serovar in the seafood of Indian origin (Heinitz et al., 2000).

2.7.2 International scenario

Seafood borne illnesses are well documented in the developed world. Each year an estimated 1.4 million cases of salmonellosis occur among humans in the United States and raw seafood was associated with large number of food borne illnesses. Report on seafood borne outbreaks over a ten years period in the USA demonstrated seafood caused 10 % of all outbreaks (Huss, 1994). Epidemiological reports showed that raw molluscan shellfish were linked to large number seafood borne illnesses. According to Centres for Disease Control and Prevention report from 1993 to 1997, 47 outbreaks and 1868 cases of illnesses in the United States were associated with consumption of shellfish. A nine years study on 11312 imported and 768 domestic seafood samples in United States showed that 7.2 % of imported and 1.3 % of domestic seafood samples were contaminated with *Salmonella* (Heintz et al., 2000). Prevalence of *Salmonella* spp. in oysters in the United States was determined by Brands et al. (2005) and reported that 7.1 % of oyster were positive for *Salmonella* and *Salmonella* Newport was found to be most predominant in oysters. A study carried out on the prevalence of *Salmonella* in live molluscs of Galicia region of Spain revealed that an overall of 1.8 % shellfish was contaminated with *Salmonella* and the further suggested that mussels and oysters presented higher incidences as compared to clam and cockles (Martinez-Urtaza et al., 2003). The British health laboratory system reported the incidence of *Salmonella* in 22 out of 566 raw shellfish analyzed (PHLS, 1993). A study by Ponce et al.

(2008), for a period of over 5 years from 2001–2005, isolated 210 *Salmonella enterica* strains from seafood samples imported to US. Strains of *Salmonella* Weltevreden were the most predominantly found among the 64 different serovars isolated. A total of 37 *Salmonella* Weltevreden isolates were characterized by pulsed-field gel electrophoresis (PFGE), plasmid profiles and antibiotic susceptibility to assess genetic diversity.

The incidences are found to be higher in the tropical Asian countries. In Vietnam, 25 % of shrimp samples were positive for *Salmonella* and different serotypes namely *Salmonella* Weltevreden, *Salmonella* Tennessee, and *Salmonella* Dessau were isolated from shrimp samples. *Salmonella* serotypes have been isolated from coastal waters (18 %) and shellfish (10 %) of Hong Kong Island and *Salmonella* isolated from diarrhoea patients were not observed in coastal waters and shellfish of same region (Yam et al., 1999). The contamination of aquaculture ponds, water bodies and coastal marine environment has increased the prevalence of *Salmonella* in seafood. Different *Salmonella* serotypes such as *Salmonella* Typhimurium, *Salmonella* Agona and *Salmonella* Senftenberg were isolated from molluscs and seawater samples of Galicia, North-western Spain (Martinez-Urtaza et al., 2004).

In Asian countries, several occasions *Salmonella* has been found in fish and shrimp ponds. Studies have shown that 25 % of Japanese eel culture ponds and 22 % of shrimp ponds were contaminated with *Salmonella* (Huss et al., 2000). Different *Salmonella* serovars have been isolated from brackish water in South east Asia (Reilly and Twiddy, 1992). A survey on 331 food samples including 55 seafood in the Malaysian markets place reported 25% incidence of *Salmonella* in raw prawns

(Arumugswamy et al.,1995). Different studies showed that *Salmonella* Weltevreden was the most prevalent serovar in seafood (Boonmar et al., 1998; Heinitz et al., 2000; Shabarinath et al., 2007).

2.8 Statistical Analysis for evaluation of detection methods

2.8.1 Kappa coefficient

The evaluation of different diagnostic tools required an estimation of the main parameters for accuracy i.e. sensitivity, specificity, positive and negative predictive values. When a diagnostic tool requires human interpretation, it is also important criteria to appreciate the degree of agreement between the methodologies (raters). The most commonly used measure of inter-rater agreement is the kappa coefficient, which measures agreements between observers beyond that expected by chance alone.

Fig. 2.1 Equation for the calculation of kappa coefficient

		Rater 2		
		Present	Absent	
Rater 1	Present	a	b	a + b
	Absent	c	d	c + d
		a + c	b + d	N

P_o = observed proportion of agreement = (a + d) / N

P_e = proportion of agreement expected by chance = $\dfrac{[(a + b)(a + c) + (b + b)(c + d)]}{N^2}$

Hence, kappa (k) = $(P_o - P_e)/(1 - P_e)$

Different statistical tools have been used to determine the relative specificity, sensitivity and to determine the diagnostic accuracy of an assay (Lehmann et al., 1995; Malorny et al., 2003a). The kappa coefficient has been widely used as chance-corrected measure of nominal agreement in a variety of application area. In the context of inter observer agreement studies, Fleiss (1975); Kraemer (1979) have provided persuasive arguments favoring the use of kappa statistics over other measure of agreement that have been proposed (Donner and Klar, 1996). The calculation of kappa value is based on difference between observed and expected agreements among raters. The interpretation of the kappa value was defined on a ' -1 to 1 scale', where 1 is perfect agreement and negative values indicate agreement less than chance. The results are generally interpreted, as having fair agreement (0.21 to 0.40), moderate agreement (0.41-0.60), substantial agreement (0.61-0.80) and perfect agreement (0.81 to 1.0) between the raters (Viera and Garrett, 2005). Generally, different techniques have been used for detection of *Salmonella* in food samples and the efficacy of individual assays was often statistically determined.

The rapid and specific detection of *Salmonella* spp. in animal feed samples were determined by PCR assay with a short culture enrichment period and results were statistical analyzed to determine the confidence levels (Lofstrom et al., 2004). The relevance of the kappa coefficient is to develop the statistical agreement values between the diagnostic assays. The kappa coefficient was calculated to determine the degree of agreement between culture, PCR, TaqMan *Salmonella* and Transia card *Salmonella* assays for detection of *Salmonella* spp. in naturally–contaminated

ground chicken, turkey, and beef (Fratamico, 2003) and observation exhibited the variable kappa coefficient values ranging from 0.28 to 0.87 and revealed the varying level of agreement between the four *Salmonella* detection techniques.

Similarly, asymptomatic *Salmonella* infections in swine were compared with culture, ELISA and broth culture-PCR assays and kappa coefficient values (0.52 to 0.94) were calculated to determine the agreement between the assays (Sibley et al., 2003). Sachse et al. (2003) determined the kappa value for PCR, ELISA and culture assays used in detection of *Chlamydia suis* from clinical specimens to highlight the statistical concordance between different methods and the kappa coefficient value was reported to be at 0.712.

2.8.2 Simpson's Index

Epidemiological molecular typing of the bacterial pathogens is carried out by a variety of techniques including ribotyping, ERIC-PCR, RAPD and PFGE analysis. To type different *Salmonella* serovars, there are often choice of methods available that have been developed independently by different groups and each method has a different level of discrimination ability. The efficiency of each typing method depends on number of factors; typability, reproducibility and discrimination. Of these characteristics, typability and reproducibility are relatively easy to quantify and often expressed in simple percentage (Hunter and Gaston, 1988). The discrimination power of a typing method is its ability to distinguish between unrelated strains. It is determined by the number of types identified by the test method and relative frequency of these types. This index was derived from elementary probability theory (Armitage and Barry, 1987) and given in the following equation:

Fig.2. 2 Equation for the calculation of discrimination index

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

Where N, total number of strains

S, total number of types

n_j, number of strains in j^{th} type

D, the numerical value of discrimination index

Hunter and Gaston (1988) suggested the use of a single numerical index of discrimination (D value), based on the probability of two unrelated samples from the test population placed in different typing groups and calculated the Simpson's index of diversity. Hunter and Gaston (1988) have recommended a D value > 0.9 for good differentiation. The Simpson's index of discrimination evaluates the efficacy of different typing methods for an organism. *Salmonella* Livingstone was compared based on three fingerprinting methods such as ribotyping, PFGE and RAPD analysis and observed the D value at 0.855, 0.766, 0.236, respectively (Eriksson et al., 2005). Similarly, four different molecular typing methods were compared to differentiate *Salmonella* spp. and calculated the discrimination value (Lim et al., 2005). The combination of different typing method improved the efficacy of epidemiological studies to subdivide *Salmonella* Mbandaka into 35 types and the index of discrimination attained at 0.947 (Hoszowski and Wasyl, 2001).

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MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Culture media

Both dehydrated bacteriological media and compounded media were used for isolation and identification of *Salmonella* from seafood.

3.1.1.1 Dehydrated Media

For the isolation and identification of *Salmonella* from seafood, the following

Table-3.1 List of dehydrated media

Sl. No.	Media	Source
1.	Bismuth Sulphite Agar	Oxoid, UK
2.	Brain Heart Infusion Agar	Difco, USA
3.	Brilliant Green Agar	Difco, USA
4.	Buffer Peptone Water	Oxoid, UK
5.	Hektoen Enteric Agar	Difco,USA & Oxoid, UK
6.	Lactose broth	Oxoid , UK
7.	Lysine Iron Agar	Difco, USA
8.	M broth	Difco, USA
9.	MacConkey Agar	Difco, USA
10.	Malonate broth	Difco, USA
11.	Methyl Red & Voges-Proskauer broth	Difco, USA
12.	Motility GI Medium	Difco, USA
13.	Muller Hinton Agar	Difco, USA
14.	Rappaport Vassiliadis broth	Difco, USA
15.	Semisolid Motility Media	Difco, USA
16.	Simmon's Citrate Agar	Difco, USA
17.	Triple Sugar Agar	Difco, USA
18.	Tryptic Soy Agar	Difco, USA
19.	Urea Agar	Difco, USA
20.	Xylose Lysine Desoxycholate Agar	Difco, USA & Oxoid, UK

dehydrated media were used during this study. The details are given in Table 3.1. Dehydrated media and chemicals were imported either directly from the manufactures or procured through their Indian agents. Oxoid (U.K.) and Difco (USA) brand dehydrated media were mainly used.

3.1.1.2 Compounded Media

The following media were compounded in the laboratory. The chemical ingredients were AR/GR grade from Merck (India), Sisco Research Laboratories (India), or Sigma (India). Biological ingredients were either from Oxoid (UK), Difco (USA) or HiMedia (India)

1. Tetrathionate broth (TT)

a) Base

Beef extract	0.9 g
Peptone	4.5g
Yeast extract	1.8g
NaCl	4.5g
$CaCO_3$	25.0g
Sodium thiosulphate	40.7g
DW	1 litre

b) Iodine solution

Iodine crystals	6 g
Potassium Iodide	5 g
Distilled water (DW)	20 ml

pH: 8.4 ± 0.2.Dissolved the ingredients in DW, heated to boil, cooled below 45°C and mixed with 20 ml of iodine solution. Mixed and tubed in 10 ml. quantities.

2. Sugar Fermentation broth

Peptone	10 g
Sodium chloride	5 g
Sugar	10 g
Phenol Red	0.018 g
Distilled water (DW)	1 litre

pH: 7.4 ± 0.2

The following sugars, viz. dulcitol, glucose, lactose, maltose, mannose, mannitol, sucrose, cellobiose, arabinose, raffinose, trehalose and xylose, and sugar derivatives viz. inositol, salicin, sorbitol were used as required. To 100 ml of the basal media, 1g of the respective sugar was added , dissolved, dispensed in 4ml quantities in 100x12mm tubes containing inverted Durham's tubes. The media was sterilized at

115°C for 20 min. In case of disaccharides and oligosaccharides viz. lactose, maltose, sucrose, cellobiose, trehalose and raffinose, filter sterilized solutions were was added to the pre-sterilized broth.

3. Lysine decarboxylase media

Peptone	0.5 g
Yeast extract	0.3 g
Glucose	0.1 g
L-Lysine	0.5 g
Bromo cresol Purple (BCP)	0.002 g
Distilled water (DW)	100 ml
pH 6.8 ± 0.2	

Weighed and dissolved the above ingredients, except BCP, in 100ml of DW adjusted the pH to 6.8, Sterilized at 115°C for 20 min

4. Amino acid utilization broth

Peptone	5 g
Yeast extract	3 g
Amino acid	10 g
Bromocresol Purple	0.02 g
Distilled water (DW)	1 litre
pH 6.8 ± 0.2	

Dissolved the ingredients in DW. Hydrochlorides of lysine, arginine, ornithine, valine and phenylalanine were used as amino acids. 100ml of each amino acid medium was prepared, pH adjusted, dispensed in 3 ml quantities in small test tubes, and sterilized at 115°C for 20 min.

5. Phenol Red Tartrate Agar

Peptone	10 g
Yeast extract	3 g
Pot. Tartrate	10 g
Phenol Red	0.024 g
NaCl	5 g
Agar	15g
Distilled water (DW)	1 litre

Dissolved the ingredients in DW and pH adjusted at 7.6 ± 0.2. Distributed the media into test tubes in 5 ml quantities and sterilized at 121 °C for 15 min.

3.1.2 Molecular biology - chemicals, reagents, and buffers.

Molecular biology grade chemicals, reagents and buffers were obtained from Sigma (India), Merck (India), SRL (India), and Bangalore Genei (India). All molecular biology chemicals and buffers were prepared in double distilled water (Millipore, Germany). List of chemicals, reagents and buffers and their preparation are given below.

(a) Electrophoresis chemical and reagents

1. Agarose (Electrophoresis grade), Sigma

2. Acrylamide/Bis-Acrylamide solution, 12%, Sigma

3. Ethidium Bromide (10mg/ml) Sigma

Weighed 100 mg of Ethidium bromide in 10 ml of pre-sterilized TAE and dissolved by stirring with magnetic stirrer for 3-4 h. Stored in dark container at 4 °C.

3 TAE buffer (50 X)

Tris base, 242 gm (Sigma)

Glacial acetic acid, 57.1 ml (Merck)

0.5 M EDTA, 100 ml (pH 8) (SRL)

Weighed 242 g of Tris, 57.1ml of glacial acetic acid and 100 ml of EDTA (0.5 M, pH 8) dissolved in 600 ml of double distilled water (Millipore). Final volume was adjusted to 1 liter with additional double distilled water and sterilized at 115°C for 20 min. Stored at room temperature and always used 1X TAE buffer.

4 Gel Loading Buffer

Bromophenol Blue, 0.25g (Sigma)

Xylone Cyanol , 0.25 g (Sigma)

Sucrose, 40 g (SRL)

TAE 1X, 100ml

Dissolved all components in 100 ml of sterile TAE and stored at 4°C.

(b) Chemical and reagents for Plasmid and genomic DNA isolation

1. Alkaline Lysis Solution I

50mM glucose (HiMedia)

25mM Tris Cl pH 8 (Sigma)

10mM EDTA pH 8 (SRL)

Solution I was prepared from standard stocks of 1M Tris, 0.5 M EDTA and 50 mM glucose in a batch of 100 ml. Sterilized the solution at 121 °C for 15 min and stored at 4 °C.

2. Alkaline Lysis Solution II

0.2N NaOH (SRL)

1 % w/v SDS (SRL)

Prepared fresh 0.2 N NaOH from 10 N stock and added 1% (w/v) SDS. Stored at room temperature.

3. Alkaline Lysis Solution III

5 M potassium Acetate, 60.0 ml (SRL)

Glacial acetic acid, 11.5 ml (Merck)

Distilled water, 28.5 ml

Dissolved 5 M (60 ml) of potassium acetate and 11.5 ml of glacial acetic acid in 28.5 ml of double distilled water (Millipore). Stored the solution at 4 °C.

4. Calcium chloride stock, 2.5M (SRL)

Dissolved 11 g of calcium chloride in 20 ml double distilled water (Millipore). Sterilized the solution by passing it through 0.22 μm filter and stored in 1 ml aliquots at 4 °C.

5. Ethanol, 70 % (Amresco)

Dissolved absolute alcohol (70 ml) was in 30 ml of double distilled water (Millipore) and stored at 4 °C.

6. TE buffer

10mM Tris-HCl (Sigma)

1mM EDTA, pH 8.0 (SRL)

Dissolved 0.12 g Tris base in 80 ml of double distilled water (Millipore) and adjusted the pH to 8.0 with conc. HCl. Tris base was mixed with 1mM EDTA (0.037 g, pH 8.0) and final volume was attained to 100 ml with additional double distilled water. Sterilized at 115 °C for 20 min and kept at room temperature.

7. Phenol: Chloroform: Isoamyl alcohol

Tris-equilibrated phenol (pH 8.0) 25ml (Sigma)

Chloroform, 24 ml (Merck)

Isoamyl alcohol, 1ml (SRL)

Equilibrated phenol, chloroform, and isoamyl alcohol was mixed in a ratio (25:24:1) and stored in dark bottle at 4 °C.

8. SDS, 20 % (SRL)

Dissolved 20 g of electrophoresis–grade SDS in 75 ml of warm (70°C) double distilled water (Millipore) and adjusted the volume to 100 ml. Stored the solution at room temperature.

(c) Reagents and buffers for PFGE analysis

1. Agarose, PFGE grade (Sigma)

1.2 % agarose gel was prepared in 0.5 X TBE buffer by heating microwave oven for 2 min.

2. Cell lysis buffer

50 mM Tris (Sigma)

50 mM EDTA (SRL)

1 % N- lauryl Sarcosine, pH 8.0 (Sigma)

Dissolved 6 g of Tris in 250 ml of double distilled water (Millipore) and 18.6 g of EDTA in 100 ml of double distilled water (Millipore), adjusted pH to 8.0. and both solutions were mixed together. To this solution, 50 ml of 10% N- lauryl Sarcosine

was added and final volume was made to 500 ml with sterile double distilled water (Millipore) and stored at room temperature.

3. Cell suspension buffer

100mM Tris (Sigma)

100mM EDTA, pH 8.0 (SRL)

Buffer was prepared from the stock of 1 M Tris (pH 8.) and 0.5 M EDTA (pH 8.0) in sterile double distilled water (Millipore) 500 ml. Stored at room temperature.

4. TBE buffer (10 X)

Tris base (Sigma)

Boric acid (SRL)

EDTA (SRL)

Dissolved 108 g of Tris base and 55 g of boric acid in 700 ml of double distilled water (Millipore). 40 ml of 0.5 M EDTA (pH 8.0) was added to this mixture and finally volume was adjusted to 1 liter with additional double distilled water (Millipore). Sterilized 10 X stock buffer and stored at room temperature. Always used 0.5 X TBE buffer for electrophoresis.

3.1.3 Enzymes, Oligos, dNTDs and DNA markers

Taq polymerase and dNTPs were used in different PCR assays and molecular fingerprinting experiments. DNA molecular weight markers were used as a reference standard to determine the size of PCR amplicons. Rnase, Proteinase K and restriction enzyme (*Xba*I) were used in plasmid, genomic DNA, and PFGE experiments. All enzymes, dNTPs, and molecular weight markers were stored at -20°C in deep freezer (Vest frost, India). PFGE DNA ladder was used as a molecular weight standard in PFGE experiments and stores at 4°C (Samsung, India). All details are given in Table 3.2.

Table 3.2 List of enzymes, oligos, dNTDs and DNA markers used

Sl.No.	Enzymes, Oligos, dNTDs and DNA ladders	Trade name (Country)
1.	dNTPs	Finnzymes (Finland)
2.	*Taq* Polymerase (Dynazyme II)	Finnzymes (Finland)
3.	Proteinase K	Finnzymes (Finland)
4.	*Rnase*	Bangalore Genei, India)
5.	Restriction Enzyme *Xba* I (30U/ml)	New England Biolab (UK)
6.	100 bp DNA ladder	Fermentas (Germany)
7.	1000 bp DNA ladder	Fermentas (Germany)
8.	PFG DNA ladder	New England Biolab (UK)

3.1.4 *Salmonella* Type cultures

The following type cultures were used as positive controls in the biochemical characterization, developments of rapid molecular detection methods, and characterization of molecular fingerprinting techniques for *Salmonella* serovars.

Table 3.3 List of *Salmonella* Type cultures used

Salmonella Serovars	Type Culture	Source
Salmonella arizonae (IIIa)	MTCC 660	IMTEC, Chandigarh
Salmonella Typhimurium	ATCC 23564	ATCC, VA, USA
Salmonella Typhi	ATCC 6539	ATCC, VA, USA
Salmonella Weltevreden	MTCC 1169	IMTEC, Chandigarh

3.1.5 Oligonucleotide primers

The following imported primers were used in the experiments related to development

Table 3.4 List of *Salmonella* specific primers used

Sl. No	Primer Sequence (5'-----3')	Brand	Reference
1.	**SRS** ST11, AGCCAACCATTGCTAAATTGGCGCA ST15, GGTAGAAATTCCCAGCCGGGTACTG	IDT, USA	Aabo et al., 1993
2.	***invA* gene** GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	IDT, USA	Rahn et al., 1992
3.	***stn* gene** CTTTGGTCGTAAAATAAGGCG TGCCCAAAGCAGAGAGATTC	IDT, USA	Makino et al., 1999
4.	***fimA* gene** CCTTTCTCCATCGTCCTGAA TGGTGTTATCTGCCTGACCA	Sigma, USA	Cohen et al., 1996
5.	**PCR ribotyping** TTGTACACACCGCCCGTCA GGTACTTAGATGTTTCAGTTC	Sigma, USA	Kostman et al., 1992
6.	**ERIC- PCR** ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGGG	IDT, USA	Versalovic et al., 1991

of *Salmonella* specific PCR, characterization of virulence genes (*invA*, *stn*, and *fimA*.), PCR-ribotyping and ERIC-PCR studies. The *invA* primer was used in real-time PCR. All primers were stored at -20°C in deep freezer (Vest frost, India) and used as per manufactures instructions.

3.1.6 *Salmonella* antisera

The following antisera were used in *Salmonella* serotyping.

Table 3.5 List of *Salmonella* antisera used

Antisera	**Source**
Salmonella O Poly A-I & Vi	Difco, USA
Individual O antisera (A,B, C1, C2, C3, D1, D2, E1, E2, E4, F, G, K & N)	Difco, USA
Salmonella H antisera, Spicer –Edwards 1, 2, 3, 4	Difco, USA
Salmonella H antisera, EN complex	Difco, USA
Salmonella H antisera, L complex	Difco, USA
Salmonella H antisera, 2, 5, 6, 7	Difco, USA
Salmonella Vi antiserum	Difco, USA

3.1.7 Major equipments

The following major instruments were used for different molecular biology experiments.

Table 3.6 List of Major equipments used

Sl. No.	Instrument	Purpose/experiments
1	Refrigerated Centrifuge, R5880 (Eppendorf, Germany)	Pelleting
2	Gel documentation system, Multilight Cabinet (Alpha Innotech Corporation, USA)	Gel Imaging
3	Pulsed field gel electrophoresis system, Gene Navigator (Amersham Biosciences, USA)	PFGE
4	Shaker Incubator (Sanyo, Japan)	Shaking Condition
5	Thermocyclers, Minicycler & Mastercycler personal (M J Research, USA & Eppendorf, Germany)	PCR assay
6	Real-Time PCR, DNA Engine, PTC-200 Peltier Thermalcycler (M J Research, USA)	Quantitative assay
7	ELISA Reader & Washer Strip Reader, SR 601, Qualisystems & Qualiwash (gsk, Qualigens, India)	ELISA assay

3.1.8 Seafood samples

Fresh and unprocessed seafood samples were collected from different fish markets and fish landing centres of Cochin (Kerala) during the period 2003 to 2007. The seafood samples included pelagic and demersal fish, crustaceans, molluscans, and cephalopods. A total of 443 seafood samples were analyzed for isolation of *Salmonella.* Details are given below in Tables 3.7.

Table 3.7 List of seafood samples used

(a) Pelagic Fish

Name	No. of samples
Rastrelliger kanagurta	9
Sardinella longiceps	9
Valamugil cunnesuis	5
Dussumieria acuta Valenciennes	4
Liza subviridis	3
Scomberomorus commerson	5
Scomberomorus guttatus	5
Scombroides lysan	6
Carangoides praeustus	3
Carangoides armatus	5
Atule mate	3
Sardinella albella	2
Mugil cephalus	3
Anodontostoma chacunda	5
Caranx sexfasciatus	3
Carangoides malabaricus	5
Sardinella gibbosa	4
Total	**79**

(b) Demersal Fish

Name	No. of samples
Lethrinus miniatus	2
Lethrinus ornatus	3
Sphyraena obtusata	4
Gerres erythrourus	1
Gerres filamentosus	3
Otolithes cuvieri	4
Cynoglossus macrostomus	4
Johnius dussumieri	1
Johnius amblycephalus	2
Nemipterus japonicus	5
Upeneus taeniopterus	3
Upeneus taeniopterus	4
Epinephelus diacanthus	5
Priaranthus Hamrur	4
Lutjanus lutjanus	3
Pampus argentus	1
Parastomateus niger	3
Total	**52**

(c) Crustaceans samples

Shrimp	No. of samples
Penaeus indicus	19
Penaeus monodon,	12
Metapenaeus dobsoni,	18
Metapenaeus affinis,	9
Parapeneopsis stylifera,	13
Acetes spp.	15
Total	**86**
Lobster	
Penulirus ornatus	8
Penulirus homarus	5
Penulirus polyphagus	12
Total	**25**
Crab	
Scylla serrata	21
Portunus spp.	17
Total	**38**

(d) Molluscs samples

Clam	No. of samples
Villorita cyprinoides	23
Marcia opima,	12
Anadara granosa,	6
Total	**41**
Mussel	
Perna viridis	18
Perna indica	13
Total	**31**
Oyster	
Crassostrea madrasensis & Saccostrea cucullata	**27**

(e) Cephalopod samples analyzed for *Salmonella*

Squid, Cuttlefish and Octopus	No. of samples
Loligo spp. & *Doryteuthis* spp.	23
Sepia spp.	21
Octopus spp.	20
Total	**64**

3.2 Methods

3.2.1 Isolation and identification of *Salmonella* from seafood samples

A total of 443 fresh, raw and unprocessed seafood samples were collected from different fish landing centres, markets, and retailers in Cochin Corporation, India, over a period of 4 years from 2003 to 2007 as detailed in Table 3.4a, b, c, d, e. All samples were collected in sterile polythene bags and immediately (1-2 h) transported to the laboratory and examined for *Salmonella*.

3.2.1.1 Isolation and identification

Salmonella cultures were isolated as per the culture method of BAM, USFDA, (Andrews and Hammack, 2001) and ISO (2000). Seafood samples included whole body part of fish, shrimp, lobster, squid, cuttlefish and octopus, whereas, soft muscle parts of crab, clam, oyster and mussel were used in the study. Each 25 g of seafood sample was homogenized with 225 ml of lactose broth (Oxoid, UK) or BPW broth in a stomacher blender (Seward, UK) at 250 rpm for 1 min. The seafood homogenate was transfer in to 500 ml Erlenmeyer flask and pre-enriched at 37°C in incubator (Kemi, India) for 24 h. The pre-enrichment was followed by selective enrichment in Rappport–Vassiliadisis (RV) and teterathionate (TT) broths. One ml of sample broth form pre-enrichment medium was pipetted in to TT both and incubated at 43 °C in a water-bath for 24 h. Simultaneously, 0.1 ml pre-enrichment broth was inoculated in to RV broth and incubated at 42 °C in a serological water-bath (Lab-line, India) for 24 h. Subsequently, selective enriched samples from RV and TT broth were streaked onto brilliant green agar (BGA), bismuth sulfite agar (BSA), Hektoen enteric agar (HEA), xylose lysine

desoxycholate (XLD) agar media. The selective plates were incubated at 37°C for 24 h. After the completion of incubation, typical pink colonies, surrounded by bright red medium were picked up from BGA plates and streaked on to BHI slants for further identification. Similarly, brown, grey to black colonies with metallic sheen, and surrounded brown to black colour, typical colonies on BSA plates, pink colonies with or without black centres on XLD medium and blue-green colonies with or without black centre on HEA medium media were selected for identification. Atleast, 2-3 typical colonies from BGA, BSA, XLD and HEA plates were selected and streaked on BHI slants for biochemical identification.

Table 3.8 List of *Salmonella* biochemical tests for *Salmonella*

Sl. No.	Tests /Media	*Salmonella* typical reaction
1.	Gram's stain	Gram negative, short rods
2.	Motility	Motile [a]
3.	TSI	Acid butt & alkaline slant
4.	LIA	Alkaline but & Alkaline slant
5.	H_2S on TSI agar	+ve
6.	Urease	-ve
7.	Lysine decarboxylase	+ve
8.	Glucose fermentation	Acid & gas!
9.	Dulcitol utilization	Acid & gas
10.	Malonate utilization	-ve *
11.	Indole test	-ve
12.	Lactose fermentation	-ve*
13.	Sucrose utilization	-ve
14.	VP test	-ve
15.	MR test	+ve
16.	Simmons citrate agar	+ve*
17.	Polyvalent antisera A-I &Vi	Agglutination +ve

[a] Some are non-motile, *Variations noted, !Variations

Before proceeding for the biochemical tests, suspected *Salmonella* cultures were purified on MacConkey agar by streak dilution method. The typical *Salmonella*

colonies (transparent and colourless) on MacConkey agar were transferred to BHI slants for further biochemical identification. *Salmonella* spp. were identified based on key biochemical reactions on triple sugar iron agar (TSI), lysine iron agar (LIA), urease, indole, malonate, lactose, dulcitol, MRVP, Simmons citrate, lysine decarboxylase and polyvalent (somatic) agglutination test. All *Salmonella* cultures were identified based on the typical reactions as shown in Table 3.8.

3.2.2 Serotyping of *Salmonella* isolates

All biochemically typical *Salmonella* isolates were serotyped based on reaction with somatic (O), flagellar (H), and capsular (Vi) antisera (Difco, USA). *Salmonella* O antigens were identified as per scheme shown in Table 3.9. *Salmonella* O and Vi antigens were identified by slide test procedure. The following steps are involved for the identification of *Salmonella* O antigens. A drop of 0.85 % saline was placed on clean glass side and loopful of test culture was transferred to the saline, mixed properly with saline to form a uniform suspension. A drop of *Salmonella* O Poly A-I &Vi antiserum was dispensed to the suspension on glass slide. The slide containing test organism and O Poly A-I &Vi antiserum was rotated for 1 min and observed the visible agglutination. Rapidly formed +++ (3+) agglutination were considered positive for serotype testing (75% positivity). The culture found positives for *Salmonella* O Poly A-I & Vi test were further tested for individual O antiserum viz. A, B, C1, C2, C3, D1, D2, E1, E2, E4, F, G, K and N by slide test procedure as described above and the cultures found negative for individual antiserum were tested for Vi antisera.

After the confirmation of the individual *Salmonella* O antisera, culture were further characterized for H (phase-I) antisera based on Spicer-Edwards antisera by

tube test procedure, whereas, L, EN and 1 complex antigens were identified, separately. Before the identification of H antigens, test cultures were consecutively sub-cultured in Motility GI medium (Difco, UK) to increase the motility of the test organism. The steps for the identification of H antigens are as followed;

Table 3.9 Scheme for identification of *Salmonella* O antigens

Test	*Salmonella* O Poly A-I & Vi				
Result	+ve				-ve
Test	Individualual O antisera tested (A, B, C1, C2, C3, D1, D2, E1, E2, E4, F, G, K and N)				↓
Result	+ve	-ve			
Test	↓	*Salmonella* antiserum Vi			
Result	↓	+		-	
Test	↓	Heated and Retested *Salmonella* antiserum Vi		↓	↓
Result	↓	+ve	-ve	↓	↓
Conclusion	Confirmation of H antigen	Not *Salmonella*	Test boiled culture with individual O antisera groups	Not *Salmonella*	Check for the rare groups (w, x, y, z, 51-61)

The test cultures were inoculated in Motility medium (Difco, USA) by stabbing slightly below the surface in 12 x 150 mm test tubes and incubated at 37C for 18- 20 h. The organisms that have migrated 50-60 mm to the bottom of the tubes were used for the test. The cultures from the bottom of the tube were transferred to BHI broth

and incubated at 35°C for 4-6 h. The incubation was followed by preparation of test culture suspension with equal volume of 0.6 % formalized saline. A 0.5 ml of culture suspension and equal amount of diluted H antisera (1:250) was added in to a 12 x 75 mm test tubes and incubated in a water bath at 50 °C for 1 h. Agglutination in tubes were recorded after the incubation. The identification of phase I, H antigens was followed by identification of phase II antigens after a phase reversal process.

