SALMONELLA IN AQUATIC ENVIRONMENT AND ITS SIGNIFICANCE

KAVITHA R. K., B.F.Sc.

Department of Fishery Microbiology

College of Fisheries, Mangalore

Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar

2007

SALMONELLA IN AQUATIC ENVIRONMENT AND ITS SIGNIFICANCE

KAVITHA R. K., B.F.Sc.

Thesis submitted to the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, in partial fulfillment of the requirements for the degree of

MASTER OF FISHERIES SCIENCES

IN

FISHERY MICROBIOLOGY

Mangalore March, 2007

Dedicated with love to

My parents

DEPARTMENT OF FISHERY MICROBIOLOGY
COLLEGE OF FISHERIES, MANGALORE
KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR

****

Certificate

This is to certify that the thesis entitled ***"Salmonella* in aquatic environment and its significance"** submitted by **Ms. Kavitha R. K.**, for the award of **Master of Fisheries Sciences** in **Fishery Microbiology** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of research work done by her during the period of study in this University under my guidance and supervision and the thesis is not previously formed the basis of the award of any degree, diploma, associateship of other similar titles.

Mangalore
March 2007

Prof. (Dr). Indrani Karunasagar
Professor and Head
Dept. of Fishery Microbiology

Approved by,

Chairman

Prof. (Dr). Indrani Karunasagar

Members

Prof. (Dr). B. A. Shamasundar

Prof. K. S. Udupa

Shri. M. M. Maragal

ACKNOWLEDGEMENTS

With deep sense of gratitude and esteem regards, I wish to place in record the scholarly guidance received from ***Dr. (Mrs). Indrani Karunasagar****, Professor and Head, Department of Microbiology, College of Fisheries, Mangalore, in planning and executing this piece of work. I ever remain indebted to Madam for her love and affection during my stay in the department.*

With profound regards, I express my obligations owed to ***Prof. (Dr). Iddya Karunasagar****, National Professor, ICAR for his invaluable suggestions and inputs during the work.*

My sincere thanks go to the advisory committee members, ***Dr. B. A. Shamasundar****, Professor, Department of Fish Processing Technology,* ***Shri. M. M. Maragal****, Associate Professor, Department of Biochemistry and* ***Shri. K. S. Udupa****, Professor and Head, Department of Fishery Statistics, College of Fisheries, Mangalore, for their encouragement and moral backup.*

I also thank ***Dr. M. N. Venugopal,*** *Professor, Department of Microbiology, College of Fisheries, Mangalore for his support during my stay in the department.*

I greatly acknowledge the institution facilities provided to me by the Dean, College of Fisheries, Mangalore.

Thanks are due to the helps received from the staff of Central Research Institute, Kasauli, Uttar Pradesh for serotyping the bacterial isolates.

I remain thankful to all my teachers for their inspirations, senior friends in the department for their kind help, classmates and junior friends for their affection, non-teaching staffs of the college for their lending hand during my stay in the college.

No words can express my heartfelt devotion and foremost indebtedness to my beloved parents whose blessings and sacrifice, to my sisters whose unlimited love made me reach this stage.

Mangalore,
March 2007

(Kavitha R. K.)

CONTENTS

Introduction

1. INTRODUCTION

Salmonella is one of the most important and widely studied bacterium responsible for food borne diseases throughout the world. Despite extensive scientific investigation and worldwide prevention policies, typhoid fever remains a major public health problem in developing countries, responsible for an estimated 17 million new infections and 6, 00,000 deaths each year (Pang, 1998; Sinha *et al.,* 1999; Parry, 2003; Crump *et al.,* 2004; Bhan *et al.,* 2005; Connor and Schwartz, 2005). Human adapted *Salmonella* serotypes like Typhi and Paratyphi result in grave systemic diseases like typhoid fever, whereas, infections with non-typhoid *Salmonella* serotypes most often lead to self-limited acute gastroenteritis. Though these serotypes are limited to gastroenteritis, approximately 5% of individuals develop bacteremia. With some underlying conditions, children are at increased risk of bacteremia, which may lead to extra intestinal focal infections. Occasionally non-typhoid *Salmonella* bacteremia can be more serious in adult patients with underlying diseases. Interestingly nontyphoidal *Salmonella* are an important cause of food borne illness in developed countries (Martinez-urtaza *et al., 2003*).

Intestinal tract of humans and animals are the principal habitat of *Salmonella*. Therefore, the pathogen can reach the environment through their excreta. *Salmonella* is found to survive for days in groundwater, pond water, seawater and for months in contaminated eggs and frozen seafood (Baudart *et al*., 2000). Infection can either be as a result of direct contact with animals (Fone and Barker, 1994) or from the consumption of contaminated food (Humphrey *et al.,* 1998). Therefore, the most common vehicles of transmission are meat, meat products, eggs and egg products that contain *Salmonella* serovars as a result of infection or faecal contamination. Seafood harvested or washed with faecal and animal waste contaminated coastal waters, also become one of the routes of human infection by *Salmonella*.

Antimicrobial resistance among non-typhoid *Salmonella* serotypes has been a serious problem worldwide. The emergence of multidrug resistant *Salmonella enterica* serotype Typhimurium, like the strains DT104 among *Salmonella* spp. might have further added to the difficulty in treating patients with such infections. Further, the reports on the outbreak of drug-resistant DT29 among cattle and humans raised concerns about the potential public health risks of the veterinary and aquaculture use of antibiotics. Since then several reports have been published on the occurrence of drug

resistant *Salmonella* spp. from various food and environmental samples, which is of greater epidemiological, microbiological and public health concern.

Aquatic environment becomes a common place of human activities such as use of water for washing domestic animals, recreation, fishing and more over a dumping ground for sewage wastes and agricultural runoff particularly in developing countries. Among this, estuaries and coastal waters receive huge amount of such waste. As a result, human and animal origin pathogens such as *Salmonella* along with other disease causing organisms make their way into the aquatic systems. Invariably, seafood harvested from such water bodies harbor human pathogens. Therefore, it becomes difficult for the seafood processors to meet the zero tolerance of *Salmonella* as the pathogen becomes part of the aquatic system. Therefore, understanding of the aquatic environments for the incidence and prevalence of *Salmonella* spp. based on precise methods of detection would help in developing strategies for ensuring the safety of processed seafood. Further, studies on antibiotic resistance and genetic diversity of the *Salmonella* isolates would strengthen us in coming out with promising control measures for seafood borne illness. Against this background, the present study was undertaken with the following objectives.

1. To study the incidence and prevalence of *Salmonella* spp. in aquatic environment associated with seafood.
2. To compare polymerase chain reaction (PCR) detection methods of *Salmonella* spp. to that of conventional methods.
3. To study the antibiogram pattern and genetic variability of confirmed *Salmonella* isolates.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Historic background

Long before the bacteriological era began, typhoid fever was defined with clinical signs and symptoms. This was the first Salmonellosis ever recognized (Le Minor, 1981). Louis (1829) grouped several enteric fevers under the name typhoid. The typhoid bacillus was first observed by Eberth (1880) in spleen sections and mesenteric lymph nodes of a typhoid patient. Later, Gaffky in 1884 succeeded in culturing the bacterium. Serodiagnosis of typhoid was possible when Widal in 1896, Grunbaum in 1896 and Widal and Sicard in 1897 showed that serum of typhoid patients agglutinates the typhoid bacillus (Le Minor, 1981).