Phase reversal of the identified Phase I of H antigens was carried in semisolid Motility medium (Difco, USA) by masking the identified phase I antigens with antisera. One ml of the 1:10 dilution of antisera (phase I) was added to a 25 ml of semisolid motility GI medium, mixed well and poured into a sterile Petri dish. After solidification, the test organism was inoculated by punching the edge of semisolid medium and incubated the media plates at 35-37°C for 24 h. At the end of incubation, culture migrated to the opposite side of the inoculation site were transferred to a BHI broth and incubated at 37°C for 4-6 h. The culture from BHI broth was used for the identification of phase II antigen by tube method as described above. The antigenic formula obtained from the *Salmonella* O, H (phase I and phase II) types were pooled together and derived *Salmonella* serovars as per Kauffmann–White Scheme (Popoff and 2005).

Salmonella isolates were serotyped at National *Salmonella* Centre (Veterinary), Indian Veterinary Research Institute, Bareilly, India and National *Salmonella* Centre, Central Research Laboratory, Kasauli, India.

3.2.3 Biotyping

3.2.3.1 Utilization of sugars

Ten most predominant *Salmonella* serovars namely *Salmonella* Weltevreden, *Salmonella* Rissen, , *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Ohio, and *Salmonella* Irumu isolated from seafood were biotyped based on utilization of different sugars. A total of 12 sugars viz., dulcitol, glucose, lactose, maltose, mannose, mannitol, sucrose, cellobiose, arabinose, raffinose, trehalose, and xylose were used in this study to determine the sugar utilization pattern. Selected *Salmonella* serovars were inoculated into individual sugar broth and incubated at 37°C for 48 h for the production of acid and gas.

3.2.3.2 Utilization of sugar derivative and other carbon sources

Similarly, ten most predominant *Salmonella* serovars i.e. *Salmonella* Weltevreden, *Salmonella* Rissen, , *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Ohio, and *Salmonella* Irumu isolated from seafood were biotyped based on utilization of inositol, salicin, sorbitol, citrate, and tartrate. Pre-sterilized inositol, salicin, sorbitol broths were inoculated with different test serovars and incubated 37°C for 48 h. The formation of pink colour indicated the utilization of inositol, salicin and sorbitol. Utilization of malonate by different serovars was studied in malonate broth with bromothymol blue indicator at 37°C for 48 h and the colour change from green to blue indicated the utilization of malonate by the organism. Citrate utilization was studied in Simmon's citrate agar. All test serovars were inoculated in Simmon's citrate agar and results were recorded after the

incubation at 37°C for 48 h. Similarly, all test cultures were inoculated onto phenol red tartrate agar by stabbing the butt and streaking the slants, followed by incubation at 37°C for 48 h and results were recorded.

3.2.3.3 Utilization of amino acids

The ten most prevalent *Salmonella* serovars isolated from seafood were characterized based on utilization of different amino acids viz., arginine, lysine, orinithine, valine, and phenylalanine in respective amino acid media. Amino acid broth was inoculated with different *Salmonella* cultures, an overlay of sterile liquid paraffin was added and incubated at 37°C. The observation was made upto 4 days of incubation. A positive amino acid utilization was indicated by distinct violet purple colour of the medium.

3.3 Determination of antibiotic resistance profile (antibiogram)

All *Salmonella* serovars were tested for antibiotic susceptibility by disc diffusion assay on Muller Hinton agar. The isolates were tested against all major commercial antibiotics viz., sulphonamides, quinolones, beta-lactams, cephalosporins, tetracyclines, aminoglycosides, macrolides, and chloramphenicol. The standard antibiotic discs were procured from HiMedia (Mumbai, India) (see Table 3.10). *Salmonella* test cultures were inoculated in to 5 ml of tryptic soy broth and incubated at 35 °C for 4-6 h. After incubation, cultures were streaked on entire surface of the pre set Muller Hinton agar plates with a sterile cotton swab and allowed the inoculum to dry. Then, ascetically antibiotic discs were applied on to the Muller Hinton plates and kept the plates for incubation at 37 °C for 14-19 h. The

results were recorded on the basis of the diameter of the inhibition zone as per NCCLS (2000) guidelines.

Table 3.10 List of antibacterial agents used in the study

Sl No.	Antibacterial Agent	Group	Disc Content (µg)
1.	Ampicillin (A)	Beta-lactam	10
2.	Carbenicillin (Cb)	Beta-lactam	100
3.	Cephalexin (Cp)	Cephalosporins	30
4.	Nalidixic acid (N)	Quinolone	30
5.	Ciprofloxacin (Cf)	Quinolone	5
6.	Chloramphenicol (C)	Chloramphenicol	30
7.	Gentamicin (G)	Aminogycoside	10
8.	Kanamycin (K)	Aminogycoside	30
9.	Streptomycin (S)	Aminogycoside	10
10.	Erythromycin (E)	Macrolide	15
11.	Oxytetracycline (O)	Tetracycline	30
12.	Sulphamethizol (Sm)	Sulphonamide	300

3.4 Molecular Typing of *Salmonella* serovars

3.4.1 Plasmid profile

Predominant *Salmonella* serovars viz., *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Ohio, and *Salmonella* Irumu were characterized for presence of small and large plasmids. Fresh culture of individual serovars were inoculated in BHI broth (5 ml) and incubated at 37°C in shaker incubator, 200 rpm (Sanyo, Japan) for 18 h. Overnight grown cultures (1.5 ml) were transferred into microfuge tubes follwed by centrigfugation at 10000 x g for 1 min at 4 °C. The supernatant was removed by aspiration leaving bacterial pellets as dry as possible. The bacterial

pellets obatined were resuspended in 100 µl of ice-cold alkaliune lysis solution I by vigorous vortexing followed by addition of 200 µl of freshly preapred alkaline lysis solution II. The contents were mixed by vortexing for 30 sec and ice cold solution III was added to it. The tubes were vortexed by keeping in an invert position for 10 sec to disperse solution III through the bacterial lysate. The tubes were kept in ice for 5 min and centrifuged at 12000 x g (Eppendorf, Germany) for 5 min at 4°C. Subsequently, double stranded DNA was precipitated by adding double the volume of ethanol at room temperature, followed by vortexing and allowed to stand for 2 min at room temperature. The ethanol precipitated aliquots were centrifuged at 12000 rpm for 12 min at 4°C. The supernatant was removed by aspiration and tubes were allowed to stand in an inverted position on tissue paper so as to drained off the fluid. The pellets were rinsed with 1 ml of 70 % ethanol at 4°C and dried the pellets in air for 20 min. The plasmid DNA was redissloved in 50 µl of TE buffer (pH 8.0) containing RNase (20 mg/ml), vorexted beifly and used for electrophoresis. Plasmid samples were analysed by electrophoresis on 0.8 % agarose gel containing ethidium bromide (0.5 µg/µl) in TAE buffer at 70 V for 4-6 h. Supercoiled DNA ladder (Promega, Germany) was used to estimate plasmid size. The resolved bands were photographed using a gel documentation system (Alpha Innotech Corporation, USA).

3.4.2 PCR-ribotyping assay

Four most predominant *Salmonella* serovars viz., *Salmonella* Weltevreden (n = 22), *Salmonella* Rissen (n = 21), *Salmonella* Typhimurium (n = 18) and *Salmonella* Derby (n = 17) isolated from seafood were fingerprinted based on PCR-ribotypes, as described below.

3.4.2.1 Preparation of genomic DNA

Isolation of *Salmonella* genomic DNA was carried out with slight modification of Ausubel et al. (1994) method. Single colony of each isolates of *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium and *Salmonella* Derby were allowed to grow in brain heart infusion broth (5 ml) without shaking at 37°C for 18 h. The pellets were obtained from 1ml of aliquot of the cultures by centrifuging at 10,000 x g (Eppendorf, Germany) for 1min at 4°C. The supernatant was removed by aspiration leaving bacterial pellets dry as possible. The bacterial pellets were resuspended in 435 µl of TE (pH 8.0) by vigorous vortexing followed by addition of 30 µl of 10 % SDS and 3 µl of proteinase (20mg/ml). The contents were mixed by vortexing and tubes were kept in water bath at 37°C for 1 h. After incubation, protein and cell debris were lysed with an equal volume of phenol : chlorofrom : isoamyl alcohol (25:24:1) followed by addition of 1/10 volume of 3 M sodium acetate (pH 5.2). The double stranded DNA was precipitated with double the volume of ice cold ethanol at room temperature followed by separation of precipitated nucleic acid by glass rod. Nucleic acid were rinsed with 1 ml of 70 % ethanol and dried the pellets in air for 20 min. Genomic DNA was redissloved in 200 µl of TE buffer (pH 8.0) containing RNase (20 mg/ml) and determined the concentration or stored at -20°C until further use.

The concentration of DNA was determined with UV-VIS spectrophotometer (Cary 100, Varian, Australia). The absorbance of DNA was taken at wavelength of 260 and 280 nm and OD at 260 nm was used for the determination of DNA concentration using the following formula (Sambrook and Russel, 2001);

Concentration of DNA= OD x 50 x DF (dilution factor)

= x μg/ml

The ratio of the absorbance (1.8) at 260 and 280 indicates the maximum level of DNA purity in the sample.

3.4.2.2 PCR-ribotyping assay

A typical 25 μl PCR reaction mixture contained 0.4 μM concentration of each primer (Table 3.4), 200μM concentration of each dNTP (Finzyme, Finland), 1X PCR buffer (20mM Tris-HCl [pH 8.2], 50 mM KCl, 1.5mM $MgCl_2$), 1U of *Taq* polymerase (Dynazyme II, Finnzyme), and 1μl of sample DNA (~ 50 ng). The amplification was carried out in a thermocycler (MJ Research, USA) at 94°C for 3 min, followed by 34 cycles of 94°C for 1min, 60°C for 1 min, and 72°C for 1 min. A final extension of 72°C for 4 min was incorporated to complete the amplification. The amplified PCR product was analyzed by electrophoresis run at 6 V/cm for 120 min on a 1.5 % agarose gel containing 0.5 μg of ethidium bromide. The resolution of each band was determined on UV-trans illuminator and gel images were captured for further analysis by gel documentation system (Alpha Innotech Corporation, USA).

3.4.3 ERIC-PCR assay

Four most predominant *Salmonella* serovars viz: *Salmonella* Weltevreden (n = 22), *Salmonella* Rissen (n = 21, *Salmonella* Typhimurium (n = 18) and *Salmonella* Derby (n = 17) isolated from seafood were molecular typed based on ERIC-PCR assay.

3.4.3.1 Preparation of DNA

DNA sample from *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium and *Salmonella* Derby were prepared as per procedure described in previous section (3.5.2.1).

3.4.3.2 ERIC-PCR assay

A 25µl of PCR mixture contained 0.4µM concentration of each primer (Table 3.4), 200µM of dNTP (Finzyme), 1X reaction buffer (20mM Tris.HCl (pH 8.0), 50mM KCl, 1.5mM $MgCl_2$), 2U of *Taq* polymerase (Dynazyme II, Finland) and 1 µl (~ 50 ng) of sample DNA to each PCR tube. DNA amplification was carried out in Mastercycler personal (Eppendorf, Germany) with the following reaction condition; initial denaturation at 95°C for 4 min, followed by 35 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 4 min. A final extension was given at 72°C for 5 min. The amplified products and their sizes were determined by electrophoresis on 2 % agarose gel and images were captured by gel documentation system (Alpha Innotech Corporation, USA). Reproducibility of the fingerprints was confirmed with successive run in duplicate from a single sample.

3.4.3.3 DNA fingerprint analysis

DNA fingerprinting pattern of ERIC-PCR was analysed with the Gel Compar II, Applied Maths BVBA, Belgium. Using the unweighted pair group method with arithmetic averages (UPGMA), cluster analysis was performed with Dice correlation method (Sneath and Sokal, 1973). The position tolerance was set at 1.0 % and minimum profile for each band was set at 5.0 %.

3.4.4 Calculation of discrimination indices for PCR-ribotyping and ERIC –PCR of *Salmonella* serovars

The discriminatory power of the fingerprinting methods was calculated using Simpson's index of diversity and expressed as the index of discrimination (Hunter and Gaston, 1988). Number of fingerprinting types obtained from PCR –ribotyping and ERIC-PCR were used for calculation of discrimination indices of *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium and *Salmonella* Derby. The index value was derived from banding patterns obtained in PCR-ribotyping and ERIC-PCR for different strains of a serovar and identical banding pattern were placed under similar profile groups. Hence, index values were calculated based on the following equation:

$$D = 1\text{-}1/N(N\text{-}1) \sum_{j=1}^{s} n_J (n_j - 1)$$

Where, N is the total number of strain, s is the number of type and n_j is the number of strains belonging to jth type.

3.4.5 Pulsed Field Gel Electrophoresis (PFGE) of *Salmonella* Weltevreden and *Salmonella* Typhi

Different strains of *Salmonella* Weltevreden and *Salmonella* Typhi isolated from seafood were analyzed based on the PFGE profile to ascertain .the genetic relatedness among different isolates of *Salmonella* Weltevreden and *Salmonella* Typhi, as follows.

3.4.5.1 Preparation of Genomic DNA in agarose plugs

Single colony of *Salmonella* Weltevreden (n=22) and *Salmonella* Typhi (n=7) from XLD (Difco, USA) plates were inoculated in 3 ml of brain heart infusion

broth (Difco, USA) and incubated at 37°C for 18-20 h. The cells were washed with cell suspension buffer (100mM Tris; 100mM EDTA, pH 8.0) by centrifugation, and the optical density of cells was adjusted to 1.50 at 610 nm (Varian 100, UV-Vis spectrophotometer, USA). A 200 µl of cell suspension was transferred to 1.5 ml microcentrifuge tube containing 40µl of Proteinase K (20mg/ml stock) from Sigma (India). An equal volume (200µl) of molten agarose (1.2 %) in Tris- EDTA buffer (10mM Tris and 0.1mM EDTA) and 1 % SDS was added to cell suspension, one sample at a time, and mixed gently by pipeting up and down two to three times. The agarose cell suspension mixture was dispensed immediately into the plugs mold (Amersham Bio sciences, USA) and allowed to solidify at room temperature for 10 to 15 min to form the agarose plugs. After solidification, the plugs were transferred to lysis buffer 1.5 ml of cell lysis buffer containing Proteinase K (20mg/ml) in a 2 ml of microcentrifuge. Lysis was allowed to continue for 2 h at 54°C. After, lysis, the plugs were washed with 15 ml of warm (54°C) sterile double distilled water (Millipore) and three times with warm TE in a shaking water bath at 54°C. The plugs were used for restriction digestion or stored at 4°C until further use.

3.4.5.2 Restriction digestion of genomic DNA

The agarose plugs were sliced in to a 2 mm slices with a sterile glass cover slip and restriction digestion of 2 mm slice from each plug was carried out with a 30 U of *Xba*I (restriction enzyme) in a 1.5 ml microcentrifuge tube at 37°C for 4 h. Prior to casting of the gel, the restriction mixture was removed from each tube and replaced with 200 µl of 0.5 X TBE.

3.4.5.3 Electrophoresis

Immediately after restriction digestion, samples were subjected to electrophoresis. The electrophoresis of the samples was performed on the Gene navigator pulsed field system (Amersham Bio sciences, USA) with 2 liters of 0.5X TBE running buffer. The electrophoresis conditions were as follows: 6 phases of interpolation mode for 48 h (5 s, 8 h; 25 s, 8 h; 45 s, 8 h; 85 s, 8 h; 105 s, 8 h; 120 s, 8 h) at 125 V to complete the separation of larger DNA fragments (> 500 kb). During the electrophoresis temperature was maintained at 12°C. After completion of electrophoresis the gel was stained for 30 min in 1 liter of sterile distilled water containing 100 µl of Ethidium bromide (10mg/ml) and destained in three washes of 30 min each by 1 liter of distilled water. Gel images were recorded with gel documentation (Alpha Innotech Corporation, USA) system for the analysis of PFGE fingerprinting pattern.

3.4.6 Characterization of *inv*A, *stn* and *fim*A virulence genes of *Salmonella* serovars

All *Salmonella* serovars viz., *Salmonella* Atakpame, *Salmonella* Brancaster, *Salmonella* Georgia, *Salmonella* Ohio, *Salmonella* Typhimurium, *Salmonella* Newport, *Salmonella* Mbandaka, *Salmonella* Oslo, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Lindenburg, *Salmonella* Kottbus, *Salmonella* Bareilly, *Salmonella* Nchanga, *Salmonella* Emek, *Salmonella* Irumu, *Salmonella* Typhi, *Salmonella* Othmarschen, *Salmonella* Rissen, *Salmonella* Riggil, *Salmonella* Takoradi, *Salmonella* Virchow, *Salmonella* Washington, *Salmonella* Weltevreden, *Salmonella* Worthington, *Salmonella* II (2 serovars), *Salmonella* IIIa, *Salmonella* IIIb, *Salmonella* VI and *Salmonella* Typhimurium (ATCC 23564) were

characterized for different virulence genes by PCR technique. The assay was carried with 1 ml of overnight culture subjected to centrifugation at 10000 X g, 2min, at 4°C in Centrifuge 5804 R (Eppendorf, Germany). The pellets were dissolved in 200µl of TE buffer [10mM Tris.HCl, 1mM EDTA (pH 8.0)]. The centrifuge tubes containing pellets were kept on boiling water bath for 10min at 100°C and immediately chilled on ice so as to disrupt the cell wall. The boiled cell contents were centrifuged at 10000 X g, 2min, at 4°C. After centrifugation supernatant containing DNA were carefully transferred to a new microcentrifuge tube and 5 µl of aliquot DNA lysate were used as a template DNA for PCR assay. Primers were specific for *inv*A , *stn* and *fim*A virulence genes and sequences has been shown in Table 3.11.

Table 3.11 Primer sequence and reaction parameters

Primer Sequence	Annealing Temperature (°C)	No. of Cycle	Product size (bp)	Reference
*inv*A GTGAAATTATCGCCACGTTC GGGCAA TCATCGCACCGTCAAAGGAA CC	64	35	284	Rahn et al., 1992
stn CTTTGGTCGTAAAATAAGGC G TGCCCAAAGCAGAGAGATTC	55	33	260	Makino et al., 1999
*fim*A CCTTTCTCCATCGTCCTGAA TGGTGTTATCTGCCTGACCA	58	25	85	Cohen et al., 1996

A 25µl of PCR mixture contained 0.4µM concentration of primer, 200µM of dNTP (Finnzyme, Finland), 1X reaction buffer (20mM Tris.HCl (pH 8.0), 50mM

KCl, 1.5mM $MgCl_2$), 1U of *Taq* polymerase (Dynazyme II, Finland) and 5 µl of sample DNA to each PCR tube. DNA amplification was carried out in a thermocylcer (Eppendorf, Germany) with the following reaction condition; initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 64°C for 30 sec, and 72°C for 30 sec for *inv*A gene and 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for *stn* gene. A final extension of of 5 min at 72°C was employed in both cases. The annealing temperature of *fim*A gene was kept at 58°C and a total of 25 cycles were used for the amplification of desired 85 bp amplicon (Cohen et al.,1996). The amplified products of *inv*A and *stn* genes were determined by electrophoresis on 2 % agarose gel whereas, 85 bp *fim*A gene product was electrophoresed on 12 % polyacrylamide gel. Gel images were captured by using Innotech Corporation (USA) gel documentation system.

3.5 Development of Rapid methods for detection of *Salmonella* serovars in seafood

3.5.1 PCR assay of *Salmonella* serovars

Salmonella serovars viz., *Salmonella* Typhimurium, *Salmonella* Newport, *Salmonella* Typhi, *Salmonella* Mbandaka, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Weltevreden, *Salmonella* II, *Salmonella* IIIa, *Salmonella* IIIb, and *Salmonella* VI were used in the development of *Salmonella* specific PCR assay. *Salmonella* Typhimurium (ATCC 23564) was used as positive control, and *Escherichia coli* and *Citrobactor* strains (from CIFT Type Culture Collection) were used as negative controls. PCR assay was as followed: *Salmonella* serovars were inoculated in 5 ml of BHI (Difco, USA) and incubated at 37°C for 24 h. One ml of overnight cultures were centrifuged at 10000X g (Eppendorf, Germany) for 2 min at

4°C and cells collected as pellets were dissolved in were dissolved in 200 µl of TE buffer [10mM Tris. HCl, 0.1mM EDTA (pH 8.0)]. The cell suspensions were kept in boiling water bath for 10 min and immediate transferred to a chilling ice bath for 5 min. Subsequently, the cell contents were subjected to centrifugation at 10000 X g for 5 min at 4°C (Eppendorf, Germany) and supernatant containing DNA were carefully transferred to a new microcentrifuge. An aliquot of 5 µl of aliquot DNA lysate was used as a template DNA in PCR assay. *Salmonella* specific primers, ST11, AGCCAACCATTGCTAAATTGGCGCA and ST-15, GGTAGAAATTCCCAGCCGGGTACTG, were in PCR assay (Aabo et al., 1993). A 25 µl of PCR mixture containing 0.4 µM concentration of each primers, 200µM of dNTP (Finnzyme, Finland), 1 X reaction buffer ((20mM Tris.HCl (pH 8.0), 50mM KCl, 1.5mM $MgCl_2$), 1U of Taq polymerase (Dynazyme II, Finland) and 5 µl of sample DNA was added in each PCR tube. PCR amplification was carried out in thermocycler (Eppendorf, Germany) with the following reaction parameters; initial denaturation at 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Finally, an extension at 72°C for 5 min was employed. The amplified PCR product and their size were determined by electrophoresis on 2 % agarose. The gel images were recorded using gel documentation system (Alpha Innotech Corporation, USA)

3.5.2 Rapid eight-hour PCR method

A total of 110 seafood samples, consisting of fish (45), shrimp (34), crab (10), mussel (8), clam (8), and edible oyster (5) samples from local fish market in and around Cochin were used in the study. Seafood samples (25 g) were homogenized with 225 ml of Lactose broth (Difco, USA) in a stomacher (Seward

Medicals, UK) at 230 rpm for 30 s. The seafood homogenates were incubated at 37°C for 0, 2, 4, 6, and 8 h and followed by extraction of template DNA from the enriched seafood samples after different period of incubation.

One ml of seafood samples collected after different incubation periods (0, 2, 4, 6, and 8 h) were subjected to low centrifugation at 1000 X g for 1 min at 4°C (Eppendorf, Germany). The supernatant was then centrifuged (10000 X g, 5min, 4°C), and pellet was washed twice with sterile normal saline (0.85% NaCl) followed by centrifugation at 10000 X g for 5 min at 4°C. The pellets were dissolved in 200 µl of TE buffer [10mM Tris.HCl, 0.1mM EDTA (pH 8.0)] and the cell suspension were kept on boiling water bath for 10 min at 100°C and immediately chilled on ice so as to disrupt the cell wall. The boiled cell contents were centrifuged at 10000 X g for 5 min at 4°C (Eppendorf, Germany) and supernatant containing DNA were carefully transferred to a new microcentrifuge and 5 µl of aliquot DNA lysate were used as a template DNA for PCR assay. *Salmonella* specific *inv*A primer sequences F- GTGAAATTATCGCCACGTTCGGGCAA and R- TCATCGCACCGTCAAAGGAACC were used (Rahn et al., 1992). A 25 µl of PCR mixture contained 0.4 µM concentration of each primer, 200µM of dNTP (Finnzymes, Finland), 1X reaction buffer (20mM Tris.HCl (pH 8.0), 50mM KCl, 1.5mM $MgCl_2$), 1U of *Taq* polymerase (Dynazyme II, Finland) and 5 µl of sample DNA to each PCR tube. DNA amplification was carried out in a thermocycler (Eppendorf, Germany) with the following reaction conditions; initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 64°C for 30 sec, and 72°C for 30 sec. A final extension of 5min at 72°C was employed for complete

amplification and amplified products were electrophoresis on 2 % agarose gel along with a 100 bp DNA standard. Gel images were captured and analyzed by gel documentation system (Alpha Innotech Corporation, USA).

Along with the PCR assay, all seafood samples were also analyzed for *Salmonella* by USFDA culture method to compare the efficiency of PCR methods. The isolation of *Salmonella* by culture methods as follows; 25g of seafood sample was homogenized with 225 ml of lactose broth in a stomacher blender (Seward Medicals, UK) at 240 rpm for 30 seconds and homogenate was incubated at 37°C for 24 h. Subsequently, 0.1 and 1.0 ml of enriched seafood sample was transferred into Rappaport -Vassiliadis (RV) and tetrathionate (TT) broth, respectively, and incubated at 42°C (RV) and 43°C (TT) for 18-24 h in water bath. At the end of selective enrichments, loopful of the broths were streaked onto xylose lysine desoxycholate agar (XLD), Bismuth sulphite agar (BSA), and Hektoen enteric agar (HEA) plates. The selective plates were incubated at 37°C for 24 - 48 h. Typical *Salmonella* colonies from each selective plate were picked up for further identification. The biochemical identification of 3-5 typical colonies were carried out as per BAM, USFDA method (Andrews and Hammack 2001). Serological confirmations of isolates were performed using poly A-I & Vi antisera (Difco, USA).

3.5.2.1 Determination of minimum limit of detection (MLD) and effect of seafood matrix on MLD

Fish, shrimp, mussel, crab, clam, and edible oyster, confirmed negative for *Salmonella* by both culture and PCR assays were used for the experiment. Each 25 g of fish, shrimp, mussel, crab, edible oyster, and clam were spiked with *Salmonella*

Typhi, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Mbandaka, *Salmonella* Bareilly, and *Salmonella* Weltevreden, respectively, in the range of 2cfu to $2x10^6$/25g. All spiked seafoods were homogenized with 225 ml of lactose broth and incubated at 37 °C for different incubation periods i.e. 0, 2, 4, 6, and 8 h. PCR assay was performed with 1 ml of spiked sample after the incubations and PCR assay was carried out as described previously (sec 3.5.2). The assay was repeated three times to ascertain the reliability and repeatability of the data. The cell count of *Salmonella* spiked into seafood samples were estimated by plating onto XLD agar.

A control PCR assay was carried out with *Salmonella* ranging from 2 to $2x10^6$ to in 250 ml of lactose broth in the absence of seafood. Lactose both (250 ml) containing different dilutions were incubated at PCR assay was carried out with 1 ml culture from each dilution The results were compared in order to determine the inhibition for PCR assay in presence of seafood.

3.5.2.2 Detection limit for *Salmonella* dead cells

Fish samples confirmed negative for *Salmonella* was spiked with heat killed (100°C, 30min) *Salmonella* Typhimurium. Six different seeding levels of *Salmonella* i.e. 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cells/25g were used for the assay. Spiked fish was homogenized with 225 ml. of lactose broth and PCR assay was carried out with 1ml of fish homogenate at 0, 2, 4, 6, and 8 h of enrichment. Duplicate trials were performed on fish sample to evaluate the reliability of the assay.

3.5.3 Comparison of culture, ELISA and PCR method for *Salmonella* detection

3.5.3.1 Sample preparation

Freshly caught fish, shrimp, crab, clam, mussel, oyster, squid, cuttlefish and octopus were collected from fish landing centres and fish retailers of Cochin (India). A total of 215 seafood samples were tested for the presence of *Salmonella* with culture (USFDA), ELISA and PCR methods.

3.5.3.2 Culture method

Seafood (25 g) samples were homogenized with 225 ml of lactose broth in a stomacher blender (Seward Medicals, UK) for 30 seconds and homogenate incubated at 37°C for 24 h. After incubation, 0.1 and 1.0 ml of enriched seafood samples were transferred into Rappaport Vassiliadis (RV) and tetrathionate (TT) broth, respectively, and incubated at 42°C (RV) and 43°C (TT) for 18-24 h in water bath. At the end of selective enrichments, loopful of the broths were streaked onto xylose lysine desoxycholate agar (XLD), bismuth sulphite agar (BSA), and Hektoen enteric agar (HEA) plates. The selective plates were incubated at 37°C for 24 - 48 h. Typical *Salmonella* colonies from each selective plates were picked up for identification. Biochemical identification of 3-5 typical colonies were carried out as per BAM, USFDA method (Andrews and Hammack 2001) and serological confirmations of isolates were done by poly A-I & Vi antisera (Difco, USA).

3.5.3.3 ELISA assay

Seafood samples were prepared for ELISA assay as per BAM, USDFA method. 25 g of seafood sample was homogenized with lactose broth (225 ml) in the stomacher blender for 30 s and incubated at 37°C for 24 h. After the incubation, 0.1

ml and 1.0 of enriched seafood samples were transferred into Rappaport-Vassiliadis (RV) and tetrathionate (TT) broth, respectively, and incubated RV broth at 42°C and TT broth at 43°C for 18-24 h. Thereafter, 1.0 ml of RV and TT sample broth, separately transferred in to M broth containing 10 μg ml^{-1} novobiocin and incubated at 42°C for 4-6 h. Following the incubation, 0.5 ml each the M both cultures (from RV and TT enrichments) were mixed together and boiled at 100°C for 20 min. The sample was cooled at room temperature and 100 μl of aliquots were used for ELISA assay. The assay was carried out with *Salmonella*–Tek (Organon Teknika Corporation, Durham, NC), a monoclonal antibody based kit for *Salmonella,* as per manufacturer's instructions. Results were read with Reader 100, Organon Teknika (USA), Microwell System at 450 nm and results were interpreted based on the cutoff value [negative control (NCX) + 0.250] as per *Salmonella*-Tek™ instruction manual. The absorbance of a sample greater or equal to the cutoff value was considered positive for the presence of *Salmonella*.

3.5.3.4 PCR assay

One ml. of overnight enriched seafood sample was subjected to low centrifugation at 1000 X g for 2 min in Centrifuge 5804 R (Eppendorf, Germany) to allow the seafood particles to settle down and supernatant was centrifuged further at 10000 X g, 5 min, 4°C to collect cell biomass. Pellet was washed twice with sterile normal saline (0.85 % NaCl) followed by centrifugation of cells at 10,000 X g, 5 min at 4°C. Crude DNA was extracted from pellet by boiling for 10min at 100°C in 200 μl of TE buffer [10 mM Tris.HCl, 0.1 mM EDTA (pH 8.0)]. Aliquots of 5 μl DNA lysate were used as template DNA for PCR assay. *Salmonella* specific *inv*A primer (5'-GTGAAATTATCGCCACGTTCGGGCAA-3' and 5'-

TCATCGCACCGTCAAAGGAACC-3') was used for detection of *Salmonella* (Rahn et al., 1992). A 25 µl of PCR mixture contained 0.4 µM concentration of each primer, 200 µM of dNTP (Finnzyme, Finland), 1 X reaction buffer (20 mM Tris.HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$), 1 U of *Taq* polymerase (Dynazyme II, Finland) and 5 µl of sample DNA to each PCR tube. DNA amplification was carried out in Mastercycler personal (Eppendorf, Germany) with the following reaction condition; initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s and a final extension of 5 min at 72°C was employed. The amplified product was electrophoresis on 2% agarose gel and product size (284 bp) was determined with 100 bp DNA molecular weight ladder (Fermentas, USA). Finally, gel image was captured using gel documentation system (Alpha Innotech Corporation, USA). For each PCR test, separately a positive (*Salmonella* Typhimurium, ATCC 23564) and negative control (sterile distilled water) were also run.

3.5.3.5 Statistical analysis of the results from 3 method

Results from the three assays were statistically compared by using software package SPSS 12.0 for Windows (SPSS Inc., Chicago, USA). These three methods were considered as raters and the kappa coefficient was calculated to test the agreement. Based on kappa coefficient, the results were interpreted, as having fair agreement (0.21 to 0.40), moderate agreement (0.41-0.60), substantial agreement (0.61-0.80) and perfect agreement (0.81 to 1.0) between the raters.

3.5.4 Real-time PCR for *Salmonella* in seafood

3.5.4.1 Isolation and quantification of DNA used as standards in real-time assay

Salmonella Typhimurium culture was grown in a 5 ml of BHI broth at 37 for 18 h and isolation of DNA was carried out as per method of Ausubel et al. (1994). 1ml of overnight culture was centrifuged at 10,000 x g (Eppendorf, Germany) for 1min at 4°C. The supernatant was removed and bacterial pellet was resuspended in 435 µl of TE (pH 8.0) by vigorous vortexing followed by addition of 30 µl of 10 % SDS and 3 µl of proteinase (20mg/ml). The contents were mixed by vortexing and tubes were kept in water bath at 37°C for 1 h. After incubation, protein and cell debris were lysed with an equal volume of phenol : chlorofrom : isoamyl alcohol (25:24:1) followed by addition of 1/10 volume of 3 M sodium acetate (pH 5.2). The double stranded DNA was precipitated with double the volume of ice cold ethanol at room temperature followed by separation of precipitated nucleic acid by glass rod. Nucleic acid were rinsed with 1 ml of 70 % ethanol and dried the pellets in air for 20 min. Genomic DNA was redissloved in 200 µl of TE buffer (pH 8.0) containing RNase (20 mg/ml) and determined the concentration. The concentration of DNA was determined with UV-VIS spectrophotometer (Cary 100, Varian, Australia). The absorbance of DNA was taken at wavelength of 260 and 280 nm and OD at 260 nm was used for the determination of DNA concentration using the following formula (Sambrook and Russel, 2001).

Concentration of DNA= OD x 50 x DF (dilution factor)

= x µg/ml

The ratio of the absorbance (1.8) at 260 and 280 indicated the level of DNA purity in the sample. The pure DNA obtained *Salmonella* Typhimurium was decimal diluted

from 500 ng/10 µl to 0.002 pg/ 10µl with sterile TE (pH8) and stored at -20°C until further use. The DNA concentration (500 ng/10 µl to 0.002 pg/ 10µl) were used as standard to determine the number of *Salmonella* in seafood samples.

3.5.4.2 Isolation of DNA from pure culture, seeded fish and shrimp samples

One ml of *Salmonella* culture was decimally diluted from $2x10^9$ to 2 cfu/ml in normal saline and DNA was extracted from the following dilutions; 2, 2x 10, $2x10^2$, $2x10^3$, 2x 10^4, 2x 10^5, 2x 10^6 cfu/ml as described above.

Fish (*Rastrelliger Kanagurta*) and shrimp (*Penaeus monodon*) confirmed to be free from *Salmonella* by conventional culture and conventional PCR methods were used for the seeding experiments. 25 g of fish and shrimp was separately blended with 225 ml of BPW in a homogenizer at 200 rpm for 30 min. A portion (10 ml) containing 1g of tissue was transferred into 50 ml flask and seven different seeding levels of *Salmonella* i.e. 2, 2x10, $2x10^2$, $2x10^3$, $2x10^4$, $2x10^5$ and $2x10^6$ cfu/ml were used for the seeding of flasks containing fish and shrimp homogenates. DNA was extracted from the seeded homogenate preparations (10 ml) from each dilution. 10 ml of fish and shrimp seeded homogenates were centrifuged at 500 rpm at 4°C for 2 min in a centrifuge (Eppendorf, Germany). The supernatants were transferred to fresh centrifuge tubes and centrifuged at 10000 rpm at 4°C for 2 min (Eppendorf, Germany). Thereafter, isolation of DNA was carried out from the pellets as described in the previous section.

3.5.4.3 Real-time PCR assay

The real-time thermocycler used in this study was a Chromo 4 Real-time system (M J Research Corp. USA). *Salmonella* specific *inv*A primer (see Table 3.10) were used for the development of real-time assay for different concentration of

DNA from pure culture and seeded fish and shrimp samples. The assay was carried out with 25 µl of real-time PCR mixture consisting of 12.5µl of 2x SYBR Green supermix (Sigma, India), 0.6 µl of 10 µM primers (each). Finally, 10 µl of the target DNA solution was added into the reaction mixture and final reaction volume was attained at 25 µl with a 1.3 µl of the sterile milli-Q water. The SYBR Green Supermix conatained the dNTP (0.4 mM of each), Taq polymerase, 6 mM $MgCl_2$, 100 mM KCl and 40 mM Tris-HCl (pH 8.4). Real-time PCR was performed with a initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 94°C for 15 s, primer annealing at 64°C for 10 s for invA and 58°C for *fim*A, and primer extention at 72°C for 20 s for both primers. The melting curve analysis of the final PCR product was carried out from 60 to 95 °C at 1°C interval to determine the *T*m value of the PCR product. All seeded experiments were repeated once again to get the reliable results.

3.5.4.4 Quantitative detection of *Salmonella* in naturally contaminated shrimp and fish samples

The quantitative detection of *Salmonella* in fish and shrimp (5 each) collected from Cochin (India) market was used for the enumeration of *Salmonella* in naturally contaminated samples. Fish and shrimp sample (25 g) was homogenized with 225 ml of buffered peptone water (BPW) in a stomacher blender for 1 min at 150 rpm. Preparation of DNA for the real-time assay was performed with 1 ml of seafood homogenate without enrichment as described in the previous section and real-time assay was performed as discussed earlier.

Simultaneously, fish and shrimp samples were analyzed by culture method for presence of *Salmonella* (Andrews and Hammack, 2001).

RESULTS & DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Isolation and identification of *Salmonella* from seafood

4.1.1 Incidence of *Salmonella* in seafood

Over a period of four years from 2003 to 2007, a total of 443 seafood samples consisting of pelagic fish (n=79), demersal fish (n=52), shrimp (n=86), lobster (n=25), crab (n=38), clam (n=41), mussel (n=31), oyster (n=27), squid (n=23), cuttlefish (n=21), and octopus (n=20) samples (see Table 3.7) from the fish markets and landing centres of Cochin were analyzed for presence of *Salmonella*. The prevalence of *Salmonella* was maximum in clams (34.1%) followed by mussel (31%), fish (30.2%) and shrimps (29.0%) samples. These values were comparatively high as compared to crab (10.5%), oyster (14.8%), squid (17.3%), and octopus (15.0 %) samples and the lowest incidence of *Salmonella* was noted in lobster samples (8.0%). The results further demonstrated that an overall of 24.3% seafood was contaminated with *Salmonella* during this period (Table 4.1). A total of 268 *Salmonella* isolates were detected and identified from different seafood. Fish and shrimp samples contributed more than 50 % of the total *Salmonella* isolates.

The prevalence of *Salmonella* in seafood indicated the widespread contamination that resulted in the presence of major health hazard in seafood. Present study demonstrated that there is a increase in the prevalence of *Salmonella* in seafood in Cochin, which was reported to be reasonably low (7.6%) during the period 1985-87 (Nambiar and Iyer, 1991). Incidence of *Salmonella* was found to be

maximum in clam and mussel samples. The reason could be that it was due to filter feeding nature of molluscs and generally harvested in shallow, near-shore estuarine or brackish waters (Huss et al., 2004). Thus, there is a strong possibility that the live animals may be contaminated with sewage-derived pathogenic bacteria including *Salmonella*. Present study also highlighted level of contamination in coastal water bodies as most of the marine bivalves were caught from the coastal marine environments, which are now harbouring bacterial pathogens like *Salmonella*. The investigation revealed that prevalence of *Salmonella* in seafood varied depending on type of seafood.

Table 4.1 *Salmonella* cultures isolated from different seafood

Sl. No.	Sample	No. tested	Incidence of *Salmonella* (%)	No. of isolates	% of total isolates
1.	Pelagic fish	79	25 (31.6)	67	25
2.	Demersal fish	52	15 (28.8)	42	15.6
3.	Shrimp	86	25 (29.0)	58	21.6
4.	Lobster	25	2 (8.0)	4	1.4
5.	Crab	38	4 (10.5)	7	2.6
6.	Clam	41	14 (34.1)	31	11.5
7.	Mussel	31	10 (32.0)	19	7.0
8.	Oyster	27	4 (14.8)	10	3.7
9.	Squid	23	4 (17.3)	11	4.1
10.	Cuttlefish	21	3 (14.2)	9	3.3
11.	Octopus	20	2 (10.0)	10	3.7
		443	108 (24.3)	268	

The prevalence was comparatively higher at 31.6, 28.8 and 29% in pelagic fish, demersal fish and shrimps, respectively, whereas, much lower level of incidence was observed in lobsters, crabs and cephalopods. Detection of higher level of contamination in fish and shrimp samples pointed out the involvement of multi-step post harvest handling process from harvest areas to the fish market consequently deteriorated the quality of seafood available in local seafood markets. Fish and shrimp available in the local markets were found the very poor in quality and food safety, as prevalence of *Salmonella* was observed to be reasonably at high levels. Similar reports on higher prevalence of *Salmonella* in shrimps were reported in south-east Asian countries viz.: Thailand and Vietnam (Boonmar et al., 1998; Phan et al., 2005). The incidence of *Salmonella* in seafood samples imported to USA from different parts of the world demonstrated that prevalence was more in seafood originated from Asia-Pacific (12.5% positive) followed by Africa (11.5%), Middle East (10%) and Southeast Asia (7.8%) countries (Heinitz et al., 2000).

The presence of *Salmonella* in seafood has been either from the contaminated coastal areas or the unhygienic surroundings where they are landed and handled. The influence of environmental factors and human activity on the presence of *Salmonella* serovars in marine environment has been studied in Spain and showed that 2.9% of Molluscs and seawater were positive for *Salmonella* (Martinez-Urtaza et al., 2004). There has been increasing evidence that certain *Salmonella* serotypes may be part of water bodies and, aquaculture system. The cumulative effect of these contaminated environments lead to increase in presence of *Salmonella* in seafood (Saheki et al., 1989; Reilly and Twiddy, 1992). The prevalence of *Salmonella* in marine bivalve i.e. clam and mussel was observed to be

high in this study, whereas, oyster was found to be less contaminated with *Salmonella*. In contrast to our observations, 10% of shellfish (oyster, clam and mussel) isolated from Hong Kong waters were contaminated with *Salmonella* (Yam et al., 1999).

A similar study by Brands et al. (2005) showed that 7.5% of oyster in the USA were positive for *Salmonella*. The incidence of *Salmonella* in cephalopods viz., squid, cuttlefish and octopus was detected low as compared to other seafood and most of the squid, cuttlefish and octopus are deep sea animals. Cephalopods samples were collected from fishing harbours in this study which had low incidences of *Salmonella* contamination. Prevalence of *Salmonella* in crab and lobster were found to be quite low as well. The crab samples were sold live in the markets and brought in live condition to the laboratory during this study. The lobsters are highly priced seafood commodity and not easily available in the local market. Highly priced seafood are handled with proper icing and care, hence, low incidences of *Salmonella* contamination were observed in crab and lobsters.

4.1.2 Seasonal variation on incidence of *Salmonella* in seafood

The incidence of *Salmonella* in Cochin seafood has been investigated during the period (2003-2007). Year-wise isolation of *Salmonella* from seafood samples are presented in Table 4.2. The results showed that the incidence of *Salmonella* in seafood was 23 out of 73 in 2003, 28 out of 97 in 2004, 34 out of 124 in 2005, 20 out of 108 in 2006, and 3 out of 41 seafood samples were positive for *Salmonella* during 2007. The presence of *Salmonella* in seafood was also found to be variable during pre-monsoon, monsoon and post-monsoon period. A total of 16/113 seafood was positive for *Salmonella* during pre-monsoon, 49/154 in monsoon and 43/176

was positive for *Salmonella* during post-monsoon period. The relative incidence of *Salmonella* in seafood in each season showed that prevalence was highest (31.8%) during monsoon season followed by post-monsoon (24.4%) and pre-monsoon (14.1%) period.

Table 4.2 Seasonal variation on incidence of *Salmonella* from seafood

Sl. No.	Year	Samples Tested	Incidence of *Salmonella*			Total prevalence (%)
			Pre-Monsoon (%)	Monsoon (%)	Post - Monsoon (%)	
1.	2003	73	3/17 (17.6)	12/21 (57.1)	8/35 (22.8)	23 (31.5)
2.	2004	97	2/26 (7.6)	17/31 (54.8)	9/40 (22.5)	28 (29.2)
3.	2005	124	6/37 (16.2)	12/33 (36.3)	16/54 (29.6)	34 (27.4)
4.	2006	108	4/22 (18.1)	6/39 (15.3)	10/47 (21.2)	20 (18.5)
5.	2007	41	1/11 (9)	2/30 (6.6)	0/0 (0)	3 (7.3)
	Total	443	16/113 (14.1)	49/154 (31.8)	43/176 (24.4)	108 (24.3)

The prevalence of *Salmonella* in seafood was found to be varying during the period of investigation (2003-2007) and the incidence of was comparatively much higher during 2003, 2004 and 2005. The reason could be cited here that more common seafood (fish, shrimp and clams) samples were analyzed during this period. This was attributed to the fact that the incidence level of *Salmonella* in fish, shrimp and clams were found to be higher as compared to other seafood. The results further highlighted that incidence of *Salmonella* was conspicuously lower during 2006 and 2007. The lowering in incidence was justified with the fact that lobsters, crab and

cephalopods were analyzed during the period (2006 and 2007) in which the incidence was observed to be lower as compared to other seafood commodities.

Prevalence of *Salmonella* serotypes in foodstuffs including poultry meat was reported to be at 38.1% in Sau Paulo, Brazil, during a period from 1996 to 2000 (Tavechio et al., 2002). In a similar study, a total of 730 fish and 276 crustaceans from the markets in Coimbatore, India were analyzed for presence of *Salmonella* during 1990 to 1992, the study revealed that prevalence of *Salmonella* in 14.25% fish and 17.39% crustaceans samples and study further demonstrated that incidence of *Salmonella* was much higher during monsoon season (Hatha and Lakshmanperumalsamy, 1997). Present study highlighted the higher level of *Salmonella* contamination in seafood samples, particularly during monsoon and post-monsoon period.

4.2 Identification of *Salmonella* and major species isolated

Identification of *Salmonella* isolates were carried out with a set of biochemical reactions as shown in Table 4.3. The results highlighted the variation from the standard pattern in biochemical utilization of lactose, dulcitol, malonate and citrate by different isolates of *Salmonella* from seafood. The variant strains for utilization of lactose were identified and a total of 4.2% of isolates were actively utilizing lactose with in 24 h. Similarly, 10 % strain variants were identified which were found negative for dulcitol. The strain variants were also observed for gas from glucose (2.9%), malonate (2.7%) and utilization of citrate (6.8%). All other biochemical tests were found to be consistently similar for *Salmonella* strains.

The biochemical test only confirms *Salmonella* strains upto subspecies level and results were in agreement with the Bergey's Manual of Systematic Bacteriology

(Brenner and Farmer III, 2005). The biochemical tests carried out for different *Salmonella* strains from seafood revealed that there is less strain variation in *Salmonella* isolates. The variation in biochemical reaction has been reported to be very low in *Salmonella* at serovars level, however, biochemical tests showed variation at *Salmonella* subspecies. The presence of diverse *Salmonella* biochemical patterns were observed for malonate, lactose and dulcitol utilization and similar variations were reported by Brenner and Farmer III (2005).

Table 4.3 Confirmatory Tests of *Salmonella* isolates (n = 268)

Sl No.	Test /Medium	Reaction	Typical Result (%)
1.	Gram Reaction	Negative	268 (100)
2.	Motility	Motile	268 (100)
3.	Triple Sugar Iron agar	Acid butt, Alk[a] slant	268 (100)
4.	Lysine Iron agar	Alk butt, Alk slant	268 (100)
5.	Urease	Negative	268 (100)
6.	Indole	Negative	268 (100)
7.	Glucose	Acid & Gas Acid & No Gas	261 (97.0) 7 (2.6)
8.	Lactose	Negative	257 (95.8)
9.	Sucrose	Negative	268 (100)
10.	Dulcitol	Positive	241 (90.0)
11.	Malonate	Negative	261 (97.3)
12.	Simmons Citrate	Positive	250 (93.2)
13.	Methyl Red (MR)	Positive	268 (100)
14.	Voges-Proskauer (VP)	Negative	268 (100)
15.	Cytochrome oxidase	Negative	268 (100)
16.	Lysine decarboxylase	Positive	268 (100)
17.	H_2S on TSI	Positive	268 (100)
18.	Serology (Poly A-I & Vi)	Positive	268 (100)

Detection of lactose fermenting (lac^+) *Salmonella* isolates in seafood was another significant observation made during this study. A total of 11 out of 268 *Salmonella*

isolates was found to be lac$^+$ in nature. It has been reported that less than 1 % of all *Salmonellae* ferment lactose (Ewing 1986).

The prevalence of lactose positives (lac$^+$) in *Salmonella enterica* subsp. *arizoane*, *Salmonella enterica* subsp. *diarizonae* and *Salmonella enterica* subsp.*indica* was reported to be 15, 85, and 22 %, respectively and the natural habitat of the *Salmonella enterica* subsp. *salamae* (II), subsp. *arizonae* (IIIa), subsp. *diarizonae* (IIIb), subsp. *houtenae* (IV) and subsp. *indica* (VI) are considered present in the cold-blooded animals and environments (Popoff and Le Minor, 2005). Thus, it is suspected that aquatic animals being cold blooded may harbour naturally lac$^+$ *Salmonella* serovars and actual incidence of lac$^+$ *Salmonella* in seafood may be much higher than the reported incidences. At present, it is very apparent that there is not many reported incidence of lac$^+$ *Salmonella* in seafood.

Several factors are responsible for lower incidences of lactose positive (lac$^+$) *Salmonella* serovars in food or seafood. Lac$^+$ *Salmonella* serovars, which are sporadic in presence and also difficult to identify, as many of the *Enterobacteriaceae* strains look similar with Lac$^+$ *Salmonella* on selective media plates; hence they escape undetected during analysis. This is because *Salmonella* isolation from different sources with routine selective and differential media utilizes non-lactose fermentation as a major biochemical property and commonly used differential plating media for isolation of *Salmonella* contains lactose. Littell (1977) has demonstrated that routine selective and differential media for *Salmonella* was not efficient enough to identify *Salmonella arizonae* group. Outbreaks of disease from lac$^+$ *Salmonella* has been reported (Falcao 1975; Dube, 1983; Camara et al., 1989; Ruiz et al., 1995). In India, *Salmonella arizonae* (IIIa) infection in infants and

children has been reported by Mahajan et al. (2003). Finally, this study has highlighted the comparatively higher prevalence of lac^+ *Salmonella* in seafood.

4.2.1 Distribution of different serovars viz-a-viz different seafood group

A total of 268 *Salmonella* isolates consisting of 32 different serovars were isolated and identified in seafood. The major serotypes identified were *Salmonella* Atakpame, *Salmonella* Brancaster, *Salmonella* Georgia, *Salmonella* Ohio, *Salmonella* Typhimurium, *Salmonella* Newport, *Salmonella* Mbandaka, *Salmonella* Oslo, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Lindenburg, *Salmonella* Kottbus, *Salmonella* Bareilly, *Salmonella* Nchanga, *Salmonella* Emek, *Salmonella* Irumu, *Salmonella* Typhi, *Salmonella* Othmarschen, *Salmonella* Rissen, *Salmonella* Riggil, *Salmonella* Takoradi, *Salmonella* Virchow, *Salmonella* Washington, *Salmonella* Weltevreden, *Salmonella* Worthington, *Salmonella* II (3,10:lv:z_6), *Salmonella* II (47:enx, z_{15}:1,6), *Salmonella* IIIa (17:-:-), *Salmonella* IIIb (38:z:-), *Salmonella* IIIb (60:r:z), *Salmonella* VI (11:b:1,7) and *Salmonella* VI (45:a:enx).

Salmonella Weltevreden was predominant serotype in seafood followed by *Salmonella* Rissen, *Salmonella* Typhimurium and *Salmonella* Derby. The number of *Salmonella* serovars and distribution of different serovars in individual seafood is given in Table 4.4. Fish samples harbored 21 different *Salmonella* serotypes followed by 15 serotypes in shrimp, 9 serotypes in clams, and 3 serovars were isolated from cuttlefish samples. *Salmonella* Ohio, *Salmonella* Worthington, *Salmonella* Washington, *Salmonella* II, *Salmonella* IIIa and *Salmonella* IIIb were isolated from fish samples, whereas, *Salmonella* Atakpame was isolated from

cuttlefish samples and *Salmonella* VI (45:a:enx) was isolated from shrimp samples. Of the different *Salmonella* serotypes isolated from seafood and *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium and *Salmonella* Derby serovars were predominantly isolated and ranked 1st, 2nd, 3rd, and 4th respectively, in seafood from Cochin area. The occurrence of *Salmonella* Weltevreden was limited to fish, shrimp, clam and mussel and was not isolated from other seafood. *Salmonella* Rissen was the second most predominant serovar in seafood and were isolated from fish, shrimp, mussel, cuttlefish, squid and lobster, followed by *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup and *Salmonella* Lindenburg (Table 4.4).

The most commonly isolated *Salmonella* serovar in seafood from Cochin was *Salmonella* Weltevreden and this observation was in concurrence with some of the earlier studies on *Salmonella* in fish and seafood from Coimbatore (Hatha & Lakshmanaperumalsamy, 1997), Mumbai (Iyer and Shrivastava, 1989b) and Mangalore fish markets (Shabarinath et al., 2007). *Salmonella* Weltevreden was ranked first in the twenty most frequent *Salmonella* serotypes isolated from seafood samples imported into USA, during a study period from 1990 to 1998 (Heinitz et al., 2000). *Salmonella* Weltevreden was most predominant serotypes in Thailand shrimps (Boonmar et al., 1998).

Table 4.4 Distribution of *Salmonella* serovars in seafood

Salmonella serotypes	Number of Isolates from										
	Fish	Shrimp	Clam	Mussel	Oyster	Crab	Lobster	Squid	Cuttlefish	Octopus	Total
S. Atakpame									2		2
S. Bareilly	6	4	4					2			16
S Braenderup	5	5			3					2	15
S. Brancaster		3	1							2	6
S. Derby	5	6	2	2	2						17
S Emek			5						2		7
S. Irumu	3		1		2		2			3	11
S. Georgia	4										4
S. Kottbus		2									2
S. Lindenburg	7	3		4							14
S. Mbandaka		9			1	3					13
S. Nchanga	5			2							7
S. Newport	2					2					4
S. Ohio	5							4			9
S. Oslo	6	1						1			8
S. Othmarschen	4					2					6
S. Rissen	8	4		3			2	1	3		21
S. Riggil	2				2				2		6
S. Takoradi	5	3									8

(Continued in next page)

Table 4.4 Distribution of *Salmonella* serovars in seafood (continued from previous page)

S. Typhi	3	3		1							7
S. Typhimurium	7	3	2	5				1			18
S. Virchow			3					2		3	8
S. Washington	4	2									6
S. Weltevreden	6	4	10	2							22
S. Worthington	3										3
Salmonella II 3,10:lv:z6	4	2									6
Salmonella II 47:enx z15:1,6	2										2
Salmonella IIIa 17:-:-	3										3
Salmonella IIIb 38:z:-	1										1
Salmonella IIIb 60:r:z	1										1
Salmonella VI 11:b:1,7		2									2
Salmonella VI 45:a:enx	1										1
Untypable	7	2	3								12
Total	109	58	31	19	10	7	4	11	9	10	268

A similar study on prevalence of *Salmonella* in food animals and human in Thailand showed that *Salmonella* Weltevreden was associated with farm animals and workers, and *Salmonella* Rissen was found to be predominantly isolated from pigs (Patungtod and Kaneene, 2006). A study spanning a period of ten years in Thailand demonstrated that *Salmonella* Weltevreden was most common serovar involved in human infections (Bangtrakulnonth et al., 2004). *Salmonella* Typhimurium was predominantly isolated from the molluscan shellfish of Galicia region of Spain (Martinez-Urtaza et al., 2003). *Salmonella* Derby was isolated from shrimp in the Mekong Delta in Vietnam (Phan et al., 2005). Quite contrary to the present observation, *Salmonella* Enteritidis, *Salmonella* Hadar and *Salmonella* Agona were most dominant serotypes prevalent in food stuffs in Sau Paulo, Brazil and frozen shrimps in Thailand (Tavechio et al., 2002; Boonmar et al., 1998).

Detection of *Salmonella* serovars such as, *Salmonella* Rissen, *Salmonella* Riggil, *Salmonella* Takoradi and *Salmonella* Othmarschen were not reported previously in seafood in India. So this appears to be the first report from India. Present study also highlighted the isolation of rare *Salmonella* serovars namely, *Salmonella* II, *Salmonella* IIIa, *Salmonella* IIIb, and *Salmonella* VI in seafood, which were not reported previously in seafood except *Salmonella arizonae* (IIIa) was isolated from fish and frog legs (Iyer and Shrivastava, 1989b). Finally, the study highlighted the widespread prevalence of *Salmonella* serovars in seafood, indicating the prevalence of contaminated coastal waters, unhygienic conditions in fish landing centres and fish markets of Cochin, contributing to the higher level of *Salmonella* contamination in seafood.

4.3 Recovery of *Salmonella* from seafood

4.3.1 Effect of pre-enrichment media

A total of 268 *Salmonella* isolates were identified from seafood and 110 out of 268 *Salmonella* strains were isolated in buffered peptone water (BPW), whereas, lactose broth (LB) found to be effective for isolation of 158 *Salmonella* isolates from seafood (Fig. 4.1). The role of two pre-enrichment broths for isolation of *Salmonella* in naturally contaminated seafood samples were found to be comparable. It is observed that 41% of the *Salmonella* isolates were detected through BPW, whereas, 59% of isolates were identified, when lactose broth was involved in pre-enrichment step. No specific trend was observed, related to the type of seafood, as both pre-enrichment broths could isolate *Salmonella* from a variety of seafoods. However, lactose broth was found to be slightly more efficient compared to BPW for isolation of *Salmonella* from seafood.

Lactose broth is widely used for the isolation of *Salmonella* in standard procedures for testing food, dairy products and other environmental samples (Andrews and Hammack, 2001). It has also been reported to be detrimental for the foods with low buffering capacity. Hence, alternative pre-enrichment media were recommended for the isolation of *Salmonella* from such foods (Angelotti, 1963). BPW was reported to be promising for isolation of *Salmonella* in food as pre-enrichment broth. Several standard methods used BPW as pre-enrichment broth for isolation of *Salmonella* from food products (Flowers et al., 1992; ISO, 2000). Thomason et al. (1977) demonstrated that BPW was 25% more efficient than the lactose both for isolation of *Salmonella* from environmental samples. Similarly, the universal broth was reported to be more useful as compared to lactose broth for

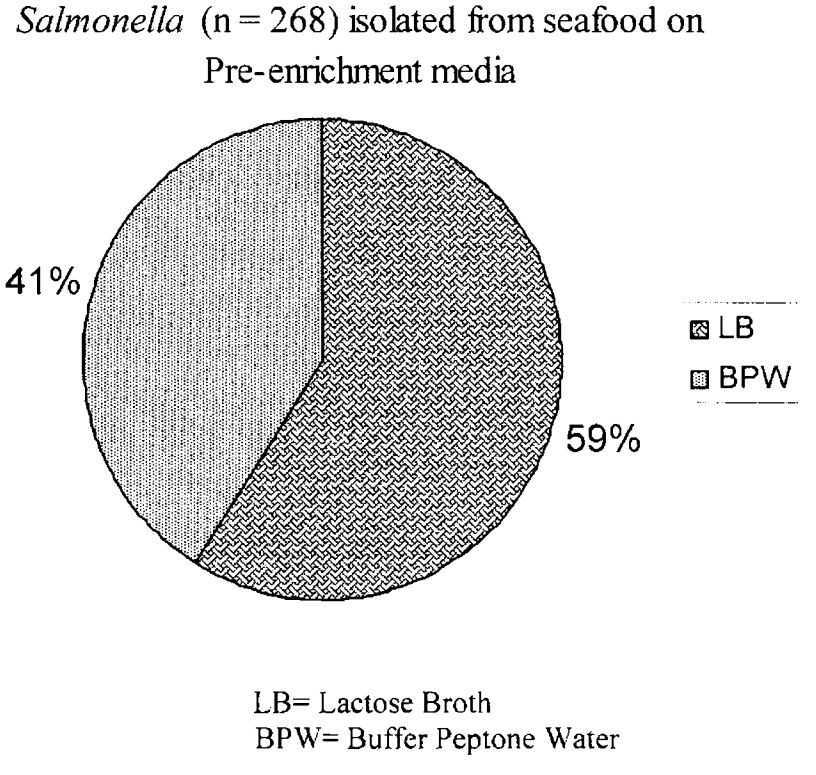
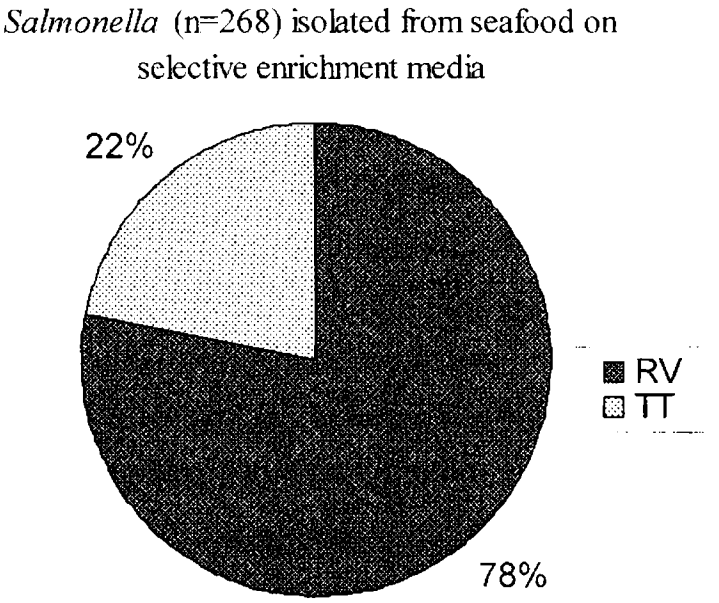


Fig. 4.1 Role of pre-enrichment media on recovery of *Salmonella* from seafood

detection of *Salmonella* Typhi in dairy foods. Hammack et al., (2006) made a comparative study to determine the effectiveness of BPW, lactose broth and universal broth media on the recovery of *Salmonella* from cantaloupes, mangoes and tomatoes and they found that BPW was more efficient for mangoes and tomatoes. The present study highlighted that lactose broth was comparatively superior compared to BPW for isolation of *Salmonella* from seafood. A collaborative study was made by 21 US and Europe laboratories on three food types (dairy, poultry and eggs) for detection of *Salmonella* by ISO 6579 and AOAC methods, using different pre-enrichment media. Their study reported that both methods were equally efficient for recovery of *Salmonella* from food products (Feldsine et al., 2003).

4.3.2 Effect of selective enrichment media

The role and efficiency of Rappaport-Vassiliadis (RV) and tetrathionate broth (TT) as selective enrichment broth were evaluated for the recovery of *Salmonella* in different seafoods. The results showed a vast difference between the two selective media for isolation of *Salmonella* from seafood. A total of 209 *Salmonella* strains out of 268 were isolated from RV broth and only 59 strains were isolated in TT both (Fig. 4.2). A study carried out on tropical seafood by Kumar et al. (2003) demonstrated that Selenite cystine broth (SC) was more efficient than tetrathionate broth (TT) in the recovery of *Salmonella* from seafood. Recently, a modification has been incorporated in AOAC protocol to replace SC with RV broth for isolation of *Salmonella* in food, including seafood. Based on the completion of AOAC pre-collaborative and collaborative studies, RV medium is now being recommended for the analysis of *Salmonella* in high microbial and low microbial load foods (Andrews and Hammack, 2001). Rappaport et al. (1956) formulated an



RV = Rappaport-Vassiliadis Medium
TT = Tetrathionate Broth

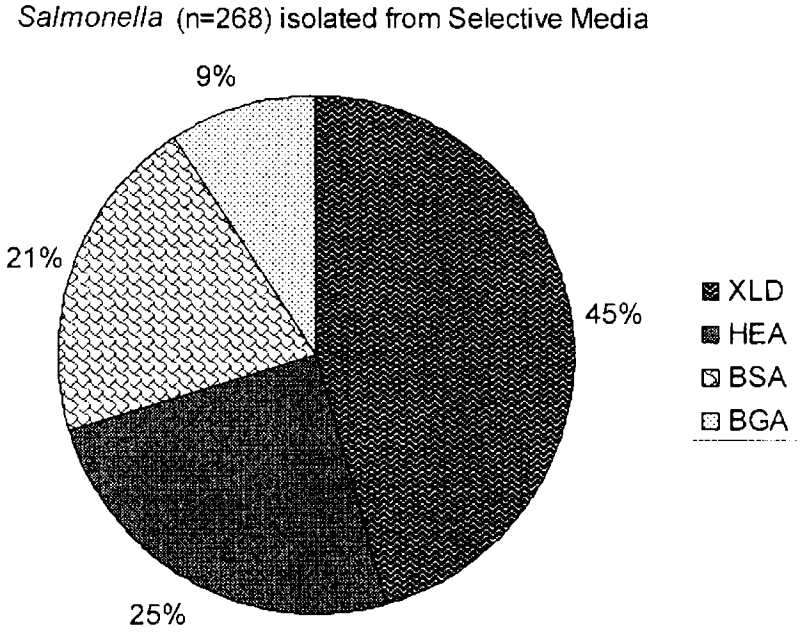
Fig. 4.2 Role of selective enrichment media involved in *Salmonella* isolation from seafood

enrichment medium for *Salmonella* that was modified by Vassiliadis et al. (1983). The Rappaport formulation, recommended incubation at 37°C and the Vassiliadis modified the method by reducing level of malachite green and also recommended incubation at 43°C. Later, work by Peterz et al. (1989) showed that incubation at 41.5° ± 0.5°C for 24 hours improved recovery of *Salmonella* spp. by RV broth.