In 1896, two isolates were recovered by Achard and Bensaude (1896) from two patients with clinical typhoid, but with negative Widal serodiagnosis. They called the disease, paratyphoid and the organism "bacille paratyphique". Other bacteria resembling paratypus bacteria were also isolated from diseased animals. Salmon and Smith in 1885 isolated such bacteria from pigs with hog cholera and assumed that they isolated the infectious agent of hog cholera (Salmon, 1885). Similar bacteria were also isolated from food borne intoxications and animal diseases.

Antigenic analysis of *Salmonella* began when Castellani (1902) described a method for absorbing antisera (Le Minor, 1981). The first antigenic scheme of White (1926) was further developed by Kauffmann (1966, 1978). The Kauffmann-White scheme contained 100 serotypes in 1941 and had about 2250 in 1988 (Le Minor and Popoff, 1988). A new bacterial genus was created to include these bacteria and was named *Salmonella* by Ligneres in 1900 in honor of Dr. Salmon who isolated the then thought to be infectious agent of hog cholera (ICMSF, 1996).

2.2. Classification

Genus *Salmonella* is a member of the family Enterobacteriaceae. It is composed of bacteria related to each other both phenotypically and genotypically with DNA base composition 50-52 mol percentage G+C (Marmur *et al.,* 1963). Based on biochemical characteristics, Kauffmann (1963) subdivided the genus *Salmonella* into four subgenera, *viz.,* subgenera I, II, III and IV with subgenus III corresponding to bacteria called *Arizona.* Second taxonomic system was proposed by Edwards and Ewing (1972) in which the genus *Salmonella* was limited to Kauffmann's subgenera I. In this system

of classification, bacteria belonging to Kauffmann subgenera III was placed as a separate genus and the Kauffmann subgenera II and IV were considered as atypical strains of either *Salmonella* or *Arizona*. Subsequently, it was found that all *Salmonella* serovars form a single DNA hybridization group. The thermal stability of DNA hybrids indicates seven subgroups. The level of relatedness among DNA subgroups is consistent with that of subspecies within one *Salmonella* species, although subspecies V is somewhat less related and could possibly represent a second species (Cross *et al.,* 1973, Le Minor *et al.,* 1982; Reeves *et al.,* 1989). Subgroups are designated as Salmonella I, II, IIIa, IIIb, IV, V and VI.

2.3. Nomenclature

Salmonella nomenclature has been controversial since the original taxonomy of the genus was not based on DNA relatedness, rather, names were given according to clinical consideration or host specificity like *Salmonella typhi*, *S. choleraesuis* etc. Later, when serological analysis initiated by White (1926) and Kauffmann (1941, 1966) who extended his work, defined the species as "a group of related fermentation phage-type" and considered that each serovar is a separate species. Since, the host specificity suggested by some of the earlier names does not exist, the names derived from the geographical origin of the first isolated strain of the newly discovered serovars was next chosen for species name, for example *Salmonella london*, *S. panama* etc.

The genus *Salmonella* initially encompassed five species *viz., Salmonella arizonae*, *S. choleraesuis* (type species of the genus), *S. enteritidis*, *S. typhi* and *S. typhimurium*. The DNA relatedness studies demonstrate that all *Salmonella* serovars form a single hybridization group with seven subspecies. Accordingly, Le Minor *et al.* (1982, 1986) proposed the name *Salmonella choleraesuis* for the single *Salmonella* species with the following seven subspecies: *Salmonella choleraesuis* subspecies *arizonae*, *S. choleraesuis* subspecies *bongori*, *S. choleraesuis* subspecies *choleraesuis*, *S. choleraesuis* subspecies *diarizonae*, *S. choleraesuis* subspecies *houtenae*, *S. choleraesuis* subspecies *indica* and *S. choleraesuis* subspecies *salamae*. The name *Salmonella choleraesuis* has been chosen, because it is the name of the type genus.

Since the name, *Salmonella choleraesuis* can lead to confusion as the specific epithet is also the name of a serovar, Le Minor and Popoff (1987) proposed to use the

name *Salmonella enterica* that has not been used previously for any serovar. Accordingly, names were given as *Salmonella enterica* subspecies enterica, *S. enterica* subspecies salamae, *S. enterica* subspecies arizonae, *S. enterica* subspecies diarizonae, *S. enterica* subspecies houtenae, *S. enterica* subspecies bongori and *S. enterica* subspecies indica.

Later in 1989, Reeves *et al.* (1989) elevated the subspecies *Salmonella enterica* subspecies bongori in rank to a species. According to rule 50a of the bacteriological code (1990 revision), the name of this new species is designated as *Salmonella bongori*. Consequently, the species *Salmonella enterica* encompasses six subspecies.

So, there are two systems of nomenclature that exist now; one which confers the rules of bacteriological code (1990 revision) and another, the proposal of Le Minor and Popoff (1987) which is followed by the large majority of the bacteriologists, publications and centre for disease control and prevention (CDC). According to this, genus *Salmonella* has two species, *viz., Salmonella bongori* and *Salmonella enterica.* The type species *S. enterica* is divided into six subspecies.

Salmonella enterica subspecies *enterica*

Salmonella enterica subspecies *salamae*

Salmonella enterica subspecies *arizonae*

Salmonella enterica subspecies *diarizonae*

Salmonella enterica subspecies *houtenae*

Salmonella enterica subspecies *indica*

Tindall *et al.* (2005) in their taxonomic note gave this recent classification to be appropriate for future references on *Salmonella*. They hoped that the recent actions of the judicial commission (2005) have allowed the nomenclature changes envisaged by Le Minor and Popoff (1987) to take effect for all future references. In addition, the taxonomic opinion of Le Minor and Popoff (1987) and Reeves *et al.* (1989) that the genus *Salmonella* should currently comprise two species and that the type species *Salmonella enterica* should be divided into six subspecies, is currently the taxonomy and nomenclature used by the WHO and other organizations (Tindall *et al.*, 2005).

2.4. General characteristics

Salmonella are Gram negative, rod shaped bacteria belonging to Enterobacteriaceae family. They are facultative anaerobes and have both respiratory and fermentative metabolism of carbohydrates. *Salmonella* are positive for catalase and negative for oxidase enzymes and produce acid and gas from glucose (ICMSF, 1996). *Salmonella* are usually motile except *S. pullorum* and *S. gallinarum*. Motile forms have peritrichous flagella. Percentage G+C content of DNA is estimated to be 50-53%. *Salmonella* may harbor phages or plasmids that code for metabolic characters like H_2S production and lactose or sucrose fermentation, which are used for identification. *Salmonella* spp. cause a variety of diseases in both humans and animals, which range from a self-limiting enterocolitis to more systemic illness such as typhoid fever. Some serotypes are host adapted such as *S. typhi*, while others, such as *S. typhimurium* and *S. entertidis* are ubiquitous and can cause disease in a large variety of hosts (Groisman *et al.*1999). The type of disease caused by these organisms depends not only on the serovar but also on the infected host. *S. typhimurium,* for example causes gastroenteritis in humans while in mice it causes a severe systemic illness, much like typhoid fever.