The efficiency of the selective media may vary with the types of food. This was proved in this study that RV both was highly efficient in isolation of *Salmonella* in seafood. Present findings were in concordance with an earlier study by Oboegbulem (1993). Their study showed that *Salmonella* was recovered in 214 / 477 (45 %) samples of chickens examined by the RV enrichment technique while there was only 29% recovery by the SC broth enrichment. So far, there are no report of comparison between RV and TT broth in meat and seafood. Detection of *Salmonellae* in food by motility enhancement in Modified Semisolid Rappaport–Vassiliadis (MSRV) medium showed equal or better results than the use of standard Rappaport–Vassiliadis (RV) broth. Also, the addition of nitrofurantoin to the modified semisolid RV (MSRV) and to xylose lysine desoxycholate (XLD) agar favours the isolation of *Salmonella* Enteritidis (de Boer, 1998).

4.3.3 Effect of selective plating media

Four different selective plating media i.e. BGA, BSA, HEA and XLD were used for isolation of *Salmonella* in seafood. The role of selective plating media was observed to be variable for the recovery of *Salmonella* from seafood. XLD was found to be the most efficient media for isolation of *Salmonella*, since, 45% of *Salmonella* strains were isolated on XLD medium, the other two differential media, viz., HEA and BSA media succeeded in the isolation of 25% and 21% *Salmonella*



XLD = Xylose Lysine Desoxycholate Agar
HEA = Hektoen Enteric Agar
BSA = Bismuth Sulphite Agar
BGA = Brilliant Green Agar

Fig. 4.3 Role of selective plating media on the recovery of *Salmonella* from seafood.

isolates from seafood samples, respectively. BGA was found to be the least effective for isolation of *Salmonella* in seafood (Fig. 4.3). Though, BSA was found to be less efficient in the isolation of *Salmonella* strains compared to XLD and HEA, but was observed to be the most efficient for isolation of *Salmonella* from diverse seafood groups. *Salmonella* strains were isolated from fish, shrimp, clam, crab, mussel, lobster, oyster, octopus and cuttlefish samples on BSA media. The XLD media was also found to be comparable to BSA in this case. Except mussel samples, it could isolate *Salmonella* from fish, shrimp, clam, crab, mussel, oyster, lobster, squid, octopus and cuttlefish samples. HEA was capable for isolation of *Salmonella* from fish, shrimp, clam and mussel, but, BGA isolated *Salmonella* from fish, shrimp and clam samples only.

Seventeen Canadian Federal, Provincial and Public Health Laboratories took part in a comparative/collaborative study that evaluated a variety of commercial media including Brilliant Green Sulpha Agar, BSA, HEA, XLD, EF-18 Agar and Rambach Agar for recovery of *Salmonella* cultures in artificially inoculated food samples. Quite contrary to the results in the present study, the qualitative testing of the six media during the comparative/collaborative study of various methods showed that EF-18 Agar recovered the greatest number of isolates and HEA ranked second, with the other agars being comparable in their recovery of *Salmonella* spp. (Warburton et al., 1994). Isolation of *Salmonella* from seafood was reported by Kumar et al. (2003) and showed that BSA and HEA were equally effective as selective plating media for fish samples and HEA was found to be more efficient for isolation of *Salmonella* in clam samples.

4.4 Biotyping of *Salmonella* isolates

4.4.1 Utilization of sugars, sugar derivatives and common carbon sources

4.4.1.1 Utilization of sugars

Ten most dominant *Salmonella* serovars viz., *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Irumu, and *Salmonella* Ohio isolated from seafood were biotyped by sugar utilization (biotype-I) pattern. The results are presented in Table 4.5. There was 100% utilization (fermentation with acid production) of arabinose, dulcitol, glucose, maltose, mannose, mannitol, raffinose, trehalose and xylose, by all the test serovars groups. However, none of the strains could utilize cellobiose, lactose and sucrose.

Biochemical profiling is a fast and accurate method for the identification of bacteria when it is performed with a set of reactions, but it is commonly disregarded as a means of grouping *Salmonella* isolates because most serovars within a given subgroup display a very similar biochemical reaction profile. As per Bergey's Manual of Systematic Bacteriology (Brenner and Farmer III, 2005), utilization of arabinose by *Salmonella* Typhi and *Salmonella* Paratyphi A was 100% and 2%, respectively. Dulcitol, utilization was 90% for *Salmonella* Paratyphi A and utilization was not reported for *Salmonella* Typhi. Similarly, distinct utilization patterns of glucose, maltose, mannose, mannitol, raffinose, trehalose and xylose by *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Choleraesuis, *Salmonella* Gallinarum, and *Salmonella* Pullorum were reported in Bergey's Manual of Systematic Bacteriology (Brenner and Farmer III, 2005).

Table 4.5 Sugar utilization pattern by *Salmonella* serovars

Salmonella Serotypes	No. of isolates	Arabinose	Cellobios	Dulcitol	Glucose	Lactose	Maltose	Mannose	Mannitol	Sucrose	Raffinose	Trehalose	Xylose	Biotype
S. Weltevreden	22	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Rissen	21	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Typhimurium	18	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Derby	17	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Bareilly	16	100	0	100	100	0	100	100	100	0	100	100	100	S1
S Braenderup	15	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Lindenburg	14	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Mbandaka	13	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Irumu	11	100	0	100	100	0	100	100	100	0	100	100	100	S1
S. Ohio	9	100	0	100	100	0	100	100	100	0	100	100	100	S1

All other serovars are reported to have similar sugar utilization pattern. Similar observation was recorded during this study that sugar biotyping pattern did not provide much variation among intra and inter *Salmonella* serovars isolated from seafood. However, a few previous studies demonstrated that serotype Typhimurium variants could be categorized by phage types and further differentiated by means of biotyping methods (Madigan et al., 1996). Until now, biochemical profiling has relied on a set of biochemical tests for which a given serotype or isolate can yield either a positive or a negative result after a given incubation time. This approach, although proven to be very valuable, does not take into account the rate or the kinetics with which the biochemical reaction takes place. Most recently, de la Torre (2005) used the biochemical kinetic data to determine the strain relatedness among *Salmonella enterica* subsp. *enterica* isolates, which may change the future biotyping assays for the determination of strain relatedness among *Salmonella* serovars. A study on biotyping, bacteriocin typing and drug resistogram of *Salmonella* Paratyphi B isolates from animals, their products and environment in India was reported by Agarwal et al. (2003). *Salmonella* Mbandaka isolates in animals and their feed in Poland have been biotyped based on utilization of glucose, mannitol, sorbitol, rhamnose, sucrose, melibiose, amygdaline and arabinose, however, limitation of *Salmonella* Mbandaka biotyping in epidemiological investigation was reported (Hoszowski and Wasyl, 2001).

4.4.1.2 Utilization of sugar derivatives and other carbon compounds

Utilization of sugar derivatives and other carbon compounds are presented in Table 4.6. There was 100 % utilization of citrate and tartrate by the 10 groups of *Salmonella* serovars. Result showed that malonate and salicin were not utilized by

any of the serovars tested. In case of inositol and sorbitol, some variations were observed in the utilization by the serovars. A total of four biotype patterns, C1, C2, C3 and C4 were obtained for sugar derivatives and other carbon sources such as citrate, malonate, sorbitol and tartrate. *Salmonella* Braenderup and *Salmonella* Mbandaka strains primarily showed more differentiation based on inositol and sorbitol utilization pattern whereas, remaining serovars produced similar biotypes (C1).

Utilization of inositol was found to be variable in *Salmonella* Weltevreden, *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Braenderup and *Salmonella* Mbandaka in this study. Gutnick et al. (1969) has showed that *Salmonella* Typhimurium utilized more than 100 compounds as a sole carbon and nitrogen source. Though, biotyping does not provide much discrimination among intraserovars for most of *Salmonella* serovars but has been often used for typing of *Salmonella* Typhimurium, *Salmonella* Derby, and *Salmonella* Mbandaka isolates. Based on fermentation of inositol, different variants of *Salmonella* have been previously reported and temperature dependent utilization of meso-inositol was used as a biotyping marker for *Salmonella* Typhimurium (Old, 1972). Guinee et al. (1972) described the utilization on rhamnose, xylose, trehalose, inositol, and tartrate by *Salmonella* spp. The ability to ferment meso-inositol by *Salmonella* Typhimurium from natural sources differentiated *Salmonella* Typhimurium strains into 21 different biotypes. A modified, two-tier system for biotyping *Salmonella* Typhimurium was developed and strains were allocated to primary types (1-32) by their reactions in five primary tests with Bitter's xylose medium, meso-inositol, L-rhamnose, d-tartrate and m-tartrate tests (Duguid et al., 1975). Strains of *Salmonella*

Typhimurium (n = 175) isolated from animals and birds in northern Japan were differentiated into 5 biovars (1, 2, 7, 10, and untypeable) by 6 kinds of fermentation tests (inositol, xylose, rhamnose, xylose, Stern's glycerol, and trehalose) of Brandis' scheme (Ishiguro et al., 1981). *Salmonella* Typhi isolated from Kolkata were biotyped and based on utilization of tartrate, 2 biotypes of *Salmonella* Paratyphi B were detected (Agarwal et al., 2003). Quite contrary to previous reports, present study did not differentiate *Salmonella* serovars based on utilization of tartrate (Table 4.6).

Table 4.6 Utilization sugar derivatives and other carbon sources pattern by *Salmonella* serovars

Salmonella Serotypes	No. of isolates	Citrate	Inositol	Malonate	Sorbitol	Salicin	Tartrate	Biotype
S. Weltevreden	22	100	90	0	100	0	100	C1[a], C2[b]
S. Rissen	21	100	100	0	100	0	100	C1
S. Typhimurium	18	100	83	0	100	0	100	C1, C2
S. Derby	17	100	94	0	100	0	100	C1, C2
S. Bareilly	16	100	100	0	100	0	100	C1
S Braenderup	15	100	85	0	80	0	100	C1, C2, C3[c], C4[d]
S. Lindenburg	14	100	100	0	100	0	100	C1
S. Mbandaka	13	100	87	0	75	0	100	C1,C2, C3, C4
S. Irumu	11	100	100	0	100	0	100	C1
S. Ohio	9	100	100	0	100	0	100	C1, C2

C1[a] citrate +, inositol +, malonate - , sorbitol + , salicin - , tartrate +
C2[b] citrate + , inositol - , malonate - , sorbitol + , salicin - , tartrate +
C3[c] citrate + , inositol + , malonate - , sorbitol - , salicin - , tartrate +
C4[d] citrate + , inositol - , malonate - , sorbitol - , salicin - , tartrate +

4.4.2 Utilization of amino acids

Predominant *Salmonella* serovars isolated in this study were further biotyped on the basis of utilization of different amino acids. The results are presented in Table 4.7. All the serovars tested utilized lysine, but none of the strains could metabolize phenylalanine and valine. There were wide variations in the utilization of arginine and orinithine. Results revealed the presence of four amino acid biotypes (A1, A2, A3, and A4) in *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, *Salmonella* Bareilly, *Salmonella* Lindenburg, *Salmonella* Mbandaka *Salmonella* Irumu, and 2 biotypes (A1 and A3) were obtained in *Salmonella* Derby and *Salmonella* Braenderup strains. (Table 4.7).

Table 4.7 Amino Acid utilization pattern by *Salmonella* serovars

Salmonella Serotypes	No. of isolates	Arginine	Lysine	Orinithine	Phenylalanine	Valine	Biotype
S. Weltevreden	22	69	100	75	0	0	A1, A2, A3, A4
S. Rissen	21	76	100	87	0	0	A1, A2, A3, A4
S. Typhimurium	18	88	100	92	0	0	A1, A2, A3, A4
S. Derby	17	100	100	96	0	0	A1, A3
S. Bareilly	16	87	100	89	0	0	A1, A2, A3, A4
S Braenderup	15	100	100	83	0	0	A1, A3
S. Lindenburg	14	92	100	73	0	0	A1, A2, A3, A4
S. Mbandaka	13	84	100	91	0	0	A1, A2, A3, A4
S. Irumu	11	63	100	74	0	0	A1, A2, A3, A4
S. Ohio	9	100	100	82	0	0	A1, A3

A1; Arginine +, Lysine +, Orinithine +, Phenylalanine -, Valine -
A2; Arginine -, Lysine +, Orinithine +, Phenylalanine -, Valine -
A3; Arginine +, Lysine +, Orinithine -, Phenylalanine -, Valine -
A4; Arginine -, Lysine +, Orinithine -, Phenylalanine -, Valine -

Utilization of amino acids such as arginine, aspartic acid, cystine and proline as a sole source of nitrogen by *Salmonella* Typhimurium has been reported by Gutnick et al. (1969). Present study highlighted the biotypes of *Salmonella* serovars based on amino acid utilization patterns and revealed that no intra-serovar *Salmonella* strains variations were observed for predominant *Salmonella* serovars from seafood. The results showed four biotypes were uniformly prevalent in all *Salmonella* serovars except *Salmonella* Derby and *Salmonella* Mbandaka. A study by Kiritani (1974) demonstrated that mutants of *Salmonella* Typhimurium were defective in transporting of branched chain amino acids. Biosynthesis of branched chain amino acids in *Salmonella* Typhimurium was studied by Epelbaum et al. (1998). They have determined the role of acetohydroxy acid synthase (AHAS) isozymes I and II in flux to different branched chain amino acids and showed that AHAS isozyme I provides the flux to valine, leucine, and pantothenate, while, isozyme II had a role in utilization of isoleucine.

4.5 Serotyping of *Salmonella* isolates

All the 256 *Salmonella* isolates were serotyped as described under material and method (3.2.2). The serotyping of *Salmonella* isolates from seafood showed that majority of *Salmonella* serovars were appeared in subspecies I. A total of 25 different serovars were identified in subspecies I and six major serogroups i.e. B, C1, C2, D1, E1 and G were identified in subspecies I. The serogroup C1 was most prevalent and followed by C2, B, E1, G, and D. Ten different serovars were identified in C1 serogroup, 6 in C2 serogroup, 3 in B serogroup, 2 in each E1 and G serogroups. The only serovar of *Salmonella* Typhi has been identified in D1

serogroup. A total of 32 different *Salmonella* serovars were identified from seafood by serotyping. The results are presented in Table 4.8.

Table 4.8 Different serotypes and their antigenic formula

Sl. No.	*Salmonella* Serovar	Number of isolates	Subspecies	Serogroup	Antigenic formula	Rank
1.	*S.* Typhimurium	18	I	B	1,4,5,12:i:1,2	3
2.	*S.* Derby	17	I	B	1,4,12:fg:-	4
3.	*S.* Brancaster	6	I	B	1, 4, 12, 27:z_{29}	13
4.	*S.* Rissen	21	I	C_1	6,7:fg:-	2
5.	*S.* Bareilly	16	I	C_1	6,7,14:y:1,5	5
6.	*S* Braenderup	15	I	C_1	6,7,14:e,h:e,n,z_{15}	6
7.	*S.* Mbandaka	13	I	C_1	6,7:z_{10}:e,n,z_{15}	8
8.	*S.* Irumu	11	I	C_1	6,7:l,v:1,5	9
9.	*S.* Ohio	9	I	C_1	6,7:b:l,w	10
10.	*S.* Oslo	8	I	C_1	6,7,14:a:e,n,x	11
11.	*S.* Virchow	8	I	C_1	6,7:r:1,2	11
12.	*S.* Othmarschen	6	I	C_1	6,7,14:g,m:-	13
13.	*S.* Riggil	6	I	C_1	6,7:g,t:-	13
14.	*S.* Georgia	4	I	C_1	6,7:b:e,n,z_{15}	14
15.	*S.* Lindenburg	14	I	C_2	6,8;i:1,2	7
16.	*S.* Takoradi	8	I	C_2	6,8:i:1,5	11
17.	*S* Emek	7	I	C_2	8,20:g,m,s: -	12
18.	*S.* Newport	4	I	C_2	6,8,20:e,h:1,2	14
19.	S. Atakpame	2	I	C_2	8,20:e,h:1,7	16
20.	*S.* Kottbus	2	I	C_2	6,8:e,h:1,5	16
21.	*S.* Typhi	7	I	D_1	9,12, [Vi]:d:-	12
22.	*S.* Weltevreden	22	I	E_1	3,10:r:z_6	1
23.	*S.* Nchanga	7	I	E_1	3,10:l,v:1,2	12
24.	*S.* Washington	6	I	G	13,22:mt:-	13
25.	*S.* Worthington	3	I	G	1,13,23:z:l,w	15
26.	*Salmonella* II	4	II	E_1	3,10:lv:z_6	14
27.	*Salmonella* II	4	II	X	47:enx, z_{15}:1,6	14
28.	*Salmonella* IIIa	3	IIIa	J	17:-:-	15
29.	*Salmonella* IIIb	1	IIIb	P	38:z:-	17
30.	*Salmonella* IIIb	1	IIIb	60	60:r:z	17
31.	*Salmonella* VI	2	VI	F	11:b:1,7	16
32.	*Salmonella* VI	1	VI	W	45:a:enx	17

(Ref; Anon. 2001. *Salmonella* antigenic scheme, Update of the Kaufmann-White Scheme. The Difco Manual, Section V, p675-787.)

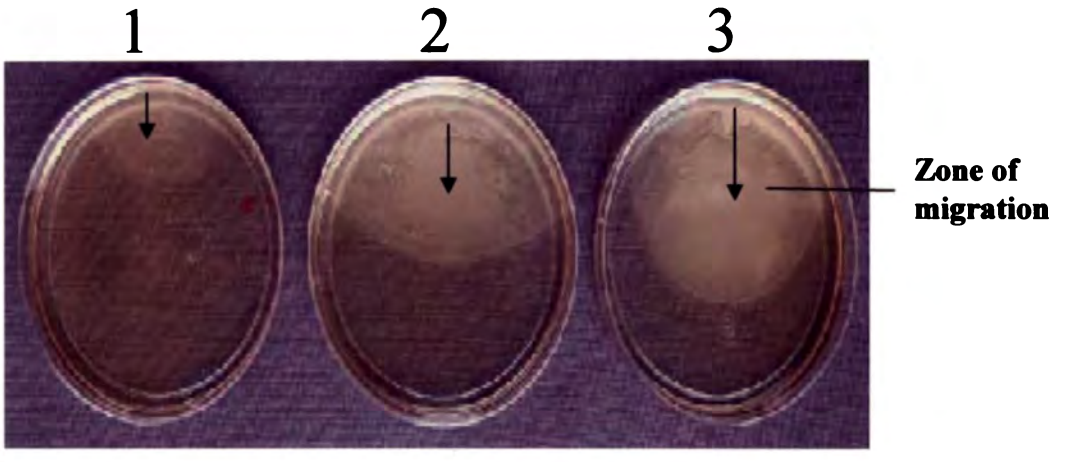


Fig. 4.4 Phase Reversal of *Salmonella* flagellar (H) antigens.

The successful completion of serotyping is dependent on phenomenon of the phase variation changes in the test organism. The cultures were subjected to the process of phase reversal in a Motility GI medium by immobilizing the flagellated (H) phase-I antigen. The cultures migrated to opposite side of the inoculation, with well expressed phase-II antigens were tested for flagellar (H) phase–II antigens (Fig 4.4). The phase variations are actively associated with motile cultures only and this phenomenon in *Salmonella* was first described by Andrewes (1922). Later on, Edwards et al. (1947) reported the phase variations in cultures of *Arizonae.* There were some irreversible phase variations also reported to be present in a few *Salmonella* serovars such as *Salmonella* Infantis, *Salmonella* Senftenberg and *Salmonella* Havana (Edwards and Ewing, 1972).

The antigenic formula has presented as somatic antigen: flagellar phase I: flagellar phase II. *Salmonella* serotyping results also showed the presence of rare *Salmonella* subspecies II, IIIa, IIIb and VI. The serogroup identified were E1 and X, J, P and 60, F and W for II, IIIa, IIIb, and VI, respectively. Less than 8% of total isolates were identified under these serogroups. Twelve *Salmonella* isolates could not be serotyped as most of them were rough in nature. A large number of *Salmonella* serovars namely, *Salmonella* Atakpame, *Salmonella* Kottbus, *Salmonella* Washington, *Salmonella* Worthington, *Salmonella* Irumu, *Salmonella* Riggil, *Salmonella* Rissen, *Salmonella* Takoradi, *Salmonella* II (3,10:lv:z_6), *Salmonella* II (47:enx, z_{15}:1,6), *Salmonella* IIIa (17:-:-), *Salmonella* IIIb (38:z:-), *Salmonella* IIIb (60:r:z), *Salmonella* VI (11:b:1,7) and *Salmonella* VI (45:a:enx) were identified for first time in India, from seafood.

Salmonella serovars were identified from seafood, in India by many authors and highlighted the presence of a large number of *Salmonella* serovars in seafood. Nambiar and Iyer (1991) found 16 different *Salmonella* serovars in fresh and frozen fish from Cochin retail trade. Similarly, Iyer and Shrivastava (1989b) reported 34 different *Salmonella* serovars in fishery products and froglegs. The study also revealed that serogroup C1 and *Salmonella* Weltevreden serovar were most prevalent in seafood. Shabarinath et al. (2007) showed the presence of mainly *Salmonella* Weltevreden from seafood in Mangalore. The findings in the present investigation are more or less in agreement with these reports. However, the number of *Salmonella* serovars involved in this study is quite large (256 numbers). Also, present investigation highlighted the presence of large number of serovars in *Salmonella* subspecies II, IIIa, IIIb and VI in seafood, which indicated the contamination in seafood from cold-blooded animals and environments.

4.6 Antibiotic resistance of *Salmonella* isolates

Salmonella serovars isolated from seafood were studied for antibiotic resistance pattern against common antibacterial drugs used in human and animal therapy. All the 256 *Salmonella* isolates were tested against the 12 antibiotics, listed in Table 3.10 (Materials and Methods) and results are presented in Table 4.9.

Results showed that all *Salmonella* serovars were resistant to erythromycin, while, all the serovars were susceptible to ampicillin, ciprofloxacin, chloramphenicol, gentamicin, and kanamycin. *Salmonella* Lindenburg, *Salmonella* Rissen, *Salmonella* Takoradi and *Salmonella* Typhi isolates were resistant towards nalidixic acid. Sixteen out of 29 *Salmonella* serovars were resistant to sulphamethizol. Resistance against oxytetracycline was observed in *Salmonella*

Othmarschen (80 %), *Salmonella* Lindenburg (71 %), *Salmonella* Derby (18 %), *Salmonella* Typhimurium (70 %) and *Salmonella* Mbandaka (50 %). Similarly, a large numbers of *Salmonella* serovars were resistant towards carbenicillin. Present study also determined the multi-drug resistance (MDR) in *Salmonella* serovars of seafood origin. The results showed MDR in 49.3 % , 31.8 % , 10 %, 0.4 % of *Salmonella* isolates towards erythromycin and sulphamethizol (2 drugs), erythromycin, sulphamethizol and carbenicillin (3 drugs), erythromycin, sulphamethizol, carbenicillin and oxytetracycline (4 drugs), erythromycin, sulphamethizol, carbenicillin, oxytetracycline and nalidixic acid (5 drugs), respectively (Table 4.10). Among *Salmonella* serovars, *Salmonella* Atakpame, *Salmonella* Takoradi and *Salmonella* Typhimurium were resistant against five antimicrobial drugs, whereas, *Salmonella* Derby, *Salmonella* Lindenburg, *Salmonella* Rissen, and *Salmonella* Typhi were resistant against four antimicrobials.

The widespread use of antibacterial substances for human and animal therapy, as well as use in aquaculture practices had resulted into development of resistance by bacteria towards antimicrobial substances. Most antimicrobial resistant *Salmonella* infections were acquired from eating contaminated foods of animal origin (Angulo et al., 2000). The antimicrobial resistance in *Salmonella* serovars isolated from different food animals and human sources has been well documented (Mirza et al., 2000; Piddock, 2002). This study has showed over all a moderate level of antibiotics resistance in *Salmonella*, isolated from seafood. The MDR in *Salmonella* serovars towards ampicillin, amoxicillin, and chloramphenicol was quite contrary to results reported elsewhere (Gebreyes et al., 2000).

Table 4.9 Antibiogram *of Salmonella* serovars (n =256)

Sl. no.	Serovars	No of isolates	Percentage of isolate resistance to antibiotics[a]											
			A	N	Cf	C	Sm	Cp	E	G	S	K	O	Cb
1.	S. Atakpame	2	0	0	0	0	100	0	100	0	50	0	100	50
2.	*S.* Bareilly	16	0	0	0	0	0	0	100	0	0	0	0	0
3.	*S* Braenderup	15	0	0	0	0	35	0	100	0	0	0	0	0
4.	*S.* Brancaster	6	0	0	0	0	0	0	100	0	0	0	0	0
5.	*S.* Derby	17	0	0	0	0	75	0	100	0	0	0	18	56
6.	*S* Emek	7	0	0	0	0	0	0	100	0	0	0	16	100
7.	*S.* Irumu	11	0	0	0	0	25	0	100	0	0	0	12	0
8.	*S.* Georgia	4	0	0	0	0	0	0	100	0	0	0	0	0
9.	*S.* Kottbus	2	0	0	0	0	0	0	100	0	0	0	0	100
10.	*S.* Lindenburg	14	0	85	0	0	100	0	100	0	0	0	0	71
11.	*S.* Mbandaka	13	0	0	0	0	83	0	100	0	0	0	50	100
12.	*S.* Nchanga	7	0	0	0	0	14	0	100	0	0	0	14	100
13.	*S.* Newport	4	0	0	0	0	0	0	100	0	0	0	0	0
14.	*S.* Ohio	9	0	0	0	0	22	0	100	0	0	0	0	11
15.	*S.* Oslo	8	0	0	0	0	0	0	100	0	0	0	0	37
16.	*S.* Othmarschen	6	0	0	0	0	20	0	100	0	0	0	80	100

[a]A, Ampicillin; N, Nalidixic acid; Cf, Ciprofloxacin; C, Chloramphenicol;G,Gentamicin; Sm, Sulphamethizol; Cp,Cephalexin; E, Erythromycin; S, Streptomycin; K, Kanamycin; O, Oxytetracycline; Cb,Carbenicillin

(Table 4.9 continued next page)

Table 4.9 Antibiogram of *Salmonella* serovars (n =256) (continued from previous page)

Sl. no.	Serovars	No of isolates	Percentage of isolate resistance to antibiotics[a]											
			A	N	Cf	C	Sm	Cp	E	G	S	K	O	Cb
17.	*S.* Rissen	21	0	71	0	0	90	0	100	0	0	0	0	57
18.	*S.* Riggil	6	0	0	0	0	80	0	100	0	0	0	0	60
19.	*S.* Takoradi	8	0	42	0	0	71	0	100	0	0	0	14	28
20.	*S.* Typhi	7	0	16	0	0	66	0	100	0	0	0	33	0
21.	*S.* Typhimurium	18	0	0	0	0	88	0	100	0	17	0	70	76
22.	*S.* Virchow	8	0	0	0	0	0	0	100	0	0	0	0	0
23.	*S.* Washington	6	0	0	0	0	0	0	100	0	0	0	0	0
24.	*S.* Weltevreden	22	0	0	0	0	81	0	100	0	0	0	0	22
25.	*S.* Worthington	3	0	0	0	0	0	0	100	0	0	0	0	0
26.	*Salmonella* II	8	0	0	0	0	75	0	100	0	0	0	0	75
27.	*Salmonella* IIIa	3	0	0	0	0	85	0	100	0	0	0	0	42
28.	*Salmonella* IIIb	2	0	0	0	0	50	0	100	0	0	0	50	50
29.	*Salmonella* VI	3	0	0	0	0	28	0	100	0	0	0	0	14

[a]A, Ampicillin; N, Nalidixic acid; Cf, Ciprofloxacin; C, Chloramphenicol; G,Gentamicin; Sm, Sulphamethizol; Cp,Cephalexin; E, Erythromycin; S, Streptomycin; K, Kanamycin; O, Oxytetracycline; Cb,Carbenicillin

Table 4.10 Multi-drug resistance (MDR) Pattern of *Salmonella* serovars (n = 256)

Sl. No.	MDR* Pattern	Resistance (%)
1.	E Sm	113 (49.3)
2.	E Sm Cb	73 (31.8)
3.	E Sm Cb O	23 (10.0)
4.	E Sm Cb O N	1(0.4)
5.	E Sm Cb O N C	Nil
6.	E Sm Cb O N C G	Nil
7.	E Sm Cb O N C G S	Nil
8.	E Sm Cb O N C G S Cf	Nil
9.	E Sm Cb O N C G S Cf A	Nil
10.	E Sm Cb O N C G S Cf A Cp	Nil
11.	E Sm Cb O N C G S Cf A Cp K	Nil

* MDR; resistance to two or more than two antimicrobial tested
E, Erythromycin; Sm, Sulphamethizol; Cb, Carbenicillin; O, Oxytetracycline; N, Nalidixic acid; C, Chloramphenicol; G,Gentamicin; S, Streptomycin; Cf, Ciprofloxacin; A, Ampicillin; Cp,Cephalexin; K, Kanamycin;

They found almost all *Salmonella* serovars to be resistant towards ampicillin, and chloramphenicol. High incidences of fluoroquinolone resistance in *Salmonella* serovars was reported from cattle in Germany and humans and farm animals in England and Wales (Frost et al., 1996; Malorny et al., 1999). In the present study, only *Salmonella* Lindenburg (85%), *Salmonella* Rissen (71%) and *Salmonella* Takoradi (42%) were resistant to nalidixic acid, however, all other *Salmonella* serovars were sensitive to both nalidixic acid and ciprofloxacin (quinolones). Nalidixic acid resistance was observed in *Salmonella* isolates originated from Tilapia and catfish in Taiwan and Thailand (Zhao et al., 2003). *Salmonella* Typhimurium isolated from man and animal in North-east India showed the resistance towards nitrofurantoin, tetracycline and chloramphenical

(Murugkar et al., 2005). A detailed study by Piddock (2002) showed that quinolones-resistant *Salmonella* can also be resistant to other antibacterial agents including chloramphenicol and tetracycline. In United States resistance to tetracycline in *Salmonella* spp. increased from 9 to 24 % between 1980 to 1990 (Lee et al., 1994).

4.7 Molecular typing of *Salmonella* isolates from seafood

4.7.1 Plasmid profiles of *Salmonella* isolates

Salmonella serovars were characterized for presence of small and large plasmids by Alkaline lysis (Sambrook and Russel, 2000) and Kado and Liu (1981) methods. The results are presented in Fig. 4.5, which shows the plasmid profile of 10 *Salmonella* serovars. Table 4.11 gives the details of *Salmonella* isolates, size, and number of plasmids isolated and their profile groups. Plasmids were isolated from *Salmonella* serovars such as *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Braenderup, Lindenburg, and *Salmonella* Mbandaka isolates (Fig. 4.5). Large Megadalton sized plasmids were isolated in *Salmonella* Typhimurium and *Salmonella* Derby isolates. A total of nine plasmid profiles were obtained from different *Salmonella* serovars associated with seafood. *Salmonella* serovars such as *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Bareilly, *Salmonella* Irumu, *Salmonella* Ohio, *Salmonella* Oslo and *Salmonella* Typhi did not harbour plasmids and serovars without plasmid were placed under profile I. *Salmonella* Typhimurium showed 3 plasmid profiles (I, IIa, and IIIa) and harboured both small and large plasmids. *Salmonella* Derby and *Salmonella* Braenderup exhibited 3 plasmids profiles, however, each

serovar harboured different plasmids of different sizes. *Salmonella* Braenderup harboured five plasmids of 1.5, 2.1, 3.5, 3.8, 4.1, and 9 kb in sizes.