2.5. Antigenic structure and serotypes

The antigenic classification or serotyping of *Salmonella* used today is a result of extensive studies of antibody interaction with bacterial surface antigens. As summarized by Le Minor (1981), the genus *Salmonella* has three kinds of major antigens *viz.,* somatic (O) or cell wall antigens, surface (Vi) antigens and flagellar (H) antigens, all with diagnostic or identifying applications. Somatic antigens are heat stable and alcoholic resistant. Cross absorption studies individualize a large number of antigenic factors, 67 of which are used for serological identification. Some *Salmonella* serovars show surface antigens. Surface antigens in *Salmonella* may mask 'O' antigens and the bacteria thus are not agglutinated with 'O'antisera. One well-known specific surface antigen, 'Vi' antigen occurs in only three *Salmonella* serovars, Typhi, Paratyphi C and Dublin. Flagellar antigens are heat labile proteins. A few *Salmonella enterica* serovars produce flagella, which always have the same antigenic specificity. Such an antigen is called monophasic. Most *Salmonella* serovars however, can alternatively

produce flagella with two different 'H' antigenic specificities. The 'H' antigen is then called diphasic. Specific serotypes were defined as a result of complex antigen variability. This resulted in the identification of over 2000 *Salmonella* serotypes.

2.6. Ecology and distribution

Salmonellae are widely distributed in the environment. The principle habitat of *Salmonella* is the intestinal tract of humans and animals (Le minor, 1981). Many *Salmonella* serovars are host adapted and are found predominantly in one particular host. Some serovars are ubiquitous (non-host adapted) and still some have unknown habitat. *Salmonella* serovars that are associated with humans usually cause grave diseases like typhoid and often are found in the blood. In humans, ubiquitous *Salmonella* organisms are mostly responsible for food borne toxic infections. *Salmonella* excretion by patients may continue long after clinical cure. *Salmonella* may survive in sewage if improperly treated and may reach riverine and coastal waters. *Salmonella* may be associated with all kinds of food. The contamination of meat may originate from animal Salmonellosis but most often it results from contamination of muscles with the intestinal contents during evisceration of animals. Vegetables and fruits can harbor *Salmonella* when fertilizers of faecal origin are used or when contaminated water is used to wash them. Domestic fowls and animals, wild birds, mammals and reptiles constitute an important reservoir of *Salmonella* (Will *et al.,* 1973).

2.7. *Salmonella* pathophysiology

The principle clinical syndromes associated with *Salmonella* infection are enteric fever and gastroenteritis (Miller and Pegues, 2000). Enteric fever is a protracted systemic illness that results from infection with exclusively human pathogens. Clinical manifestations include fever, abdominal pain, transient diarrhea and occasionally a maculopapular rash. The pathological hallmark of enteric fever is mononuclear cell infiltration and hypertrophy of the reticuloendothelial system including intestinal Peyer's patches, mesenteric lymph nodes, spleen and bone marrow. In contrast, many non-typhoid *Salmonella* strains such as enteritidis and Typhimurium infect a wide range of animal host including poultry, cattle and pigs and usually cause self-limiting enteritis in humans (Michael and Samuel, 2001).

Salmonella invade epithelial cells by a morphologically distinct process termed bacterial mediated endocytosis (Francis *et al.*, 1992). Shortly after bacteria adhere to

the apical epithelial surface, profound cytoskeletal rearrangements occur in the host cell, disrupting the normal epithelial brush border and inducing the subsequent formation of membrane ruffles that reach out and enclose adherent bacteria in large vesicles. In addition to invasion of intestinal epithelial barrier, *Salmonella* serotypes clinically associated with enteritis induce a secretary response in the intestinal epithelium and initiate recruitment and transmigration of neutrophils into the intestinal lumen (Galyov *et al.,* 1997). Once across the intestinal epithelium *Salmonella* encounters another obstacle of innate immunity, the submucosal macrophage. *Salmonella* serotypes that cause systemic infection enter macrophages and subsequently activate virulence mechanism that allow evasion of the microbicidal functions of the phagocyte permitting survival and replication in the intracellular environment (Alpuche-Aranda *et al.,* 1994).

2.7.1. *Salmonella* virulence plasmids

Many *Salmonella* serovars harbor low copy number virulence plasmids important for systemic infection (Gulig, 1990; Wallis, *et al,* 1995). *Salmonella* virulence plasmids vary in size from 50-90 kb depending on the serovars (Guiney, *et al.,* 1994). All plasmids contain the 7.8 kb *Salmonella* plasmid virulence (*spv*) locus. This locus harbors five genes designated *spv* R, A, B, C and D. Expression of *spv* genes might play a role in the multiplication of intracellular *Salmonella* (Rotger and Casadesus,1999). Other than this, proteins encoded by the virulence plasmid genes *tra*T*, rck*, and *rsk* might be involved in resistance of *Salmonella* spp. to the bacteriolytic activity of serum. Friedrich *et al.* (1993) discovered a 7 kb *pef* operon region that contained genes for plasmid-encoded fimbriae. It is thought to be important in the colonization of *Salmonella* in the host alimentary tract, thereby increasing the bacterial load in the vicinity of epithelial cell lining.

2.7.2. *Salmonella* pathogenicity islands

Pathogenicity islands represent distinct, often large regions on the chromosome that contain virulence genes (Hensel, 2004). Many of the virulence phenotypes of *Salmonella enterica* are encoded by genes on pathogenicity islands, which are referred to as Salmonella Pathogenicity Islands (SPI). At present twelve different SPIs have been described. Some of these are conserved throughout the genus *Salmonella,* while others are specific for certain serovars (Hensel, 2004).

2.7.2.1. *Salmonella* pathogenicity island 1 (SPI-1)

Salmonella Pathogenicity Island 1 (SPI-1) is the best characterized among the pathogenecity islands of *Salmonella*. It is approximately 40 kb in size and has an overall G+C content of 42%. SPI-1 contains at least 29 genes encoding various components of type three secretion system (TTSS), its regulators and secreted effectors (Collazo and Galan, 1997). One subset of SPI-1 effector proteins mediates the invasion of nonphagocytic cells by *Salmonella*, a process that involves the modification of the actin cytoskeleton and a second subset associated with enteropathogenesis (Wallis and Galyov, 2000). SPI-1 is present in *S. bongori* and all subspecies and serotypes of *S. enterica* (Ochman and Groisman, 1996).

2.7.2.2. *Salmonella* pathogenicity island 2 (SPI-2)

The *Salmonella* Pathogenicity Island 2 (SPI-2) locus is 40 kb in size and is composed of two distinct elements (Hensel *et al.*, 1999a). A portion of 25 kb is present only in *S. enterica* and essential for systemic pathogenesis. This element encodes a second, type three secretion system in *S. enterica* which is structurally and functionally distinct from that of SPI-1 and is essential for the growth of bacteria rather than its survival against host immune response (Shea *et al.,* 1999). A second distinct element of SPI-2 is a 15 kb region present both in *S. enterica* and *S. bongori*. This encodes for the tetrathionate reductase enzyme and is involved in anaerobic respiration (Hensel *et al.,* 1999b).

2.7.2.3. *Salmonella* pathogenicity island 3 (SPI-3)

Salmonella Pathogenicity Island 3 (SPI-3) is an insertion element of 17 kb with a G+C content of 47.5% (Blanc-Potard *et al.,* 1999). The major virulence function encoded by SPI-3 is the MgtCB high affinity Mg^{+2} uptake system that is required for adaptation to the nutritional limitations of the intraphagosomal habitat (Blanc-Potard and Groisman, 1997).

2.7.2.4. *Salmonella* pathogenicity island 4 (SPI-4)

Salmonella Pathogenicity Island 4 (SPI-4) is a 25 kb large island needed for intramacrophage survival and is likely to carry a type-I secretion system involved in toxin secretion (Wong, *et al* 1998).

2.7.2.5. *Salmonella* pathogenicity island 5 (SPI-5)

Salmonella Pathogenicity Island 5 (SPI-5) is a small locus of 7.6 kb size. SPI-5 encodes effector proteins for both the type three secretion system encoded by SPI-1 and SPI-2 (Hensel, 2004). SPI-5 contributes to enteric, but not to systemic Salmonellosis (Wood, *et al.*, 1998).

2.7.2.6. *Salmonella* pathogenicity island 6 (SPI-6)

Salmonella Pathogenicity Island 6 (SPI-6) is also known as Salmonella Chromosomal Island. It is a 59 kb locus and contains the *saf* gene cluster for fimbriae, *pag*N encoding an invasion and several genes of unknown function (Hensel, 2004).

2.7.2.7. *Salmonella* pathogenicity island 7 (SPI-7)

Salmonella Pathogenicity Island 7 (SPI-7) is also known as Major Pathogenicity Island. It is 133 kb in size and is specific for serovars Typhi, Dublin, and Paratyphi C. An important virulence factor encoded by SPI-7 is the Vi antigen, a capsular exopolysaccaride (Hensel, 2004).

2.7.2.8. *Salmonella* pathogenicity island 8 (SPI-8)

Salmonella Pathogenicity Island 8 (SPI-8) is a 6.8 kb locus identified in the genome sequence of *S. enterica* serovar Typhi. Putative virulence factors are bacteriocin genes, but no functional data have been reported so far (Hensel, 2004). SPI-8 appears to be specific for servoar Typhi, however, the distribution has not been investigated in detail.

2.7.2.9. *Salmonella* pathogenicity island 9 (SPI-9)

Salmonella Pathogenicity Island (SPI-9) locus of 16281 bp is located adjacent to a lysogenic bacteriophage in the chromosome of serovar Typhi. Putative virulence factors encoded by SPI-9 are a type-I secretion system and a large repeat in toxin (RTX) like protein (Hensel, 2004).

2.7.2.10. *Salmonella* pathogenicity island 10 (SPI-10)

Salmonella Pathogenicity Island 10 (SPI-10) is a large insertion of 32.8 kb. There is also a cryptic bacteriophage present within SPI-10. Virulence factors encoded by SPI-10 are the *sef* fimbriae. The distribution of *sef* fimbriae is restricted to a subset of

serovars including Typhi and Enteritidis and is considered as one of the factors that determines host specificity (Townsend *et al.,* 2001).

2.7.2.11. Salmonella genomic island (SGI)

Salmonella genomic island (SGI) of size 43 kb is found in multidrug resistant strains of *S. enterica* serovar typhimurium DT104, Paratyphi B and Agona. Within SPI-1, genes conferring the penta-resistance phenotype (resistance to tetracycline, ampicillin, chloramphenicol, streptomycin, and sulfonamides) are clustered in the multidrug resistance region composed of two integrons (Hensel, 2004). A new variant of SGI was identified in serovar Albany (Doublet *et al.,* 2004) wherein the antibiotic resistance gene cluster containing the streptomycin resistance gene was replaced by a trimethoprim resistance gene.

2.7.2.12. High pathogenicity island (HPI)

Salmonella high pathogenicity island (HPI) encodes the biosynthesis pathway for siderophore and the cognate iron uptake system. HPI detected in variety of other Gram-negative species is shown to be correlated to the ability of strains to cause septicaemic infections. The presence of HPI in subspecies IIIa, IIIb and IV of *S. enterica* was reported by Oelschlaeger *et al.* (2003). However, HPI is absent from human adopted subspecies I isolates (Hensel, 2004).

2.8. Antibiotic resistance in *Salmonella*

Antimicrobial resistance in *Salmonella* spp. is a major health problem in human and veterinary medicine worldwide. Antibiotic resistance can be the result of mutations and acquisition of resistance encoding genes (Fluit, 2005). Many antimicrobial resistance genes are associated with genetic elements called integrons, which can either be located on transposons, plasmids or chromosome (Leverstien-van *et al.*, 2003). Integrons have the ability to integrate and express genes coding for antibiotic resistance (Stokes and Hall, 1989; Hall and stokes, 1993). Class 1 integrons, the most common integron type have been detected in different *Salmonella* serovars (Fluit, 2005) and found located on the so-called *Salmonella* genomic island 1 (SGI1).

Several researchers have isolated drug resistant *Salmonella* spp. from various sources from different regions. It was found that *Salmonella typhimurium,* definitive type 104 (DT104) is an increasingly common multiple antibiotic resistant strain of *Salmonella*, that emerged rapidly throughout the world (Glynn *et al.*, 1998; Lawson *et*

al, 2004). DT104 is characterized by its resistance to ampicillin, chloramphenicol, streptomycin, sulphonamide, tetracycline and is commonly referred to as having resistance (R) type ACSSuT and has emerged as a major global health problem (Threlfall *et al*, 1994, Casin *et al.,* 1998). More than 90% of the strains of *Salmonella* isolated by Hatha and Lakshmanaperumalsamy (1997) from seafood of India were resistant to bacitracin, penicillin and novobiocin. The least resistance was observed to chloramphenicol (6.7%) and nalidixic acid (12%). In clinical isolates from India (Shanahan *et al.,* 1998), eleven of the *S. typhi* strains possessed resistance to chloramphenicol, trimethoprim and amoxicillin, while four of the isolates were resistant to each of these agents except for amoxicillin. Six of the isolates were completely sensitive to all of the antimicrobial agents tested. All the *S. typhi* isolates were susceptible to cephalosporin agents, gentamicin, amoxicillin plus clavulanic acid and imipenem. Additional resistance to quinolones such as nalidixic acid and ciprofloxacin has also been described in recent years (Piddock, 2002). One *Salmonella* isolated from frozen anchovies imported from Cambodia was resistant to six antimicrobials including ampicillin, amoxicillin, chloroamphenicol, sulfamethoxazole, tetracycline and trimethoprim (Zhao, *et al* 2003). Lailler *et al.* (2005) recorded multi-drug resistant *Salmonella* spp. up to a prevalence of 1.9% in bovine herds of western France. Harakeh *et al*. (2006) reported high percentage of resistance of *Salmonella* isolated from marine environment to cefuroxime and trimethoprim / sulfamethoxazole and a moderate resistance to cefotaxime as compared with low resistance in clinical isolates. As many as 135 strains of *Salmonella enterica* serovar Infantis isolated from poultry in Kagoshima, Japan, were examined for antimicrobial resistance by Shahada *et al*. (2006). One strain (0.7%) was resistant to ampicillin, 97% to streptomycin, 95.6% to sulphamethoxazole, 96.3% to oxytetracycline, 11.1% to kanamycin and 36.3% to ofloxacin. Multiresistant phenotypes identified were ASSuT-Km, SSuT-Km, SSuT-O and SSuT. Approximately 89% of oxytetracycline-resistant strains carried the *tet*A gene and all of the 131 streptomycin-resistant isolates carried the *aad*A1a gene. As many as 40% of the kanamycin-resistant isolates carried the *aph*A1-*lab* gene. All isolates were susceptible to chloramphenicol. Multidrug-resistant and extended-spectrum beta-lactamase (ESBL) - producing *Salmonella enterica* (serotypes Typhi and Paratyphi A) were recently isolated from blood isolates in Nepal (Pokharel *et al.,* 2006). Kariuki *et al.,* (2006) isolated non-typhoid *Salmonella* from clinical samples, which were susceptible to cefotaxime and ciprofloxacin. In the isolates of Taiwan hospitals, resistance to