Table 4.11 Plasmid Profile of *Salmonella* serovars

Salmonella serovars	Number of isolates	Plasmid size (s) (kb)	Plasmid Profile
S. Weltevreden	22	Nil	I
S. Rissen	21	Nil	I
S. Typhimurium	9	2.1 , LMD*	II a
S. Typhimurium	6	2.1, 3.8 , LMD*	III a
S. Typhimurium	3	Nil	I
S. Derby	5	LMD* (2 no.)	II b
S. Derby	8	1.9, 2.2	II c
S. Derby	4	Nil	I
S. Bareilly	16	Nil	I
S Braenderup	6	1.9, 2.2, 3, 4.5	IV a
S Braenderup	7	1.9, 2.1,3.8, 4	IV b
S Braenderup	2	1.5, 2.1, 3.5, 3.8, 4.1, 9	V
S. Lindenburg	5	4.1, 7	II b
S. Lindenburg	2	3.4, 4.1, 7	III b
S. Lindenburg	3	2.2, 2.8, 3.4	III c
S. Lindenburg	2	Nil	I
S. Lindenburg	2	1.5, 2.2,3, 4	IV d
S. Mbandaka	8	1.5, 2.2,3, 4	IV d
S. Mbandaka	5	Nil	I
S. Irumu	11	Nil	I
S. Ohio	9	Nil	I
S. Oslo	8	Nil	I
S. Typhi	7	Nil	I

* Large Megadalton Plasmid

Plasmid profile of *Salmonella* Lindenburg isolates was observed to be most diverse in nature as five different plasmid profiles (I, II b, III b, III c, and IV d) were detected and plasmid profile IV d was detected in *Salmonella* Mbandaka isolates (Table 4.11).

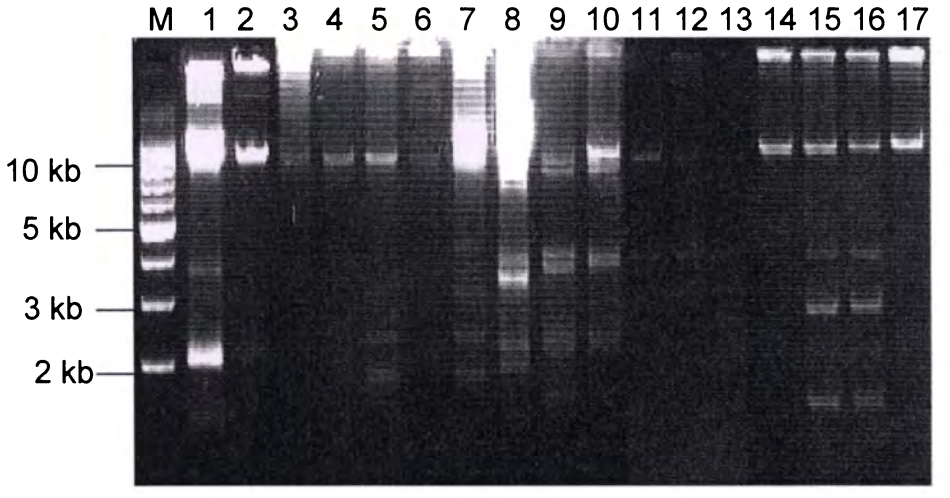


Fig. 4.5 Plasmid profile of *Salmonella* serovars
Lines 1-3; *Salmonella* Typhimurium, 4-7; *Salmonella* Derby, 8-10; *Salmonella* Braenderup, 11-15; *Salmonella* Lindenburg, 16-17; *Salmonella* Mbandaka, M; Mol. wt. marker

Plasmid profile has been used as an invaluable tool for molecular fingerprinting of *Salmonella* isolates from various sources. *Salmonella* Muenster which caused an outbreak of salmonellosis in Canada was differentiated into four major groups based on plasmid profile (Bezanson et al., 1983). In this study, large number of low molecular weight plasmids were found in *Salmonella* Braenderup, *Salmonella* Lindenburg and *Salmonella* Mbandaka. Similar results were reported by Rychlik et al. (2001) in *Salmonella* Enteritidis. They found that there low molecule weight plasmid had a role in retron reverse transcriptase and phase resistance. The presence of large plasmids in *Salmonella* Typhimurium and *Salmonella* Derby isolates is found in the present study. Similar results were reported elsewhere by Whiley et al. (1988); Liebana et al. (2001); and Tsen, (2002). *Salmonella* Enteritidis isolated from poultry and human sources in South Africa were fingerprinted based on plasmid profile and a total of 13 plasmid profile obtained in their study (Mare et al., 2001), whereas, seven plasmid profiles were detected in Brazilian isolates (Fernandes et al., 2003). Distribution and function of *Salmonella enterica* plasmid has been reported by Rychilk et al. (2006) and demonstrated the presence of two to more than 200 kb plasmids in *Salmonella enterica* species. Present study revealed that six *Salmonella* serovars such as *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Irumu, *Salmonella* Ohio, *Salmonella* Oslo and *Salmonella* Typhi did not harbour any plasmid. This observation pointed out the limitation of plasmid profile based molecular fingerprinting of *Salmonella* serovars. Present results were in agreement with the earlier studies, which reported the lower level of

discrimination power in molecular fingerprinting of *Salmonella* serovars by plasmid profile analysis (Crichton et al., 1996; Liebana et al., 2001).

4.7.2 PCR –ribotyping of *Salmonella* serovars

Four most prevalent *Salmonella* serovars in seafood namely, *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, and *Salmonella* Derby were characterized by PCR-ribotypes pattern.

Table 4.12 Serovar code, year, source, PCR-ribotype, ERIC-PCR and combined Typing profile of *Salmonella* Weltevreden (n = 22)

Sl No.	Serotype Code	Year	Source	PCR-ribotype	ERIC-PCR type	Combined[a] Type
1.	SW 339	2003	Clam	I	6	4
2.	SW 340	2003	Clam	I	1	1
3.	SW 351	2003	Fish	II	2	10
4.	SW 378	2003	Fish	I	4	2
5.	SW 379	2003	Fish	II	11	13
6.	SW 391	2003	Fish	II	11	13
7.	SW 419	2004	Clam	III	13	16
8.	SW 427	2004	Clam	I	13	8
9.	SW 452	2004	Shrimp	I	13	8
10.	SW 453	2004	Shrimp	II	12	14
11.	SW 461	2004	Fish	IV	14	18
12.	SW 477	2004	Fish	I	7	5
13.	SW 511	2005	Clam	II	3	11
14.	SW 512	2005	Clam	I	5	3
15.	SW 567	2005	Clam	III	8	15
16.	SW 571	2005	Clam	II	9	12
17.	SW 572	2005	Mussel	I	10	6
18.	SW 591	2005	Mussel	III	15	17
19.	SW 608	2006	Shrimp	I	15	9
20.	SW 613	2006	Shrimp	I	15	9
21.	SW 619	2006	Clam	I	12	7
22.	SW 627	2006	Clam	IV	16	19

[a] Combination of PCR-ribotype and ERIC- PCR results

A total of 77 *Salmonella* strains comprised of *Salmonella* Weltevreden (n=22 no), *Salmonella* Rissen (n=20), *Salmonella* Typhimurium (n=18), and

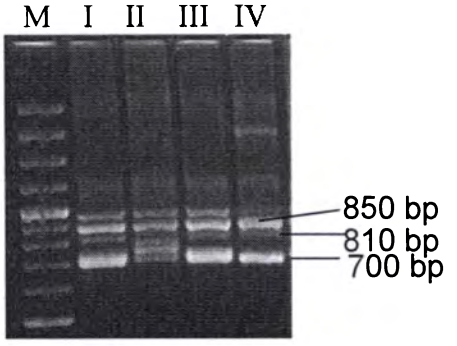


Fig.4.6 Representative PCR-ribotypes of *Salmonella* Weltevreden (n=22)
Lane I; 700 bp, 810 bp, 860 bp, 950 bp, Lane II; 710 bp, 750 bp, 800 bp, 870 bp, 950 bp, Lane III; 700 bp, 820 bp, 870 bp, 960 bp, Lane IV; 700 bp, 810 bp, 850bp.

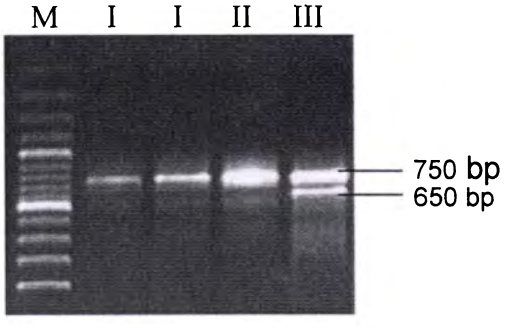


Fig.4.7 Representative PCR-ribotypes of *Salmonella* Rissen (n=20)
Lane I; 750 bp, Lane II; 740 bp, 750bp, Lane III; 650 bp, 750 bp

Salmonella Derby (n=17) were ribotyped. The representative patterns obtained are presented in the Figures 4.6, 4.7, 4.8 and 4.9. *Salmonella* Weltevreden and *Salmonella* Rissen isolates exhibited three to four band patterns ranging from 700 to 1000 bp in both serovars, whereas, 700 to 900 bp ribotype patterns were observed in *Salmonella* Typhimurium and *Salmonella* Derby.

Table 4.13 Serovar code, year, source, PCR-ribotype, ERIC-PCR and combined Typing profile of *Salmonella* Rissen (n = 20)

Sl No.	Serotype Code	Year	Source	PCR-ribotype	ERIC-PCR type	Combined[a] Type
1.	SR 360	2003	Fish	II	6	10
2.	SR 361	2003	Fish	I	5	4
3.	SR 362	2003	Fish	I	5	4
4.	SR 371	2003	Fish	II	9	12
5.	SR 372	2003	Fish	II	8	11
6.	SR 415	2004	Fish	I	7	5
7.	SR 416	2004	Fish	I	11	6
8.	SR 428	2004	Shrimp	III	7	14
9.	SR 429	2004	Shrimp	II	12	13
10.	SR 520	2005	Shrimp	II	12	13
11.	SR 521	2005	Mussel	I	13	7
12.	SR 523	2005	Mussel	I	4	3
13.	SR 548	2005	Lobster	II	4	9
14.	SR 549	2005	Shrimp	III	10	15
15.	SR 562	2005	Mussel	I	2	2
16.	SR 569	2005	Fish	III	14	16
17.	SR 571	2005	Squid	I	1	1
18.	SR 608	2006	Cuttlefish	II	3	8
19.	SR 611	2006	Cuttlefish	I	4	3
20.	SR 612	2006	Cuttlefish	III	15	17

[a] Combination of PCR-ribotype and ERIC- PCR results

There were three ribotype profiles in *Salmonella* Rissen, and four major ribotype patterns were observed in *Salmonella* Derby and *Salmonella* Weltevreden strains (Fig. 4.6 & 4.7). The most varied PCR-ribotype pattern was observed in

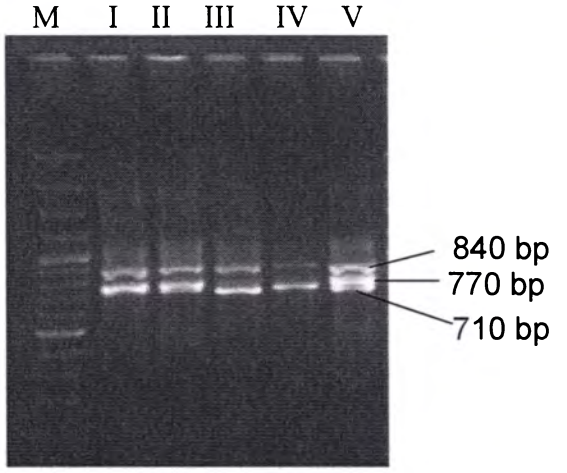


Fig. 4.8 Representative PCR-ribotypes of *Salmonella* Typhimurium isolates (n=18) Lane I; 750 bp , 850 bp, Lane II; 760 bp , 860 bp, Lane III; 710 bp, 840 bp, Lane IV; 750 bp, 900 bp, Lane V; 710 bp, 770 bp, 840 bp.

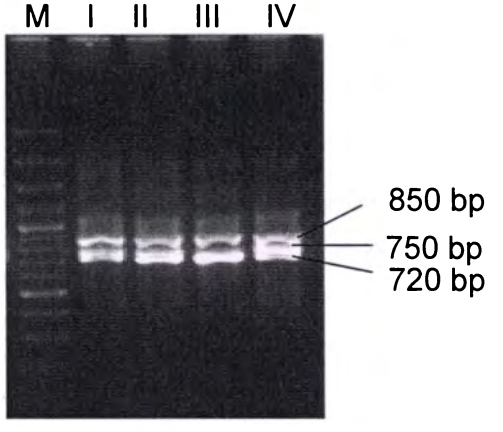


Fig. 4.9 Representative PCR-ribotypes of *Salmonella* Derby (n=17) Lane I; 720 bp, 850 bp , Lane II; 700bp, 830 bp, Lane III; 690 bp, 870 bp, Lane IV; 720 bp, 750 bp, 850 bp.

Salmonella Typhimurium isolates, in which five different banding patterns were recorded (Fig. 4.8). The PCR-ribotyping pattern (I) was detected in 9 out of 20 *Salmonella* Rissen isolates and patterns II and III were observed in seven and four *Salmonella* Rissen isolates, respectively. Similarly, PCR-ribotyping pattern (I) was most predominant *Salmonella* Weltevreden and detected in 11 of 22 isolates, followed by ribotype patterns (II, III, and IV) was observed in 6, 3, and 2 *Salmonella* Weltevreden isolates, respectively (Table 4.12). PCR-ribotype pattern I, II, and II was observed in 9, 7 and 4 *Salmonella* Rissen isolates, respectively (Table 4.13).

Table 4.14 Serovar code, year, source, PCR-ribotype, ERIC-PCR and combined Typing profile of *Salmonella* Typhimurium (n =18)

Sl No.	Serotype Code	Year	Source	PCR-ribotype	ERIC-PCR type	Combined[a] Type
1.	STM 327	2003	Fish	II	4	6
2.	STM 330	2003	Fish	II	6	7
3.	STM 432	2004	Fish	II	8	9
4.	STM 471	2004	Fish	I	3	1
5.	STM 477	2004	Mussel	II	2	4
6.	STM 480	2004	Mussel	II	1	3
7.	STM 488	2004	Mussel	II	1	3
8.	STM 523	2005	Shrimp	V	5	14
9.	STM 525	2005	Shrimp	II	7	8
10.	STM 529	2005	Shrimp	I	9	2
11.	STM 621	2006	Mussel	III	3	11
12.	STM 623	2006	Mussel	III	2	10
13.	STM 648	2006	Fish	IV	1	12
14.	STM 649	2006	Clam	IV	1	12
15.	STM 662	2006	Clam	II	3	5
16.	STM 706	2007	Fish	II	4	6
17.	STM 708	2007	Squid	IV	6	13
18.	STM 712	2007	Fish	II	8	9

[a] Combination of PCR-ribotype and ERIC- PCR results

PCR-ribotype pattern (II) was observed in 10 out of 18 *Salmonella* Typhimurium and 7 out of 17 *Salmonella* Derby isolates. The PCR-ribotype patterns I, III, IV and V were observed in 2, 2, 3, and 1 *Salmonella* Typhimurium strains, respectively (Table 4.14). The ribotype pattern I was detected in six *Salmonella* Derby strains, whereas, pattern III and IV were recorded in 3 and 1 *Salmonella* Derby strains, respectively (Table 4.15).

Table 4.15 Serovar code, year, source, PCR-ribotype, ERIC-PCR and combined Typing profile of *Salmonella* Derby (n = 17)

Sl No.	Serotype Code	Year	Source	PCR-ribotype	ERIC-PCR type	Combined[a] Type
1.	SD 339	2003	Clam	I	1	1
2.	SD 446	2004	Clam	I	1	1
3.	SD 447	2004	Fish	II	5	6
4.	SD 448	2004	Fish	I	1	1
5.	SD 479	2004	Fish	II	1	3
6.	SD 506	2005	Fish	II	2	4
7.	SD 519	2005	Shrimp	III	1	8
8.	SD 527	2005	Mussel	III	5	9
9.	SD 552	2005	Shrimp	II	1	3
10.	SD 553	2005	Shrimp	I	1	1
11.	SD 557	2005	Fish	I	2	2
12.	SD 603	2006	Mussel	III	6	10
13.	SD 611	2006	Shrimp	IV	4	11
14.	SD 612	2006	Shrimp	II	6	7
15.	SD 667	2006	Shrimp	II	3	5
16.	SD 711	2007	Shrimp	II	1	3
17.	SD 715	2007	Oyster	I	1	1

[a] Combination of PCR-ribotype and ERIC- PCR results

Molecular fingerprinting based on PCR-ribotypes provides an additional intra-serovar strain discrimination mechanism that may prove effective for the depiction of *Salmonella* contamination sources in food as well as for the epidemiological investigation of salmonellosis. Molecular fingerprinting

techniques helped in the source tracking of bacterial isolates from different sources in the same region and period, and also established the epidemiological relation among *Salmonella* serovars isolated from contaminated fish, and fish feed factories and feed ingredients (Nesse et al., 2003; Guerin et al., 2004). PCR-ribotyping method and ERIC–PCR have been successfully used in fingerprinting of *Salmonella* serovars to differentiate a strain isolated from diverse sources. Despite the fact that PCR -ribotyping had rather poor discrimination power, the former was found to be useful for establishing a link between strains isolated during 2003-06. PCR- ribotype (pattern I) was most prevalent in *Salmonella* Rissen throughout the study period in all seafoods, whereas, PCR-ribotype (Pattern I & II) was more prevalent in fish samples. Similarly, pattern I was most prevalent in *Salmonella* Weltevreden strains and very few strains were genetically related with each others. PCR-ribotype pattern II was detected in *Salmonella* Typhimurium throughout the study period i.e. 2003 to 2007. Thus, these observations pointed out the genotypic similarities among *Salmonella* Typhimurium strains isolated from different seafood samples. *Salmonella* Typhimurium strains isolated from the fish samples showed similar ribotype pattern (II) except STM 648 strain. *Salmonella* Typhimurium and *Salmonella* Derby were the most common *Salmonellae*, isolated from gastroenteritidis cases in Hong Kong and molecular epidemiological analysis of *Salmonella* Derby infection demonstrated that the two clones were most prevalent in all infections (Ling et al., 2001).

PCR- ribotyping were used for rapid intra-serovar fingerprinting of *Salmonella* Typhimurium, *Salmonella* Typhi, and *Salmonella* Enteritidis

(Altwegg et al., 1989; Baquar et al., 1994; Oslen et al., 1994). Present results were in concurrence with earlier study by Lagatolla et al. (1996) which showed four ribotype patterns in 24 *Salmonella* Derby isolates and eight patterns in 28 *Salmonella* Typhimurium isolates. Similar study on molecular typing of *Salmonella* Typhimurium and *Salmonella* Derby isolated from animal origin revealed the presence of two banding profiles in both *Salmonella* Typhimurium and *Salmonella* Derby isolates (del Cerro et al., 2002) and a manual ribotyping of *Salmonella* Enteritidis strains associated in salmonellosis outbreak in 1998 and 1999 in Canada provinces exhibited 14 unique ribotype patterns (Clark et al., 2003).

4.7.3 ERIC-PCR profile of *Salmonella* serovars

ERIC-PCR profiling of *Salmonella* Weltevreden (n=22 no), *Salmonella* Rissen (n=20) , *Salmonella* Typhimurium (n=18), and *Salmonella* Derby (n=17) was performed. The details of the primers used and the reaction conditions for ERIC-PCR is given in Table 4.16. The profiles were UPGMA cluster analysed and results are presented in figures 4.10, 4.11, 4.12 and 4.13.

Table 4.16 Primers and reactions conditions for Ribotyping and ERIC-PCR

Sl. No.	Primer Sequence	Annealing Temperature (°C)	No. of Cycle	Product size (bp)	Reference
1.	PCR ribotyping TTGTACACACCGCCCGTCA GGTACTTAGATGTTTCAGTTC	55	35	700bp - 1000bp	Kostman et al., 1992
2.	ERIC PCR ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGGG	51	35	200bp - 2000	Versalovic et al., 1991

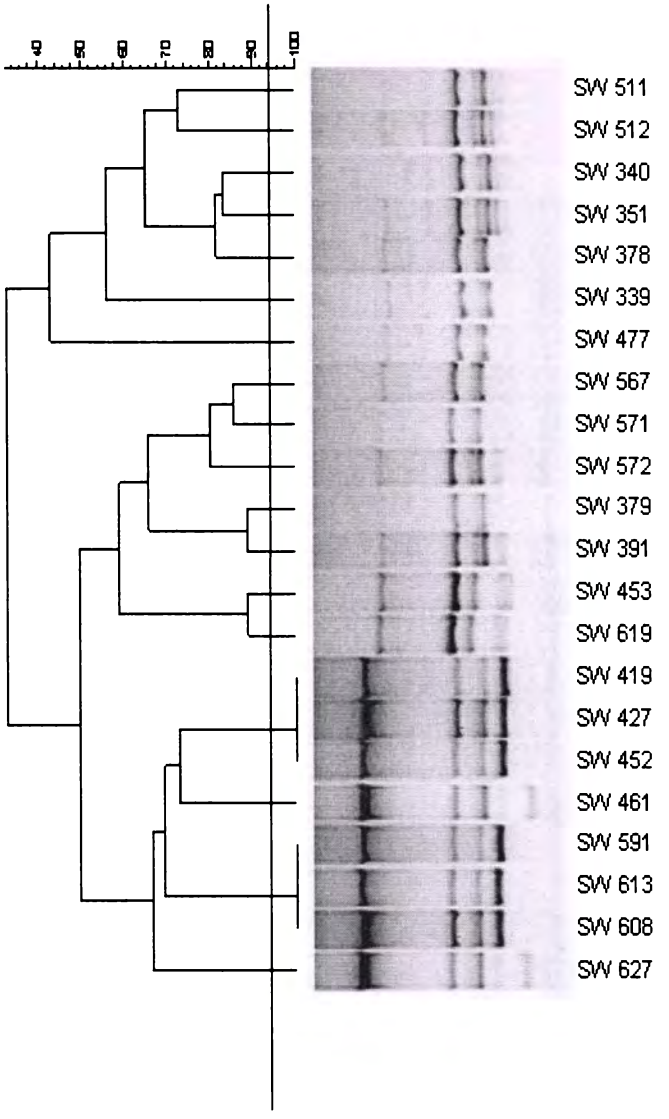


Fig.4.10 Dendrogram exhibiting the genetic relatedness among *Salmonella* Weltevreden (n = 22) isolated from seafood sources. The percentage of similarities among strains was determined using Dice coefficient and the clustering was performed by UPGMA.

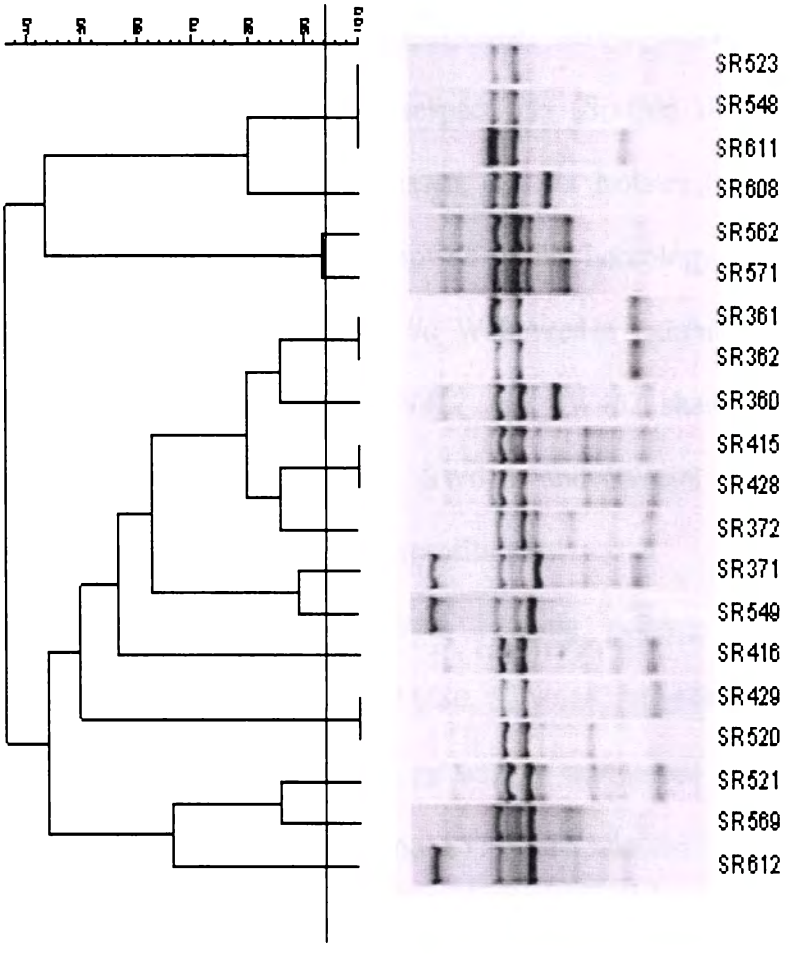


Fig. 4.11 Dendrogram exhibiting the genetic relatedness of *Salmonella* Rissen (n = 20) isolated from seafood sources. The percentage of similarities among strains was determined using Dice coefficient and the clustering was performed by UPGMA.

The level of similarity used for defining a type was set a 95 %. The minimum Dice coefficient value for ERIC-PCR was observed at 32.87 % and 36.21% for *Salmonella* Weltevreden and *Salmonella* Rissen, respectively, whereas, 44.5% and 47.5 % Dice coefficient values were obtained for *Salmonella* Typhimurium and *Salmonella* Derby, respectively. Sixteen different banding profile was observed for *Salmonella* Rissen, and six isolates (SR361, SR362), (SR429, SR520), and (SR415, SR428) showed similar homology (100 %) with in the pair. Cluster analysis of *Salmonella* Weltevreden exhibited 16 different banding patterns. Isolates SW419, SW427 and SW452 shared the common profile (profile 13). Similarly, SW591, SW613, and SW608 isolates showed 100% genetic homology with each other (profile 15).

The analysis showed 9 different banding patterns in *Salmonella* Typhimurium isolates. Four strains (STM480, STM648, STM488, and STM649) showed > 95 % similar genetic homology with in the cluster pairs. Similarly, other isolates STM 471, STM 661 and STM 662 showed identical genetic homology (Fig. 4.12). Less genetic variations were observed for 17 *Salmonella* Derby isolates identified from seafood. A total of six unique patterns were detected by ERIC-PCR with UPGMA cluster analysis. Results highlighted that the nine *Salmonella* Derby isolates namely SD339, SD446, SD448, SD479, SD519, SD552, SD553, SD711 and SD715 had > 95 % similar genetic homology, though, they were isolated in different periods and sources (Fig. 4.13). Apart from the dendrogram analysis the results of ERIC-PCR profiles for *Salmonella* Typhimurium and *Salmonella* Derby are also presented in Table 4.15

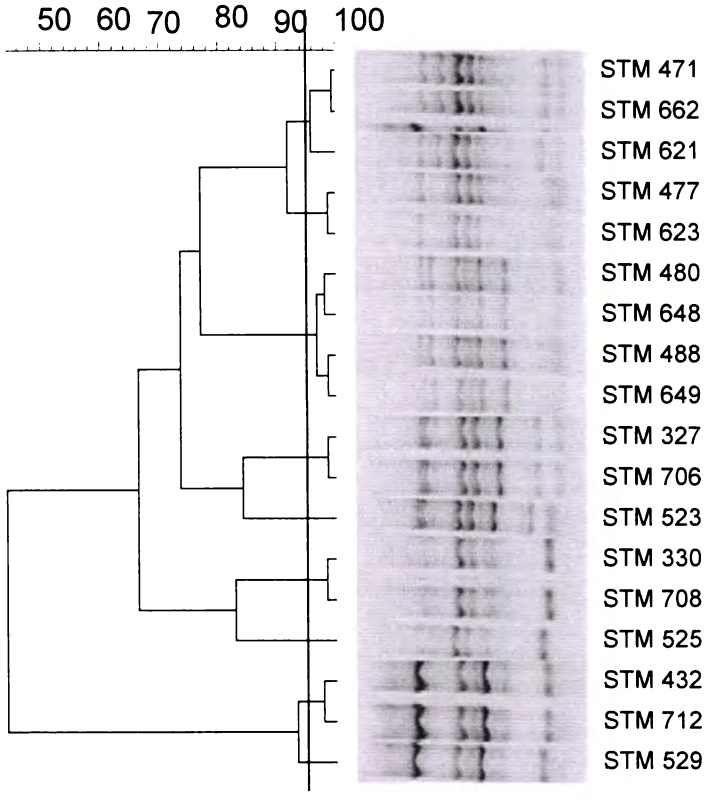


Fig.4.12 Dendrogram exhibiting the genetic relatedness of *Salmonella* Typhimurium (n = 18) isolated from seafood sources. The percentage of similarities among strains was determined using Dice coefficient and the clustering was performed by UPGMA.

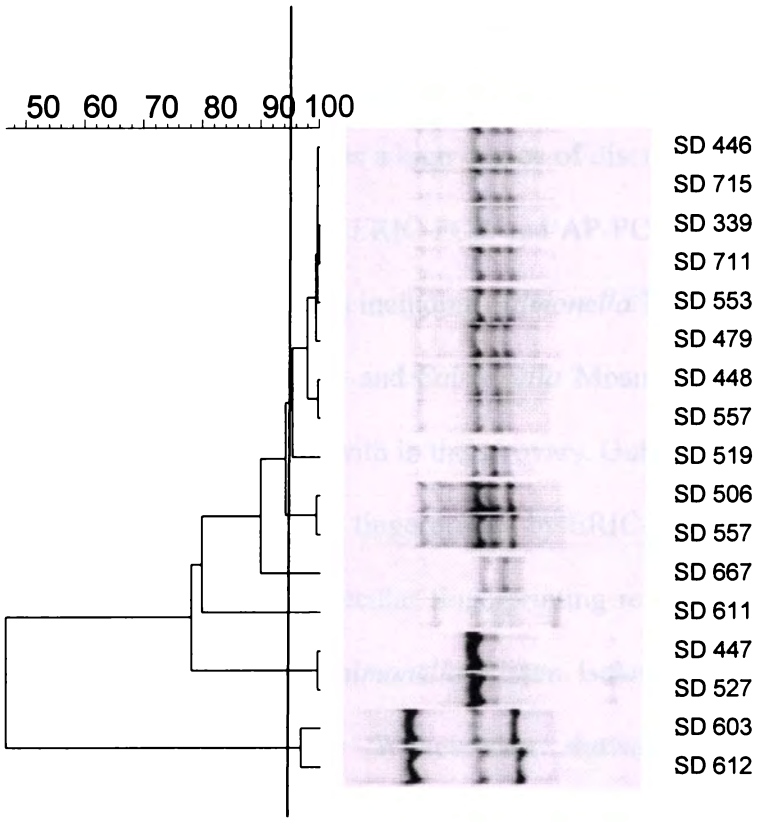


Fig. 4.13. Dendrogram exhibiting the genetic relatedness of *Salmonella* Derby (n = 17) isolated from seafood sources. The percentage of similarities among strains was determined using Dice coefficient and the clustering was performed by UPGMA.

and 4.16 and showed 9 and 6 profiles for *Salmonella* Typhimurium and *Salmonella* Derby serovars, respectively.

ERIC-PCR has been gaining importance for intra-serovar fingerprinting of *Salmonella* serovars as it provides a high degree of discrimination in a short time. Burr et al. (1998) has evaluated ERIC-PCR and AP-PCR fingerprinting methods for different *Salmonella* serovars including *Salmonella* Typhimurium, *Salmonella* Weltevreden, *Salmonella* Derby and *Salmonella* Mbandaka and highlighted the prevalence of genetic variation with in the serovars. Outbreaks and sporadic cases of *Salmonella* Panama were also fingerprinted by ERIC-PCR analysis (Soto et al., 2001). The combination of molecular fingerprinting results obtained from PCR-ribotype and ERIC-PCR for *Salmonella* Rissen isolates exhibited 16 different profiles, whereas, *Salmonella* Weltevreden showed 18 different profile. Comparatively, lower level of discrimination was observed for *Salmonella* Typhimurium and *Salmonella* Derby. Similarly, PCR-ribotypes and ERIC-PCR combined profiles exhibited fourteen and nine different patterns for *Salmonella* Typhimurium and *Salmonella* Derby isolates, respectively. In both serovars the level of discrimination was more apparent with the combination of two fingerprinting methods. In the present study a lower level of discrimination power of PCR-ribotyping was found for ERIC-PCR in *Salmonella* Rissen, *Salmonella* Weltevreden, *Salmonella* Typhimurium and *Salmonella* Derby strains in comparison with ERIC-PCR. An investigation carried out by Lim et al. (2005) compared the four molecular typing methods for differentiation of *Salmonella* spp. and exhibited that ERIC-PCR was the most efficient among the four typing methods.