ampicillin (48.5%), chloramphenicol (55.3%), streptomycin (59.0%), sulfamethoxazole (68.0%), and tetracycline (67.8%) was high, whereas resistance to all 5 antimicrobials (ACSSuT R-type) comprised 327 (41%) and was highly prevalent in *Salmonella enterica* serotype Typhimurium (72.7%, 176/242), the most common serotype (Lauderdale, *et al.,* 2006). *Salmonella* spp. highly resistant to streptomycin (54.2%) and tetracycline (35.9%) but low to gentamicin (11.5%) and sulphamethoxazole / trimethoprim (9.4%) were isolated by Adesiyun *et al.* (2007) in eggs. In their study, only 1.4% isolates of *Salmonella* were multi-resistant.

2.9. *Salmonella* in aquatic environment

The principle habitat of *Salmonella* is the intestinal tract of mammals, reptiles and birds (Pelzer, 1989). *Salmonella* reach natural environment through animal excretion (Le Minor, 1981). The aquatic environment often receives a discharge of urban, agricultural and rural sewage, which represent a permanent source of pollution. Aquatic systems can also receive storm-generated discharges during occasional events, which may transport enteric pathogenic bacteria from their natural reservoir to seawater contaminating the organism that grow in it (O'Shea and Field, 1991; Baudart *et al*., 2000). Many serovars of *Salmonella* have been isolated in natural environments contaminated by human and animal faeces, particularly in rivers, estuaries and seawater (Baudart *et al.,* 2000; Winfield and Groisman, 2003). In aquatic environment, *Salmonella* can survive for over four weeks (Roszak, *et al* 1984). *Salmonella* have several modes of survival, including formation of biofilms, resistance to low water activity, rugose formation and entry into a viable but non culturable (VBNC) state. *Salmonella* spp. may enter a VBNC state after a lengthy exposure to oligotrophic fresh water and seawater under ambient temperature. Apart from water in aquatic environments, *Salmonella* spp. are also found in higher concentrations in sediments than in overlying water (Hendricks, 1971). In the recent years, *Salmonella enterica* serovar Senftenberg appeared to be one of the main serovars in marine environment (Martinez-Urtaza *et al*., 2003). Several reports are available on *Salmonella* spp. being isolated from estuarine and marine habitats (Touron *et al.,* 2005), fish feed (Moretro *et al.,* 2003), shrimp farm (Dalsgaard and Olsen, 1995), ground water and pond water (Cho and Kim, 1999), probiotic products (Joostan *et al.,* 2006), wastewater and effluent (Espigares *et al.,* 2006). Tauron *et al.* (2005), recorded positive results for 11.3% of the sediment samples and 20% of the water samples in the waters of France. Chandran

and Hatha (2005) studied the environmental parameters influencing survivability of *Salmonella* spp. in Indian estuarine waters in a microcosm study that concluded that light plays an important role in its survival.

2.10. *Salmonella* in food

A variety of foods have been implicated as vehicles transmitting salmonellosis to humans, including poultry (Logue *et al.,* 2003; Olsen *et al.,* 2003; Vo *et al.,* 2006), beef (McEvoy *et al.,* 2003; Nagal *et al.,* 2006; Stevens *et al.,* 2006), pork (Urfer *et al.,* 2000; Bahnson *et al.,* 2006; Farzan *et al.,* 2006, Michael *et al.,* 2006), goat (Chandra *et al.,* 2006), fish and shellfish (Bhaskar *et al.,* 1995; Hatha and Lakshmanaperumalsamy, 1997; Kumar *et al.,* 2003; Shabarinath *et al.,* 2007), eggs (Suresh *et al.,* 2006), cheese (Colak *et al.,* 2007), fresh fruits and juice (Yuk and Schneier, 2006), vegetables (Gomez *et al.*, 1997) and ready to eat meat sausages (Jordan *et al.,* 2006; Mrema *et al.,* 2006). Contamination can occur at multiple steps along the food chain including production, processing, distribution, retail marketing, handling and preparation (Zhao, *et al* 2003). Melloul *et al.* (2001) demonstrated that contamination of vegetables with *Salmonella* is common in areas where raw, untreated wastewater is used for irrigation. Zhao *et al.* (2003) recorded the presence of *Salmonella* in imported food that contains vegetables, meat and spices in the U.S.

2.10.1. *Salmonella* in seafood

Salmonella are detected occasionally from fish and fishery products. *Salmonella* has been isolated from freshwater fish culture ponds in many countries. In an experiment conducted in an integrated fish farm in a South east Asian country, *Salmonella* spp. were present in 28% fish samples, pond sediment and pond water (Twiddy and Reilly, 1995). A survey in Japan has shown presence of *Salmonella* species in 21% of eel culture ponds (Saheki, *et al* 1989). In US, freshwater cultured catfish showed an overall *Salmonella* incidence of 5% (Wyatt, *et al* 1979). It is hypothesized that application of dry pellet and/or fresh chicken manure may have been an important factor causing a relatively high prevalence of *Salmonella* in water, sediment and shrimp sample (Rielly and Twiddy, 1992; Dalsgaard and Olsen, 1995). *Salmonella* have also been found in fishmeal (Hauge, 1969, Veldman *et al.*, 1995) and in fish feed factories (Nesse *et al.,* 2003). The aquatic birds and feed also contribute to this.

The marine fish collected from open sea is found to be free from *Salmonella* (Bryan, 1973), but fish collected from polluted coastal waters contained this organism (Shewan, 1962; Bryan, 1973). *Salmonella* could also be isolated from the gut of marine shrimp suggesting that *Salmonella* could survive in coastal sediments and could be present in shrimp before any pre-process handling (Nayyerahmed *et al.*,1995). Heinitz *et al.* (2000) tested seafood and shellfish around the world for the presence of *Salmonella* spp. and found that shellfish from U.S., particularly oysters had a 1.2% prevalence of *Salmonella* in domestic shellfish. In another study in the United States of America, 7.5% of the oysters sampled harbored *Salmonella* (Brands *et al.,* 2005). In the waters of North West Spain, the overall incidence of *Salmonella* was recorded at 1.8% for the samples of shellfish including mussels, oysters, clams and cockles (Martinez-Urtaza *et al.,* 2003). Harakeh *et al,.* 2006 isolated a high number of *Salmonella* from crabs of Labanese waters. In Indian seafood, Hatha and Lakshmanaperumalsamy (1997) isolated various serotypes from fishes that included *S.* Weltevreden, *S.* Typhimurium and *S.* Paratyphi B. In crustaceans the most prevalent serotype was *S.* Paratyphi B and *S.* Typhimurium. Kumar *et al.* (2003) and Shabarinath *et al.* (2007) recorded the incidences of *Salmonella* in finfish and shellfish samples. Before this, Bhaskar *et al.* (1995) had reported the contamination of cultured shrimps with *Salmonella* spp. in west coast of India. Zhao *et al.* (2003) identified *Salmonella* from a variety of imported seafood in the U.S. that included abalone, anchovies, crab, eel, catfish, ghol fish, goby, hairtail, lobster, mackerel, mahi, mahi fillet, mullet, mussels, octopus, opakapa, oyster, periwinkle, pomfret, ribbon fish, scallop, sea bream, shrimp, silver fish, sillago fish, snail, sole, squid, tailfin barb fish, thread fish and tilapia.