The cluster analysis of ERIC-PCR fingerprint revealed that nine *Salmonella* Rissen strains exhibited >95 % similarities in genetic relatedness, whereas, 12 out 22 strains *Salmonella* Weltevreden shared >95 % genetic similarities. The genetic variations in ERIC-PCR by cluster analysis were reported to be less diverse for *Salmonella* Typhimurium and *Salmonella* Derby as compared to *Salmonella* Weltevreden and *Salmonella* Rissen. These observations were in concordance with the results reported earlier on *Salmonella* Typhimurium and *Salmonella* Panama (Burr et al., 1998; Soto et al., 2001). In contrast to the great diversity of ERIC-PCR fingerprints in *Salmonella* Rissen and *Salmonella* Weltevreden in this study, Millemann et al. (1996) identified just two patterns (I and II) in 56 *Salmonella* Typhimurium and 14 *Salmonella* Enteritidis isolated from poultry. The reason could be cited here that *Salmonella* serovars were isolated from diverse non-outbreak seafood samples in this study and also highlighted the presence of diverse clones of *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium and *Salmonella* Derby in seafood.

4.7.4 Discrimination indices for PCR-ribotyping and ERIC-PCR

Discrimination indices for different PCR-ribotyping and EIC-PCR patterns were calculated as described in 3.4.4 (Materials and Methods) and results are presented in Table 4.17. Based on three different PCR-ribotype patterns, the discrimination index of PCR-ribotypes for *Salmonella* Rissen was observed at 0.668, whereas, ERIC-PCR discrimination was attained at 0.969. The combined (PCR-ribotype & ERIC-PCR) index was reached at 0.974. Similarly, lower discrimination index (0.680) was observed for *Salmonella* Weltevreden by PCR-ribotyping and combined index was recorded at 0.988. The combined

discrimination indices obtained for *Salmonella* Typhimurium and *Salmonella* Derby by different typing methods at 0.974 and 0.905, respectively (Table 4.17).

The discrimination indices of different typing patterns obtained during this study revealed that ERIC-PCR was found to be better typing method for both *Salmonella* Rissen and *Salmonella* Weltevreden from seafood. However, the combination of both PCR-ribotyping and ERIC-PCR has produced the best discrimination index values for *Salmonella* Rissen, *Salmonella* Weltevreden, *Salmonella* Typhimurium and *Salmonella* Derby.

Table 4.17 Discrimination indices of *Salmonella* Rissen and *Salmonella* Weltevreden, *Salmonella* Typhimurium and *Salmonella* Derby determined by typing methods

***Salmonella* Rissen**	No. of types	Size (%) of largest type	Discrimination Index
PCR- ribotyping	3	45	0.668
ERIC- PCR	15	15	0.969
PCR- ribotyping + ERIC- PCR	16	15	0.974
***Salmonella* Weltevreden**			
PCR- ribotyping	4	50.0	0.680
ERIC- PCR	16	13.6	0.965
PCR- ribotyping + ERIC- PCR	19	9.09	0.988
***Salmonella* Typhimurium**			
PCR- ribotyping	5	55.5	0.674
ERIC- PCR	8	22.2	0.902
PCR- ribotyping + ERIC- PCR	14	11.1	0.974
***Salmonella* Derby**			
PCR- ribotyping	4	41.7	0.714
ERIC- PCR	6	52.9	0.706
PCR- ribotyping + ERIC- PCR	11	29.4	0.905

These observations were in compliance with the reports on molecular subtyping of *Salmonella* Mbandaka and *Salmonella* Livingstone, which showed that combination of different typing method has improved the efficacy of molecular subtyping of *Salmonella* serovars (Howzowski & Wasyl, 2001; Eriksson et al., 2005). A similar study by Shabarinath et al. (2007) on strain characterization of *Salmonella* Weltevreden (12 isolates) from tropical seafood, based on ERIC–PCR and RAPD methods reported a discrimination index value of 0.56. The molecular fingerprinting studies did not establish the single route of *Salmonella* contamination in seafood, as multiple clones of *Salmonella* Rissen, *Salmonella* Weltevreden, *Salmonella* Typhimurium and *Salmonella* Derby were observed in same or different seafood throughout the study period (2003-2007). However, less genetic variations in the later two serovars pointed out that contamination routes were not many. This is amply confirmed by the observation that similar genetic clones of *Salmonella* Typhimurium and *Salmonella* Derby prevalent in seafood.

4.7.5 PFGE analysis of *Salmonella* Weltevreden and *Salmonella* Typhi

Pulsed field gel electrophoresis analysis of 22 strains of *Salmonella* Weltevreden and 7 strains of *Salmonella* Typhi was done and results are presented in Fig. 4.14 and 4.15. PFGE analysis of *Salmonella* Weltevreden strains exhibited the four main restriction patterns (X1, X2, X3 and X4) for *Xba* I restriction enzyme. X1 profile was most predominant and observed in 10 isolates (SW 339, SW 340, SW 379, SW 391, SW 419, SW 427, SW 452, SW 453, SW 461) though, these isolates were obtained from different sources and identified during different period of study. Similarly, X2, X3, and X4 patterns were observed in 8,

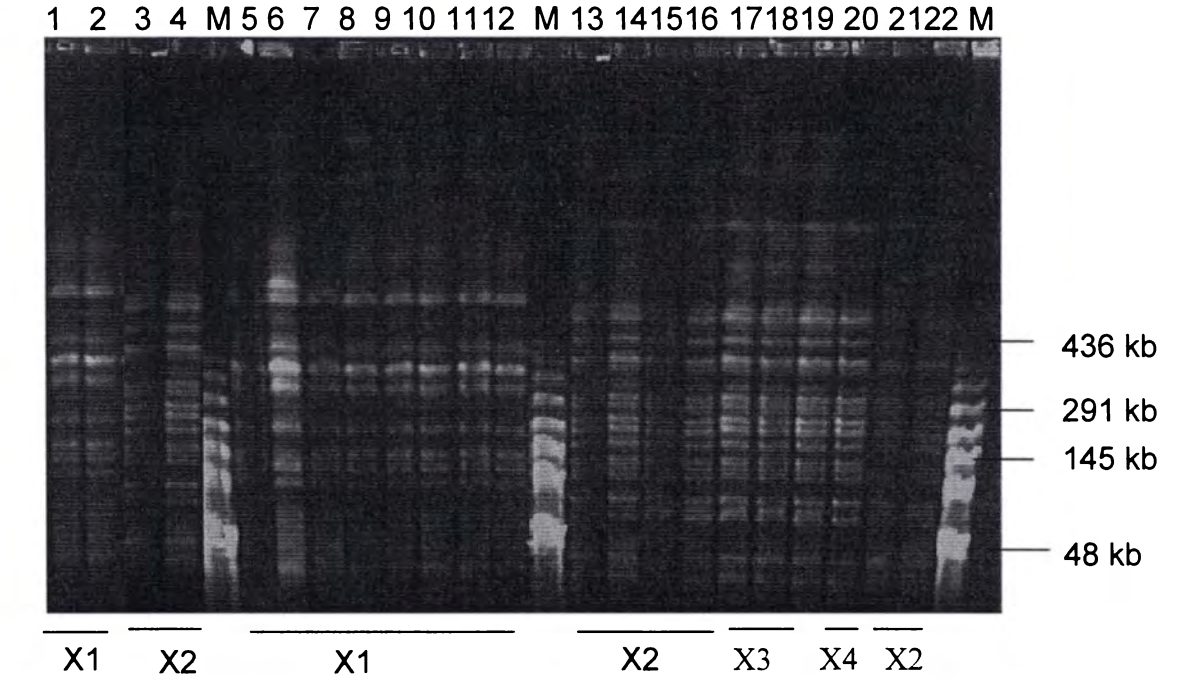


Fig. 4.14 *Xba* I based PFGE profile of *Salmonella* Weltevreden (n =22). Lanes 1-22; SW 339, SW 340, SW 351, SW 378, SW 379, SW 391, SW 419, SW 427, SW 452, SW 453, SW 461, SW 477, SW 511, SW 512, SW 567, SW 571, SW 572, SW 591, SW 608, SW 613, SW 619, SW 627, respectively, M; PFGE ladder.
X1, X2, X3, X4 (*Xba* I digested) are types of PFGE profiles.

3, 1 in *Salmonella* Weltevreden isolates, respectively (Fig. 4.14). *Salmonella* Weltevreden showed 14 to 18 restriction fragments whereas, *Salmonella* Typhi showed 12 to 14 DNA fragments. PFGE pulsotype of *Salmonella* Typhi revealed the two restriction patterns with the restriction enzyme, *Xba*I and profile X2 was identified in 5/7 isolates. Nevertheless, two *Salmonella* Typhi strains T438, T492 exhibited different restriction profile (X1).

Table 4.18 *Salmonella* Weltevreden (n=22) in each PFGE profile and their distribution among the period of isolation and seafood sources

PFGE Profile	Frequency	Year of isolation				Source			
		2003	2004	2005	2006	Fish	Shrimp	Clam	Mussel
X1	10	4	6			4	2	4	
X2	8	2		4	2	2		6	
X3	3			2	1		1		2
X4	1				1		1		

The absence of bands in the range of 175kb and the presence of extra band in the region of >436 kb were the main differences between X1 and X2 profiles. The band variation observed in this study highlighted the prevalence of mainly two PFGE profiles for seven *Salmonella* Typhi isolates (Fig. 4.15).

PFGE based fingerprinting technique is now considered the most accepted method for detection of genetic homogeneity in bacterial pathogens. Present study highlighted the genetic variations in *Salmonella* Weltevreden and *Salmonella* Typhi strains isolated from different seafood sources. *Salmonella* Weltevreden strains of one pulsotype (X1) appeared to be prevalent in all seafood during 2003

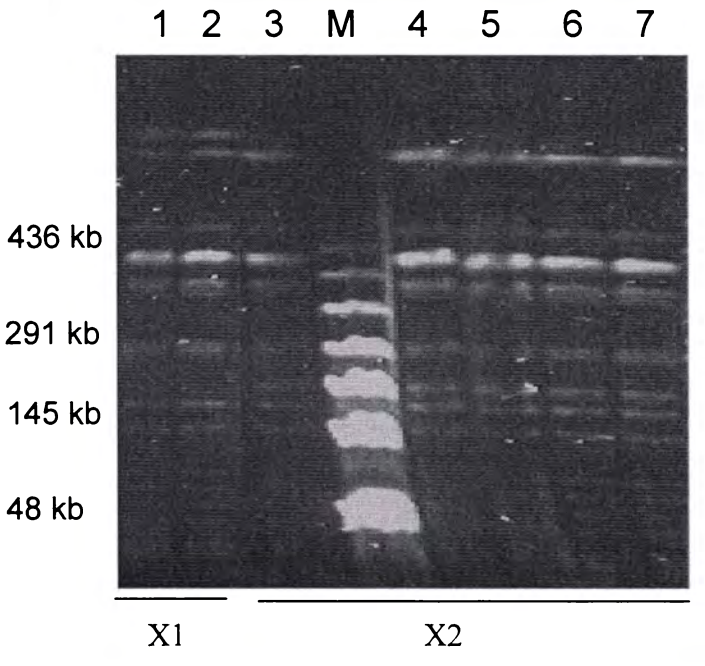


Fig. 4.15 *Xba* I based PFGE profile of PFGE profile of *Salmonella* Typhi Isolates (n=7). Lanes, 1-7, T438, T492, T514, T678, T692, T697, T717, M, PFGE ladder. X1 and X2 are PFGE profile generated by digestion with *Xba* I.

and 2004, whereas, profiles X2, X3, and X4 were predominantly found to be present during 2005 and 2006. No specific pulsotype was observed with regard to type of seafood (Table 4.18).

The genetic diversity of clinical and environmental strains of *Salmonella* in Malaysia was studied by Thong et al. (2002) by PFGE and reported 39 distinct profiles for 95 strains of *Salmonella* Weltevreden. Similarly, 15 pulsotypes were obtained for *Salmonella* Newport (n=139) in California dairy cattle (Berge et al., 2004). In this study, PFGE analysis of *Xba*I digested genomic DNA of 125 *Salmonella* Typhimurium isolates generated three distinct clusters and produced 13 to 17 fragments of 40 to 800 kb. Similar observations were recorded in this study for *Salmonella* Weltevreden isolates, which showed 14 to 18 DNA fragments of 48 kb to 530 kb. Molecular typing of prevalent *Salmonella* serovars such as *Salmonella* Derby, *Salmonella* Mbandaka, *Salmonella* Montevideo, *Salmonella* Gold Coast, and *Salmonella* Senftenberg in animals in England were fingerprinted by PFGE method and results highlighted the multiple clones of different serovars in animals (Liebana et al., 2001).

Table 4.19 *Salmonella* Typhi (n=7) in each PFGE profile and their distribution among years and seafood sources

PFGE Profile	Frequency	Year of isolation				Source		
		2004	2005	2006	2007	Fish	Shrimp	Mussel
X1	2	2				1	1	
X2	5		1	3	1		4	1

The PFGE banding pattern of *Salmonella* Typhi demonstrated that the strains, isolated from seafood had two major genetic lineages. The pulsotype profile X2 was persistent over a considerable period of time (Table 4.19). Similar results were reported by Mirza et al. (2000) who analyzed the chromosomal DNA of multi-drug resistant *Salmonella* Typhi from Asia by PFGE. They reported five different genotypic groups for all Asiatic isolates and two Indian isolates had an identical PFGE profiles. Thong et al. (1996) demonstrated the prevalence of 13 different PFGE pattern in 12 environmental (sewage and river) *Salmonella* Typhi isolates and revealed the presence of multiple clones of *Salmonella* Typhi in environmental samples. Results of the present study were in agreement with their reports. PFGE analysis of *Salmonella* Typhi isolated from seafood showed less genetic variation, though, presence of different clones of the separate ancestral genetic lineage were observed. Thus, highlighted the diverse source of *Salmonella* Typhi contamination in seafood. Overall, PFGE technique was found to be very useful in delineating the genetic variability of the *Salmonella* Weltevreden and *Salmonella* Typhi isolates from seafood.

4.7.6 Characterization of virulence genes of *Salmonella* isolates

All the 256 isolates of *Salmonella* serovars in this study viz., *Salmonella* Atakpame, *Salmonella* Brancaster, *Salmonella* Georgia, *Salmonella* Ohio, *Salmonella* Typhimurium, *Salmonella* Newport, *Salmonella* Mbandaka, *Salmonella* Oslo, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Lindenburg, *Salmonella* Kottbus, *Salmonella* Bareilly, *Salmonella* Nchanga, *Salmonella* Emek, *Salmonella* Irumu, *Salmonella* Typhi, *Salmonella* Othmarschen, *Salmonella* Rissen, *Salmonella* Riggil, *Salmonella* Takoradi,

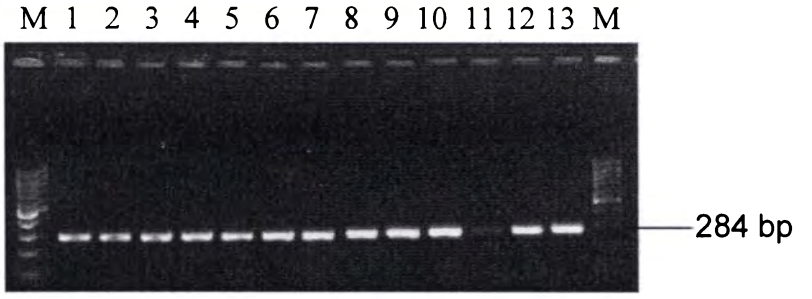


Fig. 4.16 (a) Detection of *inv*A gene. Lanes 1-13; *Salmonella* II (2 serovars), *Salmonella* IIIa, *Salmonella* IIIb, *Salmonella* VI, *Salmonella* Atakpame, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Brancaster, *Salmonella* Derby, *Salmonella* Emek, *Salmonella* Georgia, *Salmonella* Irumu, respectively, M; 100 bp DNA ladder

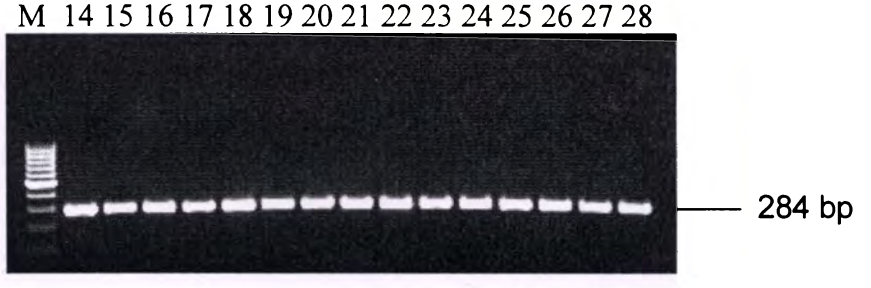


Fig. 4.16 (b) Detection of *inv*A gene. Lanes 14-28; *Salmonella* Kottbus, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Nchanga, *Salmonella* Newport, *Salmonella* Ohio, *Salmonella* Oslo, *Salmonella* Othmarschen, *Salmonella* Riggil, *Salmonella* Rissen, *Salmonella* Takoradi, *Salmonella* Typhi, *Salmonella* Typhimurium, *Salmonella* Virchow, *Salmonella* Washington, respectively, M; 100 bp DNA ladder.

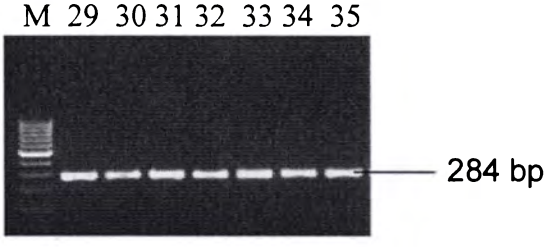


Fig. 4. 16 (c) Detection of *inv*A gene. Lanes 29- 30; *Salmonella* Weltevreden, *Salmonella* Worthington, Lane 31; *Salmonella* Typhimurium ATCC 23564, lanes 32-35; Rough Strains,
M; 100 bp DNA ladder.

Salmonella Virchow, *Salmonella* Washington, *Salmonella* Weltevreden, *Salmonella* Worthington, *Salmonella* II, *Salmonella* IIIa , *Salmonella* IIIb, and *Salmonella* VI were investigated for the presence of the three targeted virulence genes (*inv*A, *stn* and *fim*A gene). They were found to harbour these three virulence genes, corresponding to 284 bp, 260 bp and 85 bp gene amplicons, respectively (Fig. 4.16, 4.17, 4.18). Only exception was observed in case of *Salmonella arizonae* (IIIa) strains, which did not show the presence of *fim* A gene. The isolates of *Salmonella arizonae* (IIIa) were found to be negative for 85 bp *fim*A gene amplicon and a 200 bp non-specific amplicon was observed in all *Salmonella arizonae* isolates (Fig 4.18a). Weak bands were observed for *inv*A gene in *Salmonella* Emek and *Salmonella* Lindenburg strains. *Salmonella* rough strains were also detected positive for three targeted virulence genes (Fig. 4.16a & c).

The invasion (*inv*A) gene is present in *Salmonella* pathogenicity island (SPI) and found to be responsible for invasion in the gut epithelial tissue of human and animals, whereas, *stn* gene causes enterotoxic effect to epithelial cells, leading to enteric disorder (Asten and Dijk, 2005). *Salmonella* serotypes isolated from seafood harboured both invasion and enterotoxin genes as showed by PCR amplification for each virulence gene. Rahn et al. (1992) showed that two serovars *Salmonella* Litchfield and *Salmonella* Senftenburg were not harbouring *inv*A gene. However, further studies demonstrated that it was due to the natural deletion of the *inv*A gene in the Centisome 63 pathogenicity islands of environmental isolates *Salmonella* Litchfield and *Salmonella* Senftenberg chromosome (Ginocchio et al., 1997). Other studies elsewhere showed the

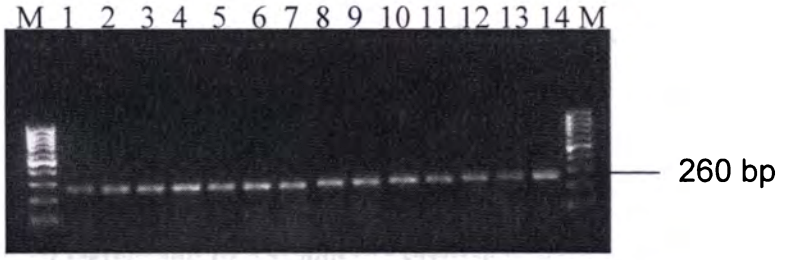


Fig. 4.17 (a) Detection of *stn* gene. Lanes 1-14; *Salmonella* II (2 serovars), *Salmonella* IIIa, *Salmonella* IIIb, *Salmonella* VI, *Salmonella* Atakpame, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Brancaster, *Salmonella* Derby, *Salmonella* Emek, *Salmonella* Georgia, *Salmonella* Irumu, *Salmonella* Kottbus, respectively, M; 100 bp DNA ladder.

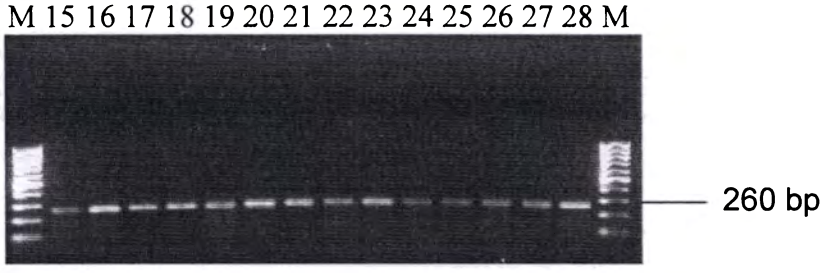


Fig. 4.17 (b) Detection of *stn* gene. Lanes 15 -28; *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Nchanga, *Salmonella* Newport, *Salmonella* Ohio, *Salmonella* Oslo, *Salmonella* Othmarschen, *Salmonella* Riggil, *Salmonella* Rissen, *Salmonella* Takoradi, *Salmonella* Typhi, *Salmonella* Typhimurium, *Salmonella* Virchow, *Salmonella* Washington, respectively, M; 100 bp DNA ladder.

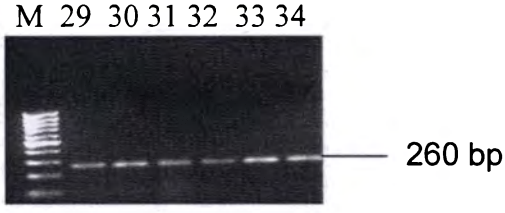


Fig.4.17 (c) Detection of *stn* gene. Lanes 29- 30; *Salmonella* Weltevreden, *Salmonella* Worthington, Lane 31; *Salmonella* Typhimurium MTCC 23564, lanes 32-34; Rough Strains, M; 100 bp DNA ladder.

presence of *inv*A gene in a collection of 630 strains, representing over 100 different serovars with an exception of *Salmonella* Senftenberg and *Salmonella* Litchfield (Rahn et al., 1992).

Table 4.20 Detect ion of *Salmonella* virulence genes (n = 256)

Sl. No.	*Salmonella* Serovars	No. of Isolate	*inv*A	*stn*	*fim*A
1.	S. Atakpame	2	+	+	+
2.	*S.* Bareilly	16	+	+	+
3.	*S* Braenderup	15	+	+	+
4.	*S.* Brancaster	6	+	+	+
5.	*S.* Derby	17	+	+	+
6.	*S* Emek	7	+[a]	+	+
7.	*S.* Irumu	11	+	+	+
8.	*S.* Georgia	4	+	+	+
9.	*S.* Kottbus	2	+	+	+
10.	*S.* Lindenburg	14	+[b]	+	+
11.	*S.* Mbandaka	13	+	+	+
12.	*S.* Nchanga	7	+	+	+
13.	*S.* Newport	4	+	+	+
14.	*S.* Ohio	9	+	+	+
15.	*S.* Oslo	8	+	+	+
16.	*S.* Othmarschen	6	+	+	+
17.	*S.* Rissen	21	+	+	+
18.	*S.* Riggil	6	+	+	+
19.	*S.* Takoradi	8	+	+	+
20.	*S.* Typhi	7	+	+	+
21.	*S.* Typhimurium	18	+	+	+
22.	*S.* Virchow	8	+	+	+
23.	*S.* Washington	6	+	+	+
24.	*S.* Weltevreden	22	+	+	+
25.	*S.* Worthington	3	+	+	+
26.	*Salmonella* II	4	+	+	+
27.	*Salmonella* II	4	+	+	+
28.	*Salmonella* IIIa	3	+	+	-
29.	*Salmonella* IIIb	2	+	+	+
30.	*Salmonella* VI	3	+	+	+

[a]Weak Positive (3 isolates), [b] Weak Positive (2 isolates)

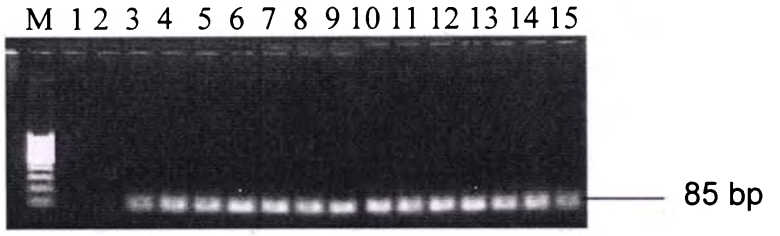


Fig. 4.18 (a) Detection of *fimA* gene. Lanes 1-15; *Salmonella* IIIa (2 strains), *Salmonella* II (2 serovars), *Salmonella* IIIb, *Salmonella* VI, *Salmonella* Atakpame, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Brancaster, *Salmonella* Derby, *Salmonella* Emek, *Salmonella* Georgia, *Salmonella* Irumu, *Salmonella* Kottbus, respectively, M; 100 bp DNA ladder.

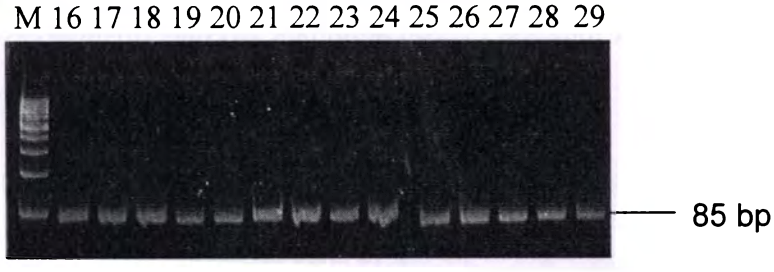


Fig. 4.18 (b) Detection of *fimA* gene. Lanes 16-29; *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Nchanga, *Salmonella* Newport, *Salmonella* Ohio, *Salmonella* Oslo, *Salmonella* Othmarschen, *Salmonella* Riggil, *Salmonella* Rissen, *Salmonella* Takoradi, *Salmonella* Typhi, *Salmonella* Typhimurium, *Salmonella* Virchow, *Salmonella* Washington, *Salmonella* Weltevreden, respectively, M; 100 bp DNA ladder.

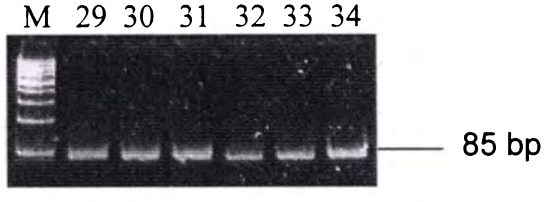


Fig.4.18 (c) Detection of *fimA* gene. Lane 29; *Salmonella* Worthington, Lane 31; *Salmonella* Typhimurium ATCC 23564, lanes 32-34; Rough Strains, M; 100 bp DNA ladder.

Salmonella enterotoxin (*stn*) gene, the gene responsible for pathogenicity in *Salmonella* serovars was characterized during this study. The *stn* gene encodes for heat labile 29 kDa enterotoxin protein in *Salmonella* serovars and elicits biological responses in both in vivo and in vitro (Hitchcock et al., 1986). Present results highlighted the prevalence of *stn* gene in all *Salmonella* serovars (30 serovars) isolated from seafood. Similar observation was reported by Prager et al. (1995) who, demonstrated that *Salmonella stn* gene was prevalent among *Salmonella enterica*, but not in *Salmonella bongori*. Distribution of virulence genes including *stn* genes were detected in *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Bareilly and *Salmonella* Paratyphi B strains isolated from man and animals in India (Murugkar et al., 2003).

Bacterial surface appendages such as fimbriae, are responsible for binding to specific receptors on epithelial cells of the host. The fimbrial protein type 1 has been implicated in *Salmonella* pathogenicity (Clegg and Gerlach, 1987). PCR based procedure have been successfully used for the detection of specific genes in *Salmonella* serovars (Swamy et al., 1996; del Cerro et al., 2002). Present study demonstrated the conspicuous absence of *fim*A gene in *Salmonella arizonae* (IIIa) isolates associated with seafood. Thus, suggested the possible genetic variation in *fim*A gene sequence of *Salmonella arizoane* (IIIa) isolated from seafood. Finally, this study revealed the invasion, enterotoxin and fimbrial genes were well present in *Salmonella* serovars isolated from seafood, thus, highlighting the virulent nature of *Salmonella* serovars associated with seafood (Table 4.20).

4.8 Development of molecular methods for rapid detection *Salmonella* in seafood

4.8.1 Development of PCR assay for *Salmonella* serovars

Salmonella specific PCR assay was developed for different *Salmonella* serovars such as *Salmonella* Typhimurium, *Salmonella* Typhi, *Salmonella* Newport, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Weltevreden, *Salmonella* II, *Salmonella* IIIa, *Salmonella* IIIb, *Salmonella* VI by a random genomic fragment primer, ST11-ST15 (Aabo et al., 1993). All *Salmonella* serovars produced desirable amplicon of 429 bp along with a positive control without any non-specific product, whereas, no amplicons were observed in case of negative controls (Fig.4.19). Present results were in concurrence with a multicenter PCR validation assay for *Salmonella* that showed specific detection of *Salmonella* serovars with ST11-ST15 primers (Malorny et al., 2003a). Detection of *Salmonella* serovars by PCR assay has been standardized with a different set of primers such as *inv*A, *stn*, *spv*C, *hil*A, and *fim*A genes (Rahn et al.,1992; Chiu and Ou, 1996; Cohen et al., 1996) and showed all primers were found to be very specific and sensitive for the detection of different *Salmonella* serovars. Ziemer and Steadham (2003) have used nine set of primers for detection of *Salmonella* spp. in intestinal-associated bacteria and revealed the three primers sets i.e. 16 rDNA, *stn*, and histidine transport operon were most useful for detection of *Salmonella* spp. Though, this study has developed the PCR for different *Salmonella* serovars, but the aim was to develop a *Salmonella* specific PCR assay that can be used for detection of *Salmonella* serovars in seafood so as to save time and labour. Though, conventional methods are still widely used for the detection

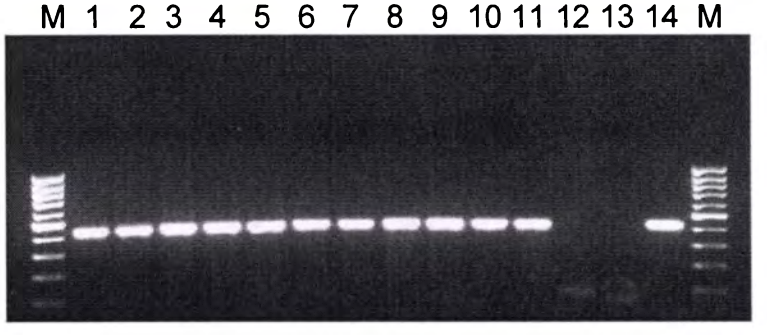


Fig. 4.19 Development of *Salmonella* specific PCR
Lanes 1-11; PCR amplification of *Salmonella* Typhimurium, *Salmonella* Typhi, *Salmonella* Newport, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Weltevreden, *Salmonella* Mbandaka, *Salmonella* II, *Salmonella* IIIa, *Salmonella* IIIb, and *Salmonella* VI, lanes; 12 and 13 negative controls (*Escherichia coli* and *Citrobacter* spp.) and lane 14; positive control (*Salmonella* Typhimurium ATCC 23564), M; 100bp DNA ladder

of *Salmonella* spp., but, detection of these pathogens depends increasingly on the availability of rapid and precise diagnostic tests for screening of large number of seafood samples. Detection of *Salmonellae* by conventional culture methods is laborious and time consuming process and in some instances found to be poor in detection with lower level of contamination (D'Aoust, 1992).