2.11. Detection of *Salmonella* in environment and seafood

The standard procedure for isolation of *Salmonella* from foods usually involves a series of steps including pre-enrichment, selective enrichment, detection on indicator agar media and characterization by biochemical and serological tests. For the pre-enrichment of *Salmonella*, when samples are contaminated with other enterobacteriaceae, Lactose broth and buffered peptone water (BPW) with an incubation temperature of 37 ^{o}C is recommended (Leusden *et al.*, 1982; Fricker, 1987; Varnam and Evans, 1991). Selective enrichment have been obtained by several workers by using modified tetrathionate broth (Kauffmann, 1935), selenite broth (North and Bartram, 1953), brilliant green–MacConkey broth (King and Metzger, 1968) and

Rappaport-Vassiliadis broth (Harvey and Price, 1982). For subsequent isolation, a number of selective plating media have been devised. Some are media that are differential and nonselective (bromocresol purple lactose agar), yet some are differential and slightly selective (MacConky agar and Drigalski agar). Most commonly used media now are hekton-enteric agar (King and Metzger, 1968), bismuth sulphite agar (Wilson and Blair, 1927), xylose-lysine-deoxycholate agar (Taylor, 1965) and brilliant green agar (Leusden *et al.*, 1982). From the selective plating, the typical colonies are generally subjected to biochemical tests based on the Bergey's manual (Sharpe and Holt, 1989).

During the last decade, serological tests have been newly developed and marketed for the classification of *Salmonella* strains. However, despite great benefits of using serology like low cost and high throughput, Nielsen (1995) showed that problems of *Salmonella* identification may not be entirely solved by this technique. Among these, the variability in response of samples and the lack of persistence of serological response can be reported.

2.11.1. Molecular methods of detection of *Salmonella*

To overcome the disadvantages involved in routine biochemical and serological methods of *Salmonella* detection, several nucleic acid based techniques like DNA hybridization and polymerase chain reaction (PCR) procedures have been developed for numerous environmental and food samples. To detect *S.* Typhi Frankel (1989) amplified flagellin gene sequences using PCR technique. For detection of *Salmonella* in export foods, Jitrapakdee *et al.* (1995) demonstrated the application of PCR. As many as three sets of primers for the detection and discrimination of all serotypes of *Salmonella enterica*, *Salmonella enteritidis* and *Salmonella* Typhimurium were designed by Soumet *et al.* (1999). Agron *et al.* (2001) developed subtractive hybridization of sequences specific for *Salmonella enterica* serovar Enteritidis. A cloth-based hybridization array system (CHAS) designed by Gauthier and Blais (2004) provided a simple, cost-effective tool for monitoring *S.* Typhimurium DT104 in foods and their production environment. Several workers have targeted various pathogenic potential genes for amplification in PCR. Oliveira *et al.* (2003) amplified three virulence genes, *inv*A, *spv*R, and *spv*C in *Salmonella* Enteritidis isolated from poultry, pigs, humans and food samples. The authors claimed that *inv*A is a good target for detecting Salmonellae in such samples. In another study, Salehi *et al.* (2005) also detected

Salmonella in broilers of Iran using *inv*A primers. Further, in India, Riyaz-Ul-Hassan *et al.* (2004) designed a set of primers for conserved *stn*, *Salmonella* enterotoxin gene to detect *Salmonella* in water, milk and blood samples.

Touron *et al.* (2005) developed a nested multiplex PCR (nm-PCR) assay to detect the presence of *Salmonella* in estuarine water and sediment samples. The target gene used was the phase 1 flagellin *fli*C chromosomal gene, present in all *Salmonella* serovars. Detection of *Salmonella enterica* serovar Typhi by selective amplification of *inv*A, *via*B, *fli*C-d and *prt* genes by PCR in mutiplex format has been reported by Kumar *et al.* (2006). Recently, real time PCR has become popular in quantifying *Salmonella* contamination in food. The methods have been standardized by many workers (Rodriguez-Lazaro *et al.,* 2003; Klerks *et al.,* 2004; Malorny *et al.,* 2003; Hein *et al.,* 2006; Patel *et al.,* 2006). In case of seafood, *inv*A gene specific nested PCR was employed to detect *Salmonella* Senftenberg in raw oysters in Spain (Vazquez-Novelle *et al.,* 2005). The detection limit of the method is less than 0.1 CFU/ml (<1 CFU/g of oyster). This procedure is shown to be an excellent tool for the sensitive detection of *S.* Senftenberg from naturally contaminated oysters, with results being obtained within 8 hours. Kumar *et al.* (2003) and Shabarinath *et al.* (2007) employed *inv*A, *inv*E, and *hns* genes to screen Indian seafoods for the presence of *Salmonella.*

2.12. Genetic characterization of *Salmonella*

The Kauffmann-White scheme, first published in 1929, divides *Salmonella* into more than 2500 serotypes according to their antigenic formulae. Serotyping methods are stable, reproducible and have high typeability; yet, there are several drawbacks, particularly the dependence on availability of antisera considering the ethics, cost and quality control measures necessary to maintain such a supply. Several molecular methodologies are currently available for studying genetic variability among strains of the same organism. These include various modifications of the polymerase chain reaction (PCR), such as the random amplification of polymorphic DNA, RAPD (Williams *et al.*, 1990; Lin *et al.,* 1996; Hilton and Penn, 1998; Guerra *et al.,* 2000; Khoodoo *et al.,* 2002), enterobacter repetitive intergenic consensus polymerase chain reaction, ERIC-PCR and arbitrarily primed polymerase chain reaction, AP-PCR (Burr *et al.,* 1998), amplified fragment length polymorphism, AFLP (Torpdahl *et al.,* 2005; Gebreyes *et al.,* 2006), repetitive palindromic extragenic polymerase chain reaction, Rep–PCR (Gebreyes *et al.,* 2006), restriction fragment length polymorphism, RFLP, pulse field gel

electrophoresis, PFGE (Martinez-Urtaza and Liebana, 2005; Torpdahl *et al.,* 2005; Gebreyes *et al.,* 2006; Kudaka *et al.,* 2006) and multilocus sequence typing, MLST (Torpdahl *et al.,* 2005). Each method has its own advantages and disadvantages based on the experimental condition and strain used for molecular differentiation. Lim *et al.* (2005) compared several of above mentioned molecular typing methods for the differentiation of *Salmonella* species and serovars and found out that combination of RAPD and ERIC-PCR to be useful for the differentiation of field-isolated *Salmonella* strains and epidemiological studies.

Material and Methods

3. MATERIAL AND METHODS

3.1. Sampling

Samples such as fish and shellfish from fish landing centre, oysters from natural beds, water and sediment from Nethravathy estuary, Mangalore and the institute fish culture farms were collected during the study for isolation of *Salmonella* spp. All the samples were collected and brought to the laboratory aseptically for isolation, identification and characterization of *Salmonella* spp. Methodology recommended by FDA (1992) was followed for the isolation.

3.2. Pre-enrichment

As much as 25 grams of sample was homogenized in 225 ml lactose broth for 2 minutes. This mixture was incubated for 24 hours at 37 °C as pre-enrichment step.

Lactose broth

Beef extract	3.0 g
Peptone	5.0 g
Lactose	5.0 g
Distilled water	1000 ml
Final pH	6.9 ± 0.2

The medium was dispensed in 225 ml aliquots into 500 ml conical flask and autoclaved at 110 °C for 10 minutes.

3.3. Selective enrichment

Approximately 1 ml of each pre-enriched sample was inoculated to 10 ml each of fluid selenite cystine broth and tetrathionate broth, while 0.1 ml was added to 10 ml of Rappaport-Vassiliadis broth. The inoculated selenite cystine broth and tetrathionate broth tubes were incubated at 37 °C for 24 hours. Rappaport-Vassiliadis broth was incubated at 43 °C in a water bath for 24 hours.

Fluid Selenite cystine broth (HiMedia, Mumbai)

Casein enzymatic hydrolysate	5.0 g
Lactose	4.0 g
Disodium phosphate	10.0 g
L-cysteine	0.01 g
Sodium hydrogen selenite	4.0 g
Distilled water	1000 ml
pH	7.0 ± 0.2

0.23 grams of dehydrated medium was dissolved in 10 ml of sterile distilled water by boiling in a water bath for 10 minutes.

Tetrathionate enrichment broth (HiMedia, Mumbai)

Casein enzymatic hydrolysate	4.3 g
Peptic digest of animal tissue	4.3 g
NaCl	6.4 g
Potassium tetrathionate	20.0 g
Crystal violet	0.005 g
Distilled water	1000 ml
Final pH	6.5 ± 0.2

0.35 grams of medium was dissolved in 10 ml of sterile distilled water by boiling in a water bath for 10 minutes.

Rappaport-Vassiliadis medium (HiMedia, Mumbai)

Papaic digest of soyabean meal	4.0 g
NaCl	7.2 g
Monopotassium phosphate	1.44 g
$MgCl_2$	36.0 g
Malachite green	0.036 g
Final pH	5.2 ± 0.2

0.492 grams of dehydrated powder was dissolved in 10 ml of sterile distilled water by boiling in a water bath for 10 minutes.

3.4. Selective plating

A loopful of inoculum, each from the above broths were streaked on hekton enteric agar, bismuth sulphite agar and xylose lysine-deoxycholate agar separately and incubated at 37 °C for 24 hours. Characteristic colonies were picked using sterile loop and maintained in trypticase soy agar (TSA) slants for biochemical tests. On hekton enteric agar, typical *Salmonella* spp. appear as blue-green to green colonies with or without black center. Rarely as yellow colonies with or without black center may be seen. On Bismuth sulphite agar, brown, black or grey with or without black centered colonies come up. Pink colonies with or without black centers on Xylose lysine deoxycholate agar are considered typical. Many will have large, glossy black centers and may appear as almost completely black. A few may appear as yellow colonies.

Bismuth sulphite agar (HiMedia, Mumbai)

Peptic digest of animal tissue	10.0 g
Beef extract	5.0 g
Dextrose	5.0 g
Na_2HPO_4 (anhydrous)	4.0 g
$FeSO_4$ (anhydrous)	0.3 g
Bismuth sulphite (indicator)	8.0 g
Brilliant green	0.025 g
Agar	20.0 g
Distilled water	1000 ml

52.33 g of this medium was suspended in 1000 ml of sterile distilled water. This was boiled to dissolve the medium completely and poured into sterile petri plates.

Xylose lysine deoxycholate agar (HiMedia, Mumbai)

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Sodium deoxycholate	2.5 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	0.8 g
Phenol red	0.08 g
Distilled water	1000 ml
Final pH	7.4 ± 0.2
Agar	13.5 g

55.18 g of medium was suspended in 1000 ml of sterile water and boiled to dissolve the media. It was then poured to sterile petriplates.

Hekton enteric agar (HiMedia, Mumbai)

Proteose peptone	12.0 g
Yeast extract	3.0 g
Bile salts No: 3	9.0 g
Lactose	12.0 g
Sucrose	12.0 g
Salicin	2.0 g
NaCl	5.0 g
Sodium thiosulphate	5.0 g

Ferric ammonium citrate	1.5 g
Bromothymol blue	0.0065 g
Acid fuschin	0.10 g
Agar	15 g
Distilled water	1000 ml

In 1000 ml of sterile distilled water, 76.67 g of above medium was suspended. The medium was then boiled to dissolve the ingredients completely and poured to sterile petri plates.

Trypticase soy agar

Tryptone	15.0 g
Soya peptone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1000 ml
Final pH	7.3 ± 0.2

The medium was autoclaved at 121 °C for 15 minutes. About 20 ml portions were then dispensed into sterile petri plates.

3.5. Biochemical tests

A series of biochemical tests were carried out to identify *Salmonella* to generic level. Minimum of five typical colonies from each of the selective agar plates were subjected to a battery of biochemical tests for the identification of *Salmonella*.

3.5.1. Oxidase test

Young test cultures were taken from the slants by glass capillary tube and placed on to pre-moistened filter paper strip with oxidase reagent. Oxidase positive colonies showed dark purple color within 10 seconds. *Salmonella* are strictly oxidase negative. All oxidase positive cultures were discarded.

Oxidase reagent

N N N N tetra methyl p-phenylene diamine dihydrochloride	10 g
Distilled water	1000 ml

Whatman filter paper No. 1 was cut into strips of 2.5x1.0 cm, sterilized, dipped in the above reagent, dried and stored in the dark at 4 °C.

3.5.2. Triple sugar iron agar test

Culture suspected to be *Salmonella* were inoculated into triple sugar iron agar (TSI) tubes by streaking on the slants and stabbing the butt. The tubes were incubated at 37 °C for 24 ± 2 hours. *Salmonella* typically produces alkaline (purple) slants and acid (yellow) butt with or without the production of H_2S (blackening of butt).

Triple sugar iron agar test (TSI), (HiMedia, Mumbai)

Peptone	10.0 g
Tryptone	10.0 g
Yeast extract	3.0 g
Beef extract	3.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferrous sulphate	2.0 g
NaCl	5.0 g
Sodium thiosulphate	3.0 g
Phenol red	0.0024 g
Agar	12.0 g
Distilled water	1000 ml
Final pH	7.2 ± 0.2

The medium was heated gently with occasional agitation, boiled for 1 to 2 minutes until the agar was dissolved. The medium was then dispensed into test tubes and autoclaved at 121 °C for 15 minutes. The medium was prepared as slants.

3.5.3. Urease test

With a sterile loop, growth of test culture was inoculated into tubes of urea broth and incubated at 37 °C for 24 hours. An un-inoculated urea broth tube and an inoculated broth without urea solution were maintained as controls. Purple red color of the tube upon incubation indicated positive reaction. Since *Salmonella* are urease negative, all positive cultures were discarded.

Urea broth base (Himedia, Mumbai)

Monopotassium phosphate	91.0 g
Dipotassium phosphate	95.0 g

Yeast extract	1.0 g
Phenol red	0.1 g
Distilled water	950 ml
Final pH	6.8 ± 0.2

The medium was dissolved, distributed in tubes in 3 ml volumes into test tubes and autoclaved at 121 °C for 15 minutes. After cooling to room temperature, 160 µl of filter sterilized 40% urea solution was added to the test tubes aseptically and mixed well.