4.8.2 An eight-hour pre-enrichment PCR method for detection of *Salmonella* in seafood

A total of 110 seafood samples, consisting of fish, crab, shrimp, mussel, edible oyster and clams were tested for *Salmonella* by PCR assay, as described in the section 3.5.1 (Materials and Methods), along with controls by USFDA method. At interval of 0, 2, 4, 6, 8 h of pre-enrichment in lactose broth, PCR assays were done. The results after 8 h of pre-enrichment showed that 37 out of 110 samples were positive for *Salmonella* by PCR method, while only 27/110 were positive for *Salmonella* USFDA method (Table 4.21). It can be seen from the table that after 2 h of pre-enrichment, 5/110 samples were positive for *Salmonella*. Similarly, after, 4 h, *Salmonella* was detected in 18 samples and after 6 h, 31 samples were found to be positive for *Salmonella* by PCR method.

The pre-enrichment step has played significant role in detection of *Salmonella* serovars from seafood by PCR. With the incorporation of pre-enrichment step prior to PCR assay has not only improved the detection efficiency by multiplication of the live cells, but also reduced the incidence of false positive arising due to *Salmonella* dead cells. At zero hour PCR, all seafood samples were found negative for *Salmonella,* though, *Salmonella* was present in the seafood. Detection efficiency of PCR was increased with the increase in pre-enrichment

period and by end of 8 h pre-enrichment maximum samples (33.6 %) showed positive result (Fig. 4.20).

Table 4.21 Detection of *Salmonella* by PCR at different pre-enrichment period

Sl. No.	Seafood Name	No. of Sample tasted	Positive by PCR (%) Pre-enrichment in LB for					Positive by USFDA Method (%)
			0 h	2 h	4 h	6 h	8 h	
1.	Fish	45	0	0	5 (11.1)	11 (24.4)	13 (28.8)	9 (20.0)
2.	Shrimp	34	0	3 (8.8)	7 (20.5)	10 (29.4)	12 (35.2)	9 (26.4)
3.	Crab	10	0	0	0	2 (20.0)	2 (20.0)	2 (20.0)
4.	Clam	8	0	0	2 (25.0)	4 (50.0)	4 (50.0)	3 (37.5)
5.	Mussel	8	0	1(12.5)	2 (25.0)	2 (25.0)	4 (50.0)	2 (25.0)
6.	Oyster	5	0	1(20.0)	2 (40.0)	2 (40.0)	2 (40.0)	2 (40.0)
	Total	110	0	5 (4.5)	18 (16.3)	31 (28.1)	37 (33.6)	27 (24.5)

The result also pointed out the limitation of the conventional culture method. It was also observed that there was less difference in results obtained from 6 to 8 h PCR pre-enrichment followed by PCR. Detection of *Salmonella* serovars by PCR in seafood after 24 to 48 hrs of enrichment has been reported by Kumar et al. (2003). This method has an advantage to detect viable and active *Salmonella* as low as 2 cfu /25g of seafood by 8 h PCR assay.

4.8.2.1 Determination of minimum limit of detection (MLD) and effect of seafood matrix on MLD

Homogenate of fish, shrimp crab, clam, mussel, and edible oyster spiked with viable *Salmonella* cells ranging from 10^6 cfu to 2 cfu /250ml gave 284 bp *Salmonella* specific amplicon by 8 h pre-enrichment PCR method from all dilutions of fish homogenate (Fig. 4.21a). Similar results were obtained from all spiked shrimp, crab, clam, mussel, and edible oyster samples (Fig. 4.21b, c, d, e, f). Each seafood was inoculated with different *Salmonella* serovars, and all serovars were detected by specific 284 bp amplicon for *inv*A primer. PCR amplicons were also obtained after 4 h of enrichment from these homogenate inoculated with *Salmonella* viable cell in the range of 10^6, 10^5 , 10^4 , 10^3cfu/250 ml, but 8 h pre-enrichment showed detectable amplicon at lower inoculum level (10^3 10^2 10, 2cfu/250ml). Similarly, after 4 h of enrichment, PCR amplicons were detected from shrimp crab, clam, mussel, and edible oyster samples spiked with 10^6, 10^5, 10^4 , 10^3cfu/250 ml. Whereas, lower spiking levels (10^3 10^2 10, 2cfu/250ml) produced positive results only after 8 h of pre-enrichment period. The results of three repetitive experiments were consistently similar and detected *Salmonella* (2 cfu/250ml) in each experiment for crab, clam, mussel, and edible oyster samples. This study has evaluated the sensitivity of performance of 8 h pre-enrichment-PCR assay for *Salmonella* in seafood and successfully detected 2cfu/250ml in variety of seafood samples. Present results were comparable with the PCR assay carried out for *Salmonella* without the presence of seafood, thus, ruled out the inhibition by seafood matrices during PCR assay (Fig. 4.22).

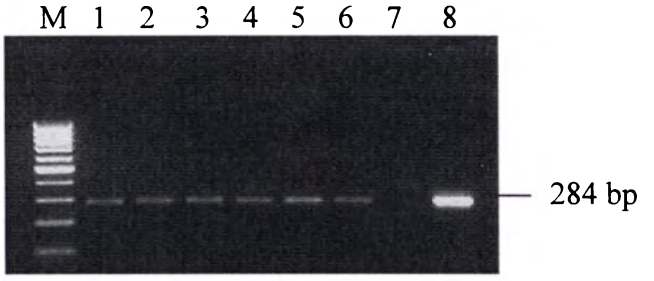


Fig. 4.20 8 h Pre-enrichment PCR for *Salmonella* from naturally contaminated seafood. Lanes 1 to 6 contain each positive from fish, shrimp, crab, clam, mussel, edible oyster, respectively, lane 7; *E.coli* (negative control) , lane 8; *Salmonella* Typhimurium (positive control), M; 100 bp DNA ladder.

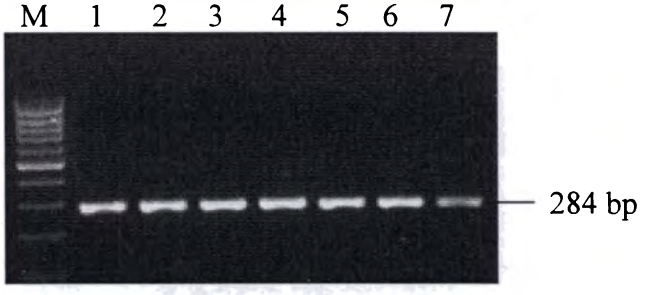


Fig. 4.21 (a) 8 h Pre-enrichment PCR for *Salmonella* in fish. Lanes 1 to 7 inoculated respectively with $2x10^6$, 10^5, 10^4, 10^3, 10^2 20, 2, cfu /250ml of fish homogenate , M; 100bp DNA ladder.

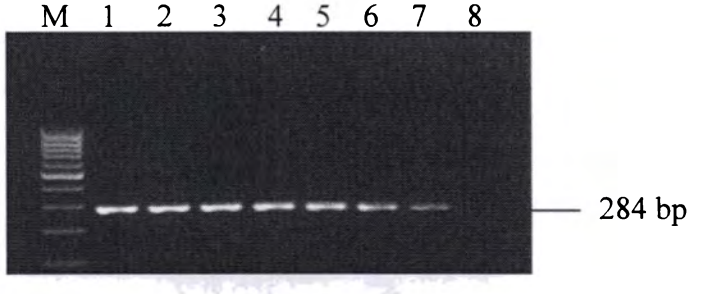


Fig. 4.21 (b) 8 h Pre-enrichment PCR for *Salmonella* in shrimp. Lanes 1 to 8 inoculated respectively with $2x10^6$, 10^5, 10^4, 10^3, 10^2 20, 2, 0 cfu /250ml of shrimp homogenate, M; 100 bp DNA ladder.

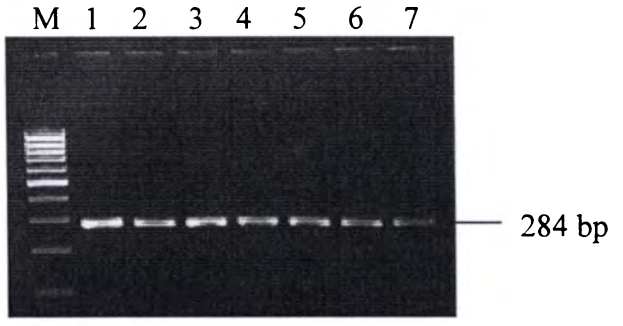


Fig. 4.21 (c) 8 h Pre-enrichment PCR for *Salmonella* in crab. Lanes 1 to 7 inoculated respectively with $2x10^6$, 10^5, 10^4, 10^3, 10^2, 20, 2 cfu /250ml of crab homogenate, M; 100 bp DNA ladder.

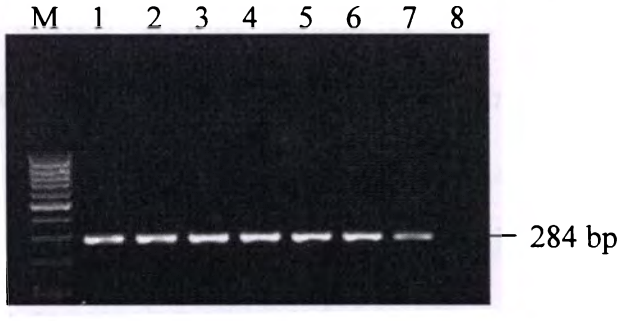


Fig. 4.21 (d) 8 h Pre-enrichment PCR for *Salmonella* in **clam**. Lanes 1 to 8 inoculated respectively with $2x10^6$, 10^5, 10^4, 10^3, 10^2, 20, 2, 0 cfu /250ml of clam homogenate, M; 100 bp DNA ladder.

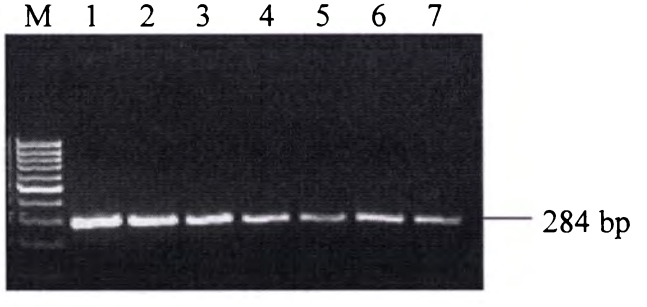


Fig. 4.21 (e) 8 h Pre-enrichment PCR for *Salmonella* in mussel. Lanes 1 to 7 inoculated Respectively with $2x10^6$, 10^5, 10^4, 10^3, 10^2 20, 2, cfu /250ml of mussel homogenate, M; 100 bp DNA ladder.

Similar results were reported in some of the earlier studies. Makino et al. (1999) detected 1 cell per gram of food samples by PCR and Ferretti et al. (2001) has successfully detected 1cfu/100 ml of food homogenate by 6-h pre-enrichment PCR assay. The degree of specificity and sensitivity of the 8 h pre-enrichment PCR assay of the contaminated samples was very significant and detected *Salmonella* from homogenate inoculated with 10cfu/250ml and 2cfu/250ml with formation of intensely clear amplicons. In the case of naturally contaminated seafood, the intensity of amplicon bands were found to be weaker than spiked samples (Fig. 4.20 & 4.22). This could be due to the fact that in the spiked cases, fresh and actively growing *Salmonella* cells were introduced in seafood homogenate, whereas in the natural samples, *Salmonella* cells may be stressed due to unfavourable conditions in the food matrix (Tietjen and Fung, 1995). Present study also indicates that this 8 h pre-enrichment PCR assay can detect most of the commonly occurring *Salmonella* serovars *viz.*, *Salmonella* Typhi, *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Mbandaka, *Salmonella* Bareilly, and *Salmonella* Weltevreden in seafood. Results of this study was in concurrence with the findings of Malorny et al. (2003a) showed that *inv*A primers can detect almost all *Salmonella* serovars, without any interference of nonspecific product in *Salmonella* related strains.

4.8.2.2 Detection limit for *Salmonella* dead cells in seafood samples

The PCR assay for *Salmonella* was carried out in fish homogenates spiked with *Salmonella* dead (heat killed) cells viz.: 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cells/250 ml. The assay was performed as described in section 3.5.1.2 (Materials and Methods). The results are given in Table 4.22. Results showed that

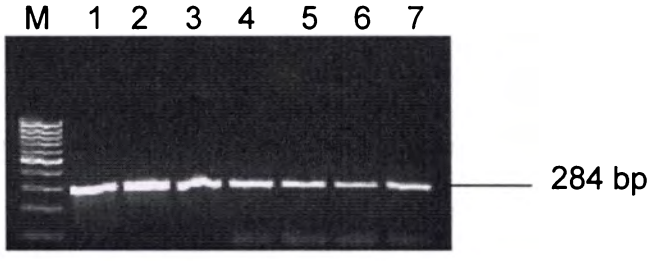


Fig. 4.21 (f) 8 h Pre-enrichment PCR for *Salmonella* in oyster.
Lanes 1 to 7 inoculated Respectively with $2x10^6$, 10^5, 10^4, 10^3, 10^2 , 20, 2, cfu /250ml of oyster homogenate, M; 100 bp DNA ladder.

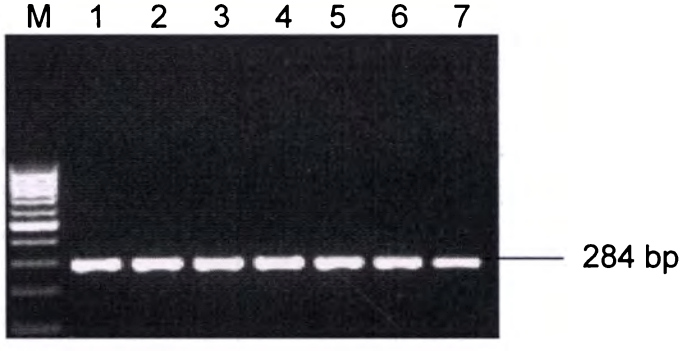


Fig. 4.22 8 h Pre-enrichment PCR for *Salmonella* without seafood
Lanes 1 to 7 inoculated respectively with $2x10^6$, 10^5, 10^4, 10^3, 10^2 , 20, 2 cfu /250ml of lactose broth, M; 100bp DNA ladder.

Salmonella dead cells in fish homogenate seeded at and above 10^5, 10^6 and 10^7 CFU/250 ml were detected by 0h, 2h, 4h, 6h, and 8 h of pre-enrichment PCR. However, dead cells at level 10^4/ 250 ml were detected initially (0 h) , but subsequently (by 2h,4h, 6h , and 8 h pre-enrichment) did not give positive result for *Salmonella*. It can be seen from Table 4.22 that *Salmonella* dead cells 10^2, 10^3/250 ml were not detected by PCR at all (0 to 8h) pre-enrichment periods.

The genuine concern about PCR assay for pathogens is the inability to recognize viable and dead cells in a food sample. Similar results were reported in earlier studies. Fach et al. (1999) has detected 10^6 cfu/25g *Salmonella* dead cells in food samples with PCR based commercial kit after 18 h pre-enrichment period.

Table 4.22 Detection of *Salmonella* dead cells (heat killed) in fish homogenate

Sl. No.	Fish Homogenate Inoculated (CFU/250ml)	PCR Result [a] at pre-enrichment period				
		0 h	2 h	4 h	6 h	8 h
1	$2x10^2$	-	-	-	-	-
2	$2x10^3$	-	-	-	-	-
3	$2x10^4$	+[b]	-	-	-	-
4	$2x10^5$	+	+	+	+	+[b]
5	$2x10^6$	+	+	+	+	+
6	$2x10^7$	+	+	+	+	+

[a] Duplicate, [b] Weak positive

This 8 h pre-enrichment PCR assay detected amplicons from *Salmonella* dead cells at and above seeding level $2x10^5$ cfu/25g of seafood samples, a level rarely achieved in naturally contaminated seafood samples. Hence, incorporation of

enrichment step prior to PCR rules out any possibility of detecting dead cells leading to false positive results in naturally contaminated seafood and creating a false alarm for processors and consumers.

4.8.3 Comparison of Culture, ELISA and PCR methods for detection of *Salmonella* from seafood

4.8.3.1 Comparison of Culture, ELISA and PCR methods

A total of 215 seafood samples were tested for *Salmonella* by the culture, ELISA and PCR methods. The results are presented in Table 4.23. Result showed that out of 83 samples of fish, 20 (24.0 %) were positive for *Salmonella* by culture method, and 23 (27.7 %) samples were positive by ELISA assay. When tested by PCR, 30 (36.1 %) of the fish samples were positive for *Salmonella.* In case of shrimp samples 11/58 (18.9 %), 15/58 (25.8 %), 20/58 (34.4 %) were positive by Culture, ELISA and PCR method, respectively.

Table 4.23 Summary of results from culture, ELISA and PCR methods for detection of *Salmonella*

Seafood Type	No. of sample	Positive by		
		Culture method	ELISA	PCR method
Fish	83	20/83 (24.0%)	23/83 (27.7%)	30/83 (36.1%)
Shrimp	58	11/58 (18.9%)	15/58 (25.8%)	20/58 (34.4%)
Crab, Clam, Mussel, Oyster	42	9/42 (21.4%)	7/42 (16.6%)	15/42 (35.7%)
Squid, Cuttlefish, Octopus	32	6/32 (18.7%)	6/32 (18.7%)	3/32 (9.3%)
Total	215	46/215 (21.3%)	51/215 (23.7%)	68/215 (31.6%)

The ELISA method proved to be less sensitive for crab, clam, mussel and oyster samples, only 16.6 % of samples being positive for *Salmonella* by ELISA, while, culture and PCR assays detected 21.4 %, and 35.7 %, respectively. PCR assay was found to be less sensitive (only 9.3 %) as compared to culture (18.7 %) and ELISA (18.7 %) methods for detection of *Salmonella* in cephalopods consisting of squid, cuttlefish and octopus. It has to be noted that all fish and shrimp samples detected positive for *Salmonella* by culture method were also positive by ELISA and PCR assays. But, similar observation was not obtained for crab, clam, mussel, oyster, squid, cuttlefish, and octopus samples. However, overall, out of 215 samples tested, 21.3% tested positive for *Salmonella* by Culture method, 23.7% by ELISA and 31.6% by PCR assay.

Establishment of an effective post harvest control program for *Salmonella* in seafood necessitates reliable, accurate and sensitive methods to assess the presence of *Salmonella* in seafood at different levels of handling. In this study, three different methods that are approved by USFDA and other agencies were compared for detection of *Salmonella* from naturally contaminated seafood samples. Traditionally, detection of *Salmonella* in food by culture method uses multiple enrichment steps, followed by identification based on series of biochemical reactions. This approach, however, found to be laborious and time consuming. The chief advantage of PCR and ELISA assays over the culture method is that these assays are simple and can handle large number of samples, simultaneously. The selection of a diagnostic test depends not only on test features such as sensitivity and speed, but also on extrinsic factors like food type, stress on organism and interpretation of results (Rijpens et al., 1999). The findings of this study revealed that PCR detected more number of seafood

samples as *Salmonella* positive than culture and ELISA methods. The results were in agreement with several similar studies reported elsewhere (Fratamico, 2003; Sachse et al., 2003). However, a study carried out by Croci et al. (2004) demonstrated the efficiency of standard culture method as equivalent to PCR and ELISA assays. The efficiency of ELISA method was observed to be higher as compared to the culture during the present study. Similar observation was reported by Schneid et al. (2006) who evaluated an indirect ELISA with culture method for the detection of *Salmonella* in chicken meat and found that ELISA method was much superior than the culture method.

In the present study, PCR assay has utilized *inv*A gene for the detection of *Salmonella* in seafood. Rahn et al. (1992) reported that *inv*A assay using the primer set 139-141 produced the perfect amplification in a wide range of *Salmonella* subspecies and serovars without any non-specific amplification among closely related genera. *Inv*A gene based diagnostic PCR assay for different *Salmonella* serovars in food samples has been found most suitable, based on inter-laboratory accuracy study (Malorny et al., 2003a). The study also indicated that nature of seafood exerted a profound influence on the method of detection. While ELISA or culture methods showed low efficiency, compared to PCR assay for detection of *Salmonella* in finfish and shellfish, the reverse effect was observed for cephalopod samples. The deviation in results in the case of cephalopods indicated the presence of PCR inhibitors in cephalopods. A parallel study proved that cephalopod ink was responsible for the inhibitory effect in *Salmonella* PCR assay. It has been reported previously that the diagnostic accuracy in the naturally contaminated samples varied among the matrix categories and was affected by the presence of certain PCR inhibitors (Malorny et al.,

2003b). The present study indicated that both culture and ELISA methods are comparable in efficiency for most of the seafood samples tested. ELISA assay included in the study emerged as an alternative to culture method for detection of *Salmonella* in seafood. Additionally, ELISA was more rapid and could handle more samples at a time. Between culture and ELISA methods, a slight increase was noted in *Salmonella* detection by ELISA, while a contrary observation has been reported by Sachse et al. (2003).

4.8.3.1 Statistical analysis of the results of *Salmonella* detection by the three methods

The data obtained from three different methods for *Salmonella* detection were statistically analyzed using kappa coefficient values and all values were significant ($P<0.01$) for culture, ELISA and PCR methods. Analysis of data using kappa coefficient values demonstrated that there was substantial to excellent agreement

Table 4.24 Kappa coefficient values showing agreement between culture, ELISA, and PCR method for fish and shrimp samples

Fish (n = 83)	Culture method	ELISA	PCR
Culture method	----	0.877*	0.695*
ELISA	0.877*	---	0.811*
PCR	0.695*	0.811*	---
Shrimp (n = 58)			
Culture method	----	0.760*	0.663*
ELISA	0.760*	---	0.635*
PCR	0.663*	0.635*	---

*Significant ($P<0.01$)

between culture, ELISA and PCR assays (kappa coefficient values ranging from 0.695 to 0.877) for fish samples (Table 4.24).

The perfect agreements were observed in between culture and ELISA methods, PCR and ELISA methods for fish samples whereas, substantial agreement was observed in between culture and PCR methods. The agreement between assays was found to be substantial (kappa value; 0.663 to 0.760) in shrimp samples. Fair agreement (kappa value; 0.385) was observed between culture and ELISA assays for crab, clam, mussel and oyster samples. Almost perfect agreement (kappa value; 1.0) was recorded between culture and ELISA methods in squid, cuttlefish and octopus samples, whereas, substantial agreement was observed between PCR and ELISA assays for cephalopods (Table 4.25).

Table 4.25 Kappa coefficient values showing agreement between culture, ELISA, and PCR method for crab, clam, mussel, oyster, squid, and cuttlefish and octopus samples

Crab, clam, mussel and oyster (n = 42)	Culture method	ELISA	PCR
Culture method	----	0.385*	0.614*
ELISA	0.385*	---	0.491*
PCR	0.614*	0.491*	---
Squid, cuttlefish and octopus (n =32)			
Culture method	----	1.000*	0.765*
ELISA	1.000*	---	0.765*
PCR	0.765*	0.765*	---

* Significant (P <0.01)

The statistical analysis signified more agreement between culture and ELISA assays, but the sensitivity of PCR for *Salmonella* in seafood cannot be ruled out. Almost perfect agreement (kappa value; 0.8 to 1.0) was recorded between culture and ELISA methods in fish and cephalopod samples which indicated the effectiveness of both methods in these samples. Fair to substantial agreements observed between culture, ELISA and PCR assays for shrimp, crab, clam, mussel and oyster indicated the low level of agreement among three methods. In concurrence with the present study, Fratamico (2003), compared culture, PCR, TaqMan *Salmonella*, and Transia Card *Salmonella* assays for detection of *Salmonella* spp. in naturally –contaminated ground chicken, turkey and beef and exhibited kappa coefficient values ranging from 0.67 to 0.87 for different detection methods.

Overall, results showed that the ELISA assay was more sensitive than culture method though, PCR assay was the most sensitive among the three methods used for detection of *Salmonella* in seafood. These results suggested that PCR assay would detect *Salmonella* more efficiently than ELISA or conventional culture methods. If sensitivity is the criteria used for the selection of method, the PCR method proved to be most efficient for detection of *Salmonella* in seafood. The culture method though time consuming, has the advantage that the different serovars could be isolated for further studies. If, large scale monitoring of *Salmonella* is envisaged, PCR is the method of choice. In conclusion, these studies suggested that no single method is safe for detection of *Salmonella* in seafood and it is reasonable to incorporate dual tests based on different principle and procedure for routine analysis of *Salmonella* in seafood. The results also stress the need for more widespread validation of these

diagnostic tests against different seafood to ascertain the efficacy of the individual test.

4.8.4 Quantitative detection of *Salmonella* in seafood by real-time PCR assay

4.8.4.1 Real-time assay of pure and quantified DNA isolated from *Salmonella* Typhimurium

Real-time PCR assay was developed for pure DNA isolated from *Salmonella* Typhimurium. In subsequent assays, the decimal dilutions of pure DNA was used as a standards for detection of *Salmonella* DNA in unknown test samples. The minimum detection sensitivity was 0.005 pg (picogram) of the pure DNA in a Real-time PCR reaction (Fig. 4.24). The linear range of detection spanned from 7 log cycles of pure DNA ranging from 5000 pg to 0.005 pg. One cell of *Salmonella* Typhimurium DNA corresponds to 0.005 pg and the minimum detection level of the newly developed real-time PCR was at 0.005 pg. There was no amplification when the amount of the DNA was further reduced to 0.002 pg (Fig. 4.23). The results showed that level of detection from real-time PCR for *Salmonella* was up to 0.005 pg. The Tm value for *inv*A gene was observed at 86 ± 1°C. (Fig. 4.25).

4.8.4.2 Quantification of *Salmonella* in pure culture, and *Salmonella* spiked fish and shrimp homogenates.

Real-time PCR assay was carried out for DNA extracted from 2 to $2x10^6$ cfu/ml of *Salmonella* pure culture (without seafood) showed linear plot of Ct values and DNA derived from 2 to 10^6 cfu/ml (Fig.4.26 & 4.27). The real-time PCR assay for *Salmonella* in shrimp homogenates, showed linear plot of Ct values against DNA derived from 20 to 10^6 cfu/g of *Salmonella* (Fig. 4.28). The

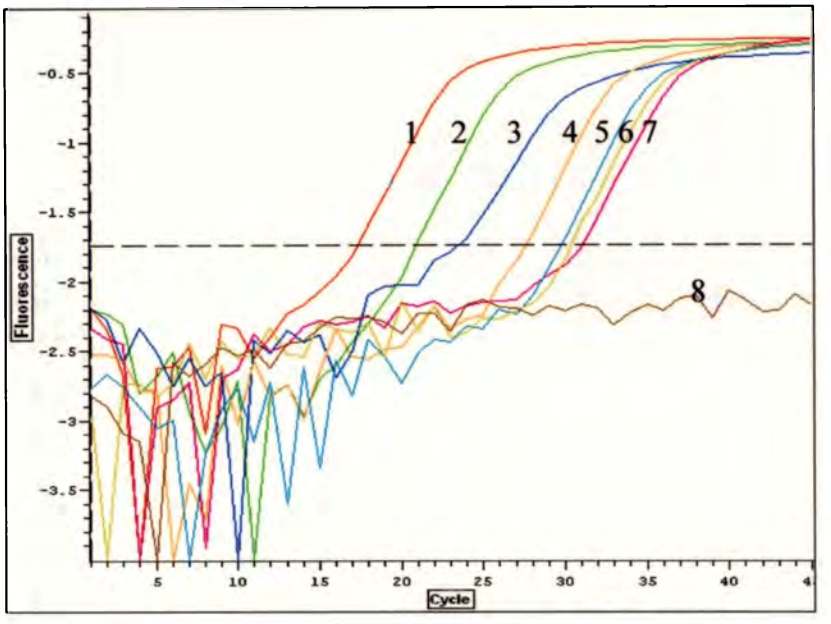


Fig.4.23 Real-time assay for serial diluted pure DNA of *Salmonella*. Curves 1-8 represents 5000, 500, 50, 5, 0.5, 0.05, 0.005, 0.002 pg of DNA, respectively.

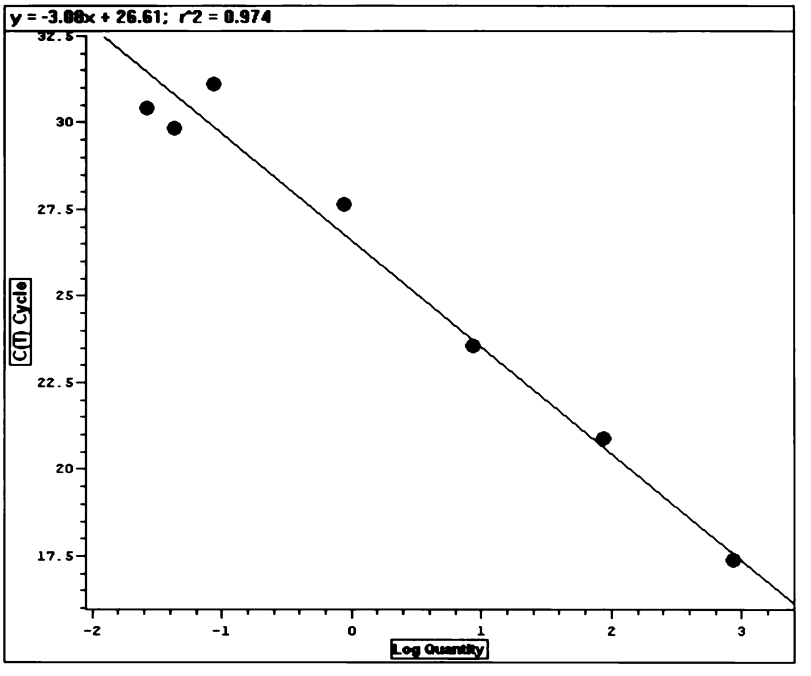


Fig. 4.24 Standard curve showing Ct value plotted against concentration of DNA (5000 pg -0.002 pg).

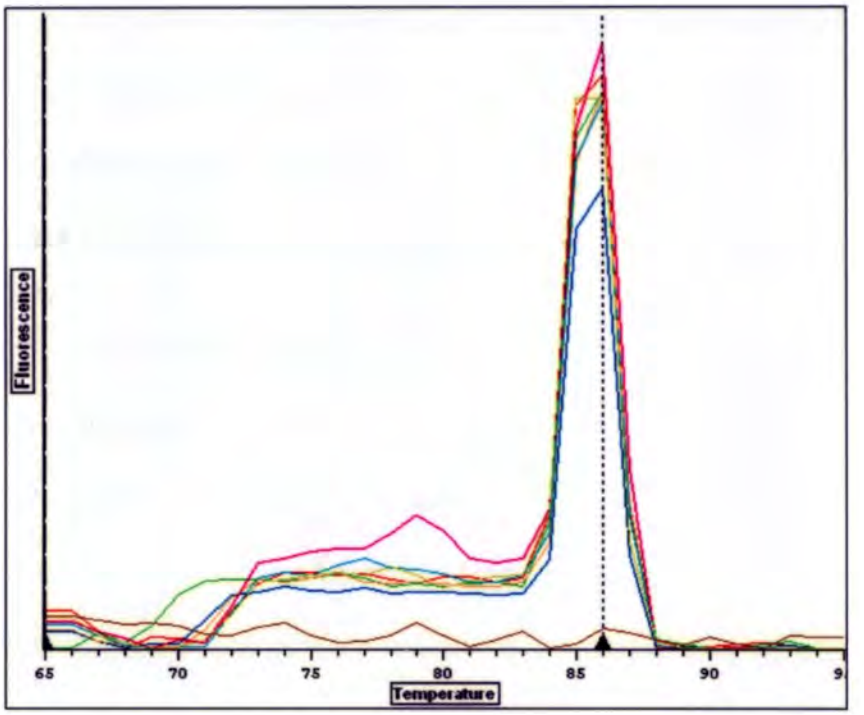


Fig. 4.25 Melting curve (Tm) analysis of real-time PCR products. Amplification derived from pure *Salmonella* DNA (5000 pg to 0.002 pg).

linear plot of Ct value and DNA from different dilution of fish samples spiked with *Salmonella* showed the level of detection in the fish homogenates inoculated with 2x10, $2x10^2$, $2x10^3$, $2x10^4$, $2x10^5$, $2x10^6$cfu/g are presented in Figures 4.29 & 4.30. The minimum level of detection of *Salmonella* in spiked fish and shrimp samples was found to be 20 cfu/g.

4.8.4.3 Quantification of *Salmonella* load in naturally contaminated fish and shrimp

The standard plot for *Salmonella* quantification was prepared using the known concentration of *Salmonella* DNA (5000 to 0.005 pg). Each time during the real-time PCR assay, the standard curve was extrapolated to determine the concentration of *Salmonella* DNA in seafood samples. The log concentration of DNA amplified was plotted against the Ct value. The quantitative data on *Salmonella* from naturally contaminated fish and shrimp showed the level of *Salmonella* load in fish and shrimp samples (Table 4.26) and representative samples amplified by real-time assay are given in Fig. 4.31. The lowest *Salmonella* load was detected in shrimp (*Metapenaeus dobsoni*) which was 0.25 pg/g of tissue, whereas, the highest *Salmonella* load (9000 pg/g) was detected in shrimp sample, corresponding to 50 cells to $1.8x10^6$ cells/g. The melting curve (Tm) value for seafood samples showed the nonspecific amplifications for *Salmonella* negative samples (Fig. 4.32). Except shrimp (*Metapenaeus dobsoni*) sample, the fish and shrimp samples positive by real-time assay were also detected positive by culture method (Andrews and Hammack, 2001).

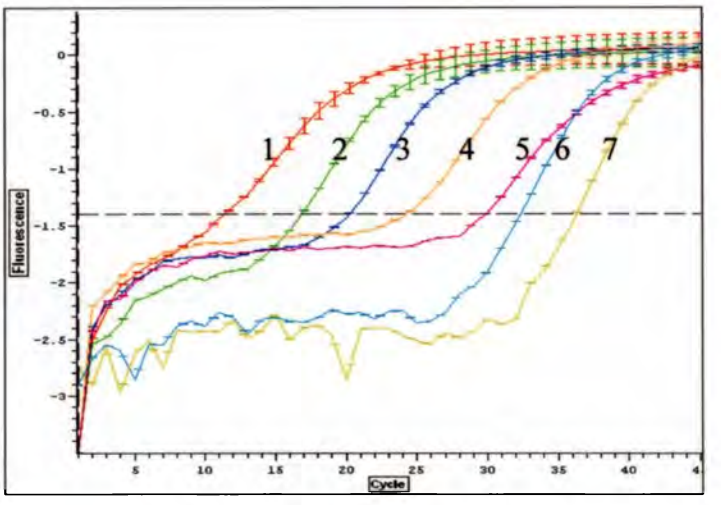


Fig. 4.26 Real-time assay in duplicate for *Salmonella* pure culture. Curves 1-7 represents amplification of DNA derived from *Salmonella* ($2x10^6$ to 2 cfu/ml).

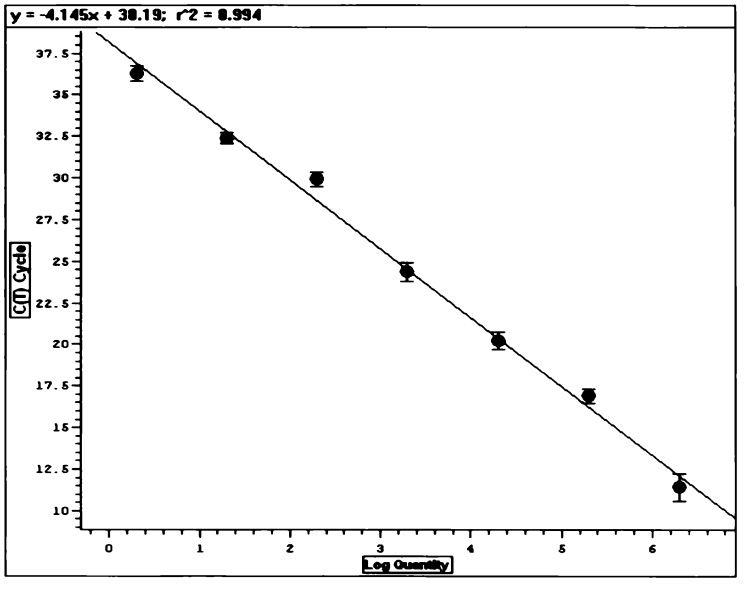


Fig. 2.27 Ct value plotted against concentration of DNA.
Amplification derived from $2x10^6$ to 2 cfu/ml of *Salmonella* in duplicate assays and showed the Standard deviation.

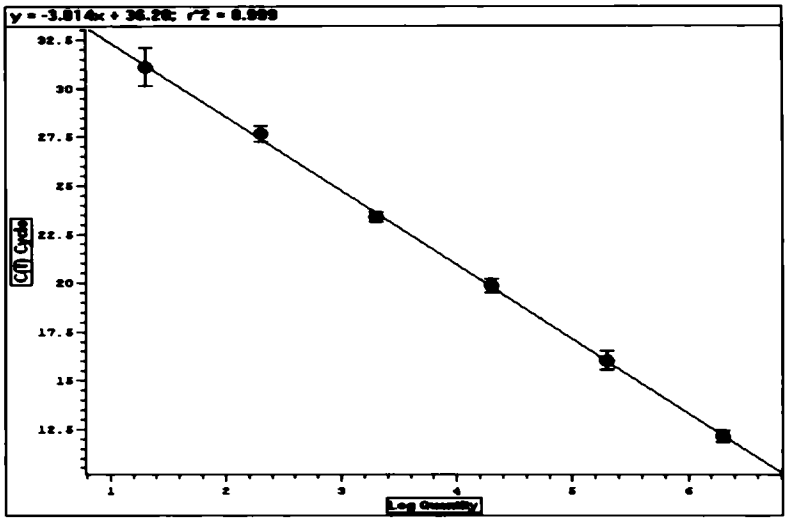


Fig. 4.28 Detection of *Salmonella* in seeded shrimp homogenates by real-time PCR. Ct value are plotted against log cfu/g and data are reported as means and standard deviation of two repeat experiments

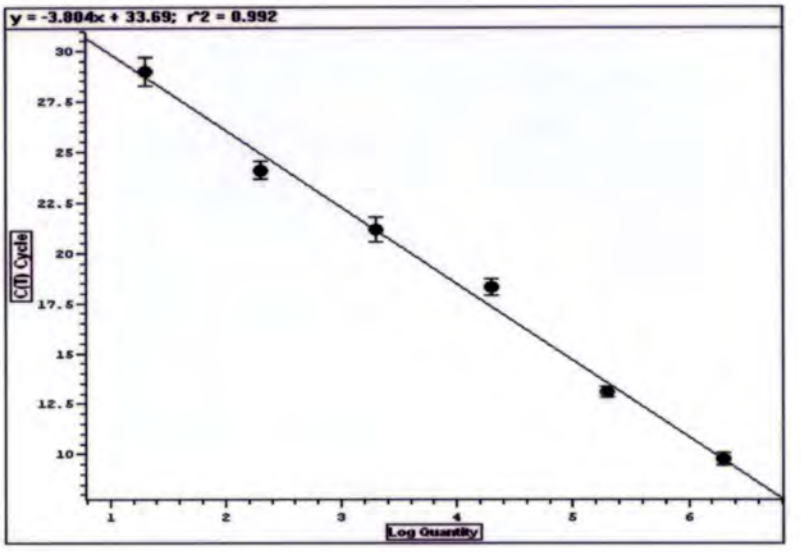


Fig. 4.29 Standard graph showing Ct value plotted against concentration of DNA. Amplification derived from *Salmonella* ($2x10^6$ to 20 cfu/g) in fish tissue. The graph also showed the Standard deviation from repeat experiments.

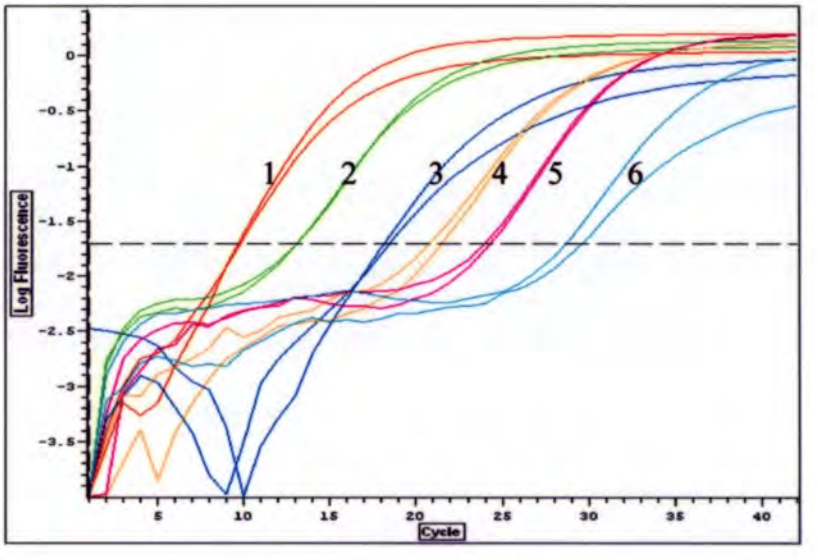


Fig. 4.30 Real-time assay in duplicate for *Salmonella* in spiked fish samples. Curves 1-6 represents amplification of DNA derived from *Salmonella* in fish tissue ($2x10^6$ to 20 cfu/g), respectively.

Table 4.26 Quantification of *Salmonella* in naturally contaminated fish and shrimp samples

Sl.	Sample	Real-time assay	Genome Equivalent (pg)/g of tissue	Culture method (USFDA)
1.	*Rastrelliger kanagurta*	Positive	31	Positive
2.	*Sardinella longiceps*	Positive	288	Positive
3.	*Penaeus monodon,*	Positive	9000	Positive
4.	*Rastrelliger kanagurta*	Negative	0	Negative
5.	*Sardinella longiceps*	Negative	0	Negative
6.	*Rastrelliger kanagurta*	Negative	0	Negative
7.	*Metapenaeus dobsoni,*	Positive	0.25	Negative
8.	*Penaeus monodon,*	Negative	0	Negative
9.	*Metapenaeus dobsoni,*	Negative	0	Negative
10.	*Penaeus monodon,*	Positive	0.52	Positive

Real-time (quantitative) PCR is increasingly being used for quantitative detection of pathogens in foods. There are still a few challenges with the widespread use of Real-time PCR for quantitative diagnostics. The detection limits are mainly determined by the amount of DNA that is present for amplification in the real-time reaction assay. Hence, extraction of DNA of the samples requires utmost care and precision. The other factor involved is inhibition from the food matrices. It may take a few more years to harmonize the real-time assay for *Salmonella* in seafood with other quantitative methods of detection. Real-time PCR method has been reported to be the most efficient and suitable quantitative method for enumeration of *Salmonella* in food and feed samples (Malorny et al., 2008). The real-time PCR assay developed in this study was found to be very useful and it could detect *Salmonella* spiked in fish and shrimp

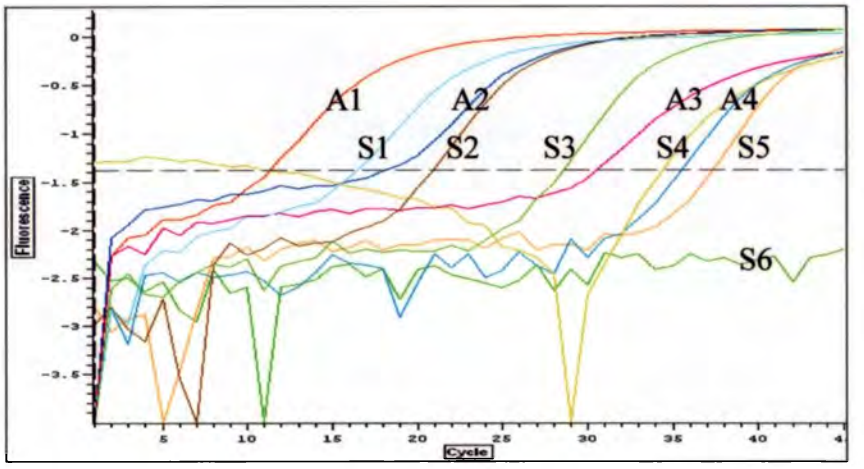


Fig. 4.31 Real-time assay for naturally contaminated fish and shrimp samples. Curves A1-A4 pure DNA used as a standard (5000 pg to 0.005 pg), curves S1-S4 representative positives from fish and shrimp samples, and S5 & S6 representative negatives.

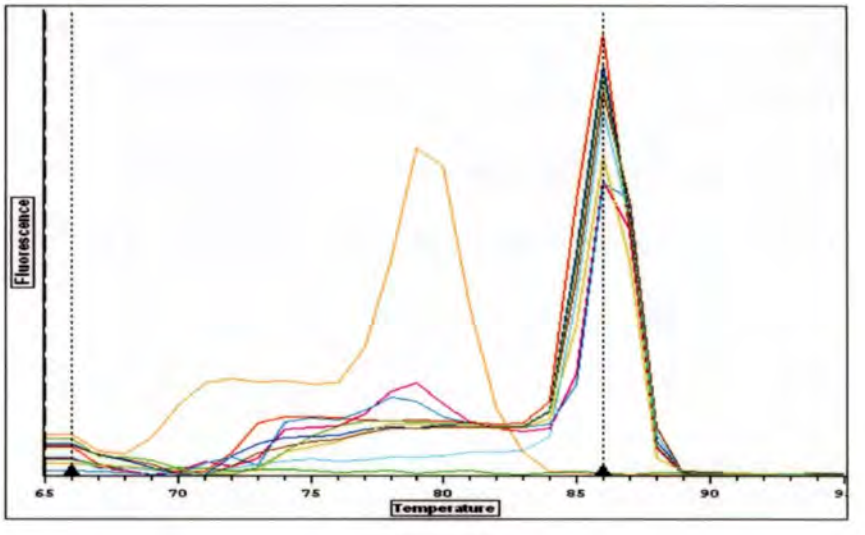


Fig. 4.32 Melting curve (Tm) analysis of real-time PCR products. Amplification derived from naturally contaminated fish and shrimp samples.

samples at a low level of 20 cells/g., whereas, in pure culture dilution detection limit was 2 cfu/ml. The results were in agreement with the Malorny et al. (2004) study. They have reported the detection of *Salmonella* (5 cfu/reaction) by real-time PCR assay. However, Wang and Levin (2006) showed the quantitative detection of *Vibrio vulnificus* in clam meat at 100 cfu/g by the quantitative PCR assay. The minimum detection limit for *Listeria monocytogenes* in water and skimmed milk was at 6 to 60 cfu/ml (Nogva et al., 2000). Eyigor (2002) and Malorny et al. (2004) compared the real-time PCR assay with conventional culture method for quantitative detection of *Salmonella* in contaminated food samples. The sensitivity of this study was far superior to some of the earlier stated study because, in this study the extraction of the genomic DNA was carried out with enzymatic digestion, followed by phenol: chloroform method. Though, this method is cumbersome and time-consuming, it is considered to be the most efficient method of DNA extraction from bacteria.

The quantitative information of *Salmonella* in naturally contaminated fish and shrimp samples showed the varying level of *Salmonella* contamination load in seafood. The results highlighted that real-time assay detected as low as 0.25 pg/g of *Salmonella* genome equivalents and seafood samples showed *Salmonella* load <100 cfu/g in various seafood sample. Hence, this method would be useful for detection of low level of *Salmonella* contamination in seafood.

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SUMMARY & CONCLUSION

5. SUMMARY AND CONCLUSION

Salmonella serovars are important food borne pathogens and often isolated from seafood worldwide. The incidences of *Salmonella* in seafood have been reported in India and abroad. Present study was mainly focused on *Salmonella* serovars in seafood of Cochin. This investigation consisted of three main parts. The first part deals with the prevalence and distribution of *Salmonella* serovars in seafood. The second part deals with biochemical and molecular characterization of *Salmonella* serovars isolated from seafood and the final part covered the development of rapid and sensitive molecular methods for detection of *Salmonella* from seafood. The important findings of this study are summarized as follows.

5.1 Prevalence of *Salmonella* in seafood

A total of 443 seafood samples consisting of pelagic fish (n=79), demersal fish (n=52), shrimp (n=86), lobster (n=25), crab (n=38), clam (n=41), mussel (n=31), oyster (n=27), squid (n=23), cuttlefish (n=21), and octopus (n=20) samples from the fish markets and landing centres of Cochin over a period of 4 years, from 2003 to 2007 were analyzed for presence of *Salmonella*. Isolation and identification of *Salmonella* from seafoods was carried out by BAM, USFDA and ISO methods. The prevalence of *Salmonella* was maximum in clams (34.1%) followed by mussel (31%), fish (30.2%) and shrimps (29.0%) samples. These values were higher, compared to prevalence of *Salmonella* in crab (10.5%), oyster (14.8%), squid (17.3%), and octopus (15.0 %) samples and the lowest incidence of *Salmonella* was

noted in lobster samples (8.0 %). The results further demonstrated that of a total of 443 seafood samples analyzed, an overall of 24.3% seafood were contaminated with *Salmonella*. The study also showed that lactose broth was comparatively superior for the isolation of *Salmonella* from seafood, compared to BPW. A comparison of different selective media indicated the Rappaport Vassiliadis (RV) and xylose lysine desoxycholate (XLD) agars were the most efficient media in the recovery of *Salmonella* in seafood.

5.2 Identification of *Salmonella* serovars from seafood

All *Salmonella* isolates were serotyped with antisera as per the Scheme for identification of *Salmonella* O, H and Vi antigens (Difco, USA). A total of 268 *Salmonella* isolates consisting of 32 different serotypes were isolated and identified in seafood. The major serotypes identified were *Salmonella* Atakpame, *Salmonella* Brancaster, *Salmonella* Georgia, *Salmonella* Ohio, *Salmonella* Typhimurium, *Salmonella* Newport, *Salmonella* Mbandaka, *Salmonella* Oslo, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Lindenburg, *Salmonella* Kottbus, *Salmonella* Bareilly, *Salmonella* Nchanga, *Salmonella* Emek, *Salmonella* Irumu, *Salmonella* Typhi, *Salmonella* Othmarschen, *Salmonella* Rissen, *Salmonella* Riggil, *Salmonella* Takoradi, *Salmonella* Virchow, *Salmonella* Washington, *Salmonella* Weltevreden, *Salmonella* Worthington, *Salmonella* II (3,10:lv:z_6), *Salmonella* II (47:enx, z_{15}:1,6), *Salmonella* IIIa (17:-:-), *Salmonella* IIIb (38:z:-), *Salmonella* IIIb (60:r:z), *Salmonella* VI (11:b:1,7) and *Salmonella* VI (45:a:enx). *Salmonella* Weltevreden was the predominant serotype in seafood, followed by *Salmonella* Rissen, *Salmonella* Typhimurium and *Salmonella* Derby. Twelve isolates were found untypable. The results of serotyping highlighted the presence of diverse

serovars prevalent in seafood and also pointing out a need for more robust serotyping facility in the country as some of the isolates could not be serotyped in India.

5.3 Biotyping of *Salmonella* based on utilization of sugars and amino acids

Bio-typing of *Salmonella* isolates was done based on Bergey's manual of systematic Bacteriology. Ten most predominant *Salmonella* serovars such as *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Ohio, *Salmonella* Irumu isolated from seafood were biotyped based on utilization of different sugars. A total of 12 sugars viz., dulcitol, glucose, lactose, maltose, mannose, mannitol, sucrose, cellobiose, arabinose, raffinose, trehalose, and xylose were used in this study to determine the sugar utilization pattern. All *Salmonella* serovars formed biotype S1 pattern, based on utilization of arabinose, dulcitol, glucose, maltose, mannose, raffinose, trehalose, and xylose sugars. The results further showed that none of the serovars utilized cellobiose, lactose, and sucrose. Utilization of other carbon sources such as inositol, salicin, sorbitol, citrate, and tartrate were found to be variable for different serovars.

The ten most prevalent *Salmonella* serovars isolated from seafood were characterized based on utilization of different amino acids viz., arginine, lysine, orinithine, valine, and phenylalanine. Results revealed the presence of four amino acid biotypes (A1, A2, A3, and A4) for *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, *Salmonella* Bareilly, *Salmonella* Lindenburg, *Salmonella*

Mbandaka *Salmonella* Irumu, and 2 biotypes (A1 and A3) were obtained in *Salmonella* Derby and *Salmonella* Braenderup strains.

5.4 Antibiotics resistance in *Salmonella* serovars

All *Salmonella* serovars were assayed for antibiotic susceptibility by disc diffusion assay on Muller Hinton agar. The isolates were tested against all major commercial antibiotics viz., sulphonamides, quinolones, beta-lactams, cephalosporins, tetracyclines, aminoglycosides, macrolides, and chloramphenicol. Results showed that all *Salmonella* serovars were 100% resistant to erythromycin. But, antibiotic resistance was not observed against ampicillin, ciprofloxacin, chloramphenicol, gentamicin, and kanamycin. *Salmonella* Lindenburg, *Salmonella* Rissen, *Salmonella* Takoradi and *Salmonella* Typhi isolates were resistant towards nalidixic acid. Sixteen out of 29 *Salmonella* serovars were resistant against sulphamethizol. Present study also determined the multi-drug resistance (MDR) in *Salmonella* serovars of seafood origin and results highlighted MDR in 49.3% , 31.8% , 10%, 0.4% of *Salmonella* isolates towards erythromycin and sulphamethizol (2 drug), erythromycin, sulphamethizol and carbenicillin (3 drug), erythromycin, sulphamethizol, carbenicillin and oxytetracycline (4 drug), erythromycin, sulphamethizol, carbenicillin, oxytetracycline and nalidixic acid (5 drug), respectively.

5.5 Plasmid profiling of *Salmonella* isolates

Predominant *Salmonella* serovars viz., *Salmonella* Weltevreden, *Salmonella* Rissen,, *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Ohio, and *Salmonella* Irumu were characterized for presence of small

and large plasmids by Alkaline lysis (Mini preparation) and Kado and Liu (1981) methods. Plasmids were isolated from *Salmonella* serovars such as *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Braenderup, *Salmonella* Lindenburg, and *Salmonella* Mbandaka isolates. Large Megadalton plasmids were isolated in *Salmonella* Typhimurium and *Salmonella* Derby isolates. A total of nine plasmid profiles were obtained from different *Salmonella* serovars associated with seafood. *Salmonella* serovars such as *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Barielly, *Salmonella* Irumu, *Salmonella* Ohio. *Salmonella* Oslo, and *Salmonella* Typhi did not harbour plasmids. Serovars without plasmid were placed under profile I. *Salmonella* Typhimurium showed 3 plasmid profiles (I, IIa, and IIIa) and harboured both small and large plasmids. *Salmonella* Derby and *Salmonella* Braenderup exhibited 3 plasmids profiles, however, each serovar harboured different plasmids of different sizes. *Salmonella* Braenderup harboured five plasmids of 1.5, 2.1, 3.5, 3.8, 4.1, and 9 kb in sizes. Plasmid profile of *Salmonella* Lindenburg isolates was observed to be most diverse in nature as five different plasmid profiles (I, II b, III b, III c, and IV d) were detected and plasmid profile IV d was detected in *Salmonella* Mbandaka isolates.

5.6 PCR-ribotyping of *Salmonella* serovars

Four most predominant *Salmonella* serovars viz., *Salmonella* Weltevreden (n = 22), *Salmonella* Rissen (n = 21), *Salmonella* Typhimurium (n = 18) and *Salmonella* Derby (n = 17) isolated from seafood were fingerprinted based on PCR-ribotyping pattern. *Salmonella* Weltevreden and *Salmonella* Rissen isolates exhibited three to four band patterns ranging from 700 to 1000 bp in both serovars, whereas, 700 to 900 bp ribotype patterns were observed in *Salmonella*

Typhimurium and *Salmonella* Derby. There were three ribotypes profile in *Salmonella* Rissen, and four major ribotype patterns were observed in *Salmonella* Derby and *Salmonella* Weltevreden strains.

5.7 ERIC-PCR assay for *Salmonella* serovars

Salmonella Weltevreden (n = 22), *Salmonella* Rissen (n = 21, *Salmonella* Typhimurium (n = 18) and *Salmonella* Derby (n = 17) isolated from seafood were molecular typed based on ERIC-PCR assay. DNA fingerprinting pattern of ERIC-PCR was analyzed with the Gel Compar II, Applied Maths BVBA, Belgium. UPGMA cluster analysis of ERIC-PCR profile in *Salmonella* Rissen and *Salmonella* Weltevreden showed the clonal variation with in the serovars. The level of similarity used for defining a type was set a 95%. The minimum Dice coefficient value for ERIC-PCR was observed at 32.87 % and 36.21 % for *Salmonella* Weltevreden and *Salmonella* Rissen, respectively, whereas, 44.5 and 47.5 % Dice coefficient values were obtained for *Salmonella* Typhimurium and *Salmonella* Derby, respectively. Sixteen different banding profile was observed for *Salmonella* Rissen, and six isolates (SR361, SR362), (SR429, SR520), and (SR415, SR428) showed similar homology (100%) with in the pair.

5.8 Discrimination indices of different typing methods

The discriminatory power of the fingerprinting methods was calculated using Simpson's index of diversity and expressed as the index of discrimination (Hunter and Gaston, 1988). Based on three different PCR-ribotype patterns the discrimination index of PCR-ribotypes for *Salmonella* Rissen was observed at 0.668, whereas, ERIC-PCR discrimination was attained at 0.969. The combined (PCR-ribotype & ERIC-PCR) index was reached at 0.974. Similarly, lower discrimination

index (0.680) was observed for *Salmonella* Weltevreden by PCR-ribotyping and combined index was recorded at 0.988. The combined discrimination indices obtained for *Salmonella* Typhimurium and *Salmonella* Derby by different typing methods was at 0.974 and 0.905, respectively.

5.9 PFGE analysis of *Salmonella* Weltevreden and *Salmonella* Typhi isolates

Different strains of *Salmonella* Weltevreden and *Salmonella* Typhi isolated from seafood were analyzed based on the pulsed field gel electrophoresis (PFGE) profile to ascertain the genetic relatedness among different isolates of *Salmonella* Weltevreden and *Salmonella* Typhi. PFGE analysis of *Salmonella* Weltevreden strains exhibited four main restriction patterns (X1, X2, X3 and X4) for *Xba* I restriction enzyme. *Salmonella* Weltevreden showed 14 to 18 restriction fragments whereas, *Salmonella* Typhi showed 12 to 14 DNA fragments. PFGE pulsotype of *Salmonella* Typhi revealed the two restriction patterns with *Xba*I and profile X2 was identified in 5/7 isolates. PFGE analysis demonstrated the intra serovar strain variation, hence, highlighted the multiple clones of the test isolates present in seafood.

5.10 Characterization of virulence genes in *Salmonella* serovars

All *Salmonella* serovars viz., *Salmonella* Atakpame, *Salmonella* Brancaster, *Salmonella* Georgia, *Salmonella* Ohio, *Salmonella* Typhimurium, *Salmonella* Newport, *Salmonella* Mbandaka, *Salmonella* Oslo, *Salmonella* Braenderup, *Salmonella* Derby, *Salmonella* Lindenburg, *Salmonella* Kottbus, *Salmonella* Bareilly, *Salmonella* Nchanga, *Salmonella* Emek, *Salmonella* Irumu, *Salmonella* Typhi, *Salmonella* Othmarschen, *Salmonella* Rissen, *Salmonella* Riggil, *Salmonella*

Takoradi, *Salmonella* Virchow, *Salmonella* Washington, *Salmonella* Weltevreden, *Salmonella* Worthington, *Salmonella* II (2 serovars), *Salmonella* IIIa, *Salmonella* IIIb, and *Salmonella* VI were *Salmonella* VI from seafood harboured three targeted virulence genes (*inv*A, *stn* and *fim*A gene) and produced desirable 284 bp, 260 bp and 85 bp gene amplicons, respectively. Exceptions were also observed, *Salmonella arizonae* (IIIa) strains did not exhibit the presence of *fim*A gene and weak *inv*A gene was observed in *Salmonella* Emek and *Salmonella* Lindenburg isolates.

5.11 Development of an eight hour PCR method for detection of *Salmonella* in seafood

Detection of *Salmonella* serovars from seafood was carried out with a different enrichment period, *viz.* 0, 2, 4, 6, and 8 h prior to PCR assay. All seafood samples were negative for *Salmonella* by PCR at zero hour. After 2 h enrichment, shrimp, mussel and edible oyster samples were positive for *Salmonella* and, an overall 5 % of seafood were found to be positive for *Salmonella*. With increase in enrichment periods to seafoods to 4, 6, and 8 h, showed improvement in detection rate as 14, 28, and 34 % respectively. The eight hour PCR exhibited 37/110 seafood samples positive for *Salmonella*, while, 27/110 seafood samples were positive for *Salmonella* by USFDA culture method. The results revealed that the newly developed 8 h PCR method was more sensitive than the culture method.

5.12 Comparison of culture, ELISA and PCR methods

A total of 215 seafood samples from different fish market and landing centers of Cochin were analyzed for the presence of *Salmonella* by culture (USFDA), ELISA and PCR methods. Results from the three assays were statistically analysed using software package SPSS 12.0 for Windows (SPSS Inc., Chicago,

USA). These three methods were considered as raters and the kappa coefficient was calculated to test the agreement. Based on kappa coefficient, the results were interpreted, as having fair agreement (0.21 to 0.40), moderate agreement (0.41-0.60), substantial agreement (0.61-0.80) and perfect agreement (0.81 to 1.0) between the raters. The comparison of different detection methods such as culture, ELISA and PCR showed that PCR was most sensitive for detection of *Salmonella* in seafood.

5.13 Development of the real-time assay for quantitative detection of *Salmonella* in seafood

Real-time PCR assay for was developed for the quantitative detection of *Salmonella* in seafood. Assay was first developed for DNA from pure *Salmonella* culture and seeded fish and shrimp samples. The quantitative detection of *Salmonella* in naturally contaminated shrimp and fish samples was carried out. The minimum detection sensitivity was 0.005 pg of the pure DNA in a PCR reaction, which corresponds to the genome of one *Salmonella* cell. The linear range of detection spanned from 7 log cycles of pure DNA ranging from 0.5 μg to 0.005 pg and further dilution did not provide any specific product. The minimum detection level in spiked seafood was 20 cfu/g. This method could quantify the level of *Salmonella* load in the naturally contaminated samples and observed that the natural contamination level of 0.25 to 9000 pg of *Salmonella* genome/g in seafood samples. The results obtained from the real-time PCR highlighted the presence of *Salmonella* cells <100 cfu/g of seafood samples. This assay would be ideal for rapid enumeration of *Salmonella* in seafood.

Suggestions for further research

1. Molecular source tracking of *Salmonella* serovars in seafood to identify the origin of *Salmonella* contamination in seafood.
2. Determine the prevalence of inherent *Salmonella* serovars in marine animals.
3. Expression studies of the *Salmonella* virulence genes to determine the pathogenicity levels in different *Salmonella* serovars.

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List of Publications

1. **Rakesh Kumar**, P.K. Surendran, and Nirmala Thampuran. 2008. Evaluation of culture, ELISA assays for the detection of *Salmonella* in seafood. ***Letters in Applied Microbiology***, 46, 221-226.

2. **Rakesh Kumar**, P.K. Surendran, and Nirmala Thampuran. 2008. An eight hour PCR for detection technique of *Salmonella* serovars in seafood. ***World Journal of Microbiology and Biotechnology***, 24, 627-631.

3. **Rakesh Kumar**, P.K. Surendran, and Nirmala Thampuran. 2008. Molecular fingerprinting of *Salmonella enterica* subsp. *enterica* Typhimurium and *Salmonella enterica* subsp. *enterica* Derby isolated from tropical seafood in South India. ***Molecular Biotechnology***, 40, 95-100.

4. **Rakesh Kumar**, P.K. Surendran, and Nirmala Thampuran. 2009. Detection and characterization of virulence factors in lactose positive and lactose negative *Salmonella* serovars isolated from seafood. ***Food Control***, 20, 376-380.

5. **Rakesh Kumar**, P.K. Surendran, and Nirmala Thampuran. 2009. Distribution and genotypic characterization of *Salmonella* serovars isolated from tropical seafood of Cochin, India. ***Journal of Applied Microbiology,*** 106, 515-524.