3.5.4. Indole test

Small amount of growth from test cultures were inoculated into tryptone broth and incubated for 24 ± 2 hours at 37 °C. Production of indole from tryptophan was tested by adding 0.5 ml of Kovac's reagent to the culture tubes. Positive reaction is seen as deep pink ring at the surface. Since *Salmonella* gives negative reaction, all positive cultures were discarded.

Tryptone broth

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Distilled water	1000 ml
Final pH	7.1 ± 0.2

The medium was dispensed into test tubes and sterilized by autoclaving at 121 °C for 15 minutes.

Kovac's reagent

p-dimethyl amino benzaldehyde	5 g
Isoamyl alcohol	750 ml
Conc. HCl	250 ml

3.5.5. Methyl Red - Voges Proskauer (MR-VP) test

Test cultures were inoculated into MR-VP broth and incubated for 72 ± 2 hours at 37 °C. After 72 hours 2.5 ml of culture was taken in a clean test tube and 5-6 drops of methyl red indicator was added. *Salmonella* gave positive test indicating diffused red color in medium for MR test. Distinct yellow colour was considered as negative reaction and such cultures were discarded as they are not *Salmonella*. VP test was performed by adding 0.6 ml of α naphthol to the tube containing cultures in MR-VP broth and shaken well. Then 0.2 ml of 40% KOH solution was added and shaken and results were

read after 4 hours. Development of pink to port wine color throughout the medium was considered positive test. *Salmonella* gave VP negative reaction.

Methyle Red - Voges Proskauer (MR-VP) broth (HiMedia, Mumbai)

Glucose	5.0 g
Peptone	7.0 g
K_2HPO_4	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

The medium was dispensed in 5 ml volumes and autoclaved at 110 °C for 10 minutes.

Methyl Red reagent

Methyl red	0.2 g
Ethyl alcohol	600 ml

The reagent was made up to 1000 ml with distilled water.

Voges - Proskauer reagent

Solution A	α- naphthol	50.0g
	Absolute alcohol	1000 ml
Solution B	40% KOH	

3.5.6. Lysine iron agar test

Lysine iron agar (LIA) slants were inoculated with small amount of growth from TSA tubes by stabbing the butt and streaking the slants. Inoculated tubes were incubated for 48 ± 2 hours at 37 °C. *Salmonella* typically produce alkaline (purple) reaction in butt and acidic reaction in slants.

Lysine iron agar (Himedia, Mumbai)

Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
L Lysine hydrochloride	10.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulphate	0.04 g
Bromocresol purple (anhydrous)	0.02 g
Agar	15.0 g
Distilled water	1000 ml
Final pH	6.7 ± 0.2

The ingredients were dissolved by heating and 4 ml proportions were dispensed into test tubes and autoclaved at 110 °C for 10 minutes. The medium was prepared as 4 cm butt and 2.5 cm slant.

3.5.7. Simmons citrate test

Test cultures were inoculated on Simmons citrate agar by streaking on the slants and stabbing the butt. The tubes were incubated for 24 ± 2 hours at 37 °C. Presence of growth usually accompanied by color change from green to blue was considered positive reaction. Most cultures of *Salmonella* are citrate positive.

Simmons citrate agar

Sodium citrate dihydrate	2.0 g
NaCl	5.0 g
K_2HPO_4	1.0 g
$NH_4H_2PO_4$	1.0 g
$MgSO_4$	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml
Final pH	6.9 ± 0.2

The medium was heated gently with occasional agitation, boiled for 1-2 minutes until agar was dissolved, dispensed into test tubes and autoclaved at 121 °C for 15 minutes. The medium was prepared as slants.

3.5.8. Phenol Red Dulcitol test

Phenol red dulcitol broth was inoculated with small amount of growth culture and incubated for 48 ± 2 hours at 37 °C. Most *Salmonella* spp. gave positive test indicated by gas formation in Durham's tube and the production of acid, indicated by color change in the medium from red to yellow. Production of acid without gas sometimes, may also be interpreted as positive reaction.

Phenol Red Dulcitol broth

Peptone	10.0 g
Sodium chloride	5.0 g
Beef extract	1.0 g
Phenol red	0.018 g
Dulcitol	5.0 g
Distilled water	1000 ml

The medium was dissolved in distilled water and dispensed into tubes containing inverted Durhan's tubes and autoclaved at 110 °C for 10 minutes.

3.5.9. Malonate test

A loopful of 24 hour culture was transferred to malonate broth and incubated for 48 ± 2 hour at 37 °C. Most *Salmonella* give negative reaction (green or unchanged color) for this test. Distinct blue color was considered as positive test.

Malonate broth

Yeast extract	1.0 g
$(NH_4)_2SO_4$	2.0 g
KH_2PO_4	0.4 g
K_2HPO_4	0.6 g
Sodium malonate	3.0 g
Sodium chloride	2.0 g
Glucose	0.25 g
Bromothymol blue	0.025 g
Distilled water	1000 ml
Final pH	6.7 ± 0.2

The medium was dissolved by heating, dispensed into test tubes and autoclaved at 110°C for 10 minutes.

After testing the cultures for above mentioned various biochemical tests, the presumed *Salmonella* cultures were stored in semisolid butts of trypticase soy agar medium. The butts with 0.5% agar were stabbed with the cultures and incubated for 24 hours. Later, about 0.5 ml of sterile liquid paraffin was added to cut off the oxygen supply. The tubes were maintained at room temperature. Whenever the cultures were required, a loopful of cultures from the butt were streaked and purified on TSA plates.

3.6. Polymerase Chain Reaction (PCR)

The isolates identified as presumptive *Salmonella* spp. by conventional biochemical tests were further confirmed by polymerase chain reaction (PCR) using *Salmonella* specific *hns* primer. *Hns* positive isolates were also tested for genes coding for invasion associated proteins, *inv*A and *inv*E.

3.6.1. Preparation of bacterial crude lysate

Presumed *Salmonella* cultures were grown overnight in Luria bertani (LB) broth at 37 °C with agitation. About 50 µl of the broth culture was transferred to fresh

microcentrifuge tube containing 450 µl of sterile 1X TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). The tubes were heated on a dry bath to lyse cells at 100 °C for 10 minutes. Immediately, the tubes were placed on ice to facilitate the release of the DNA from the cells. This was used as DNA template for PCR.

Luria Bertani broth (HiMedia, Mumbai)

Casein enzymic hydrolysate	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g
pH	7.5 ± 0.2
Distilled water	1000 ml

25 grams of Luria bertani broth dehydrated powder was dissolved in 1000 ml of distilled water. The medium was dispensed into test tubes and autoclaved at 121 °C for 15 minutes.

3.6.2. Preparation of enrichment lysate

About 1.5 ml of enriched samples from respective enrichment broths were taken into fresh sterile microcentrifuge tubes after the incubation. The tubes were centrifuged at 800 g for settling the debris of food sample. The supernatant was transferred to fresh tubes and centrifuged at 8000xg for 10 minutes to pelletise the bacteria. The supernatant was discarded and the pellet was suspended in 100 µl of sterile 1X TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). The tubes were then heated at 98 °C for 15 minutes on a dry bath for lysing. After lysing the tubes were quickly placed on ice to facilitate release of DNA from the lysed cells. This was used as enrichment lysate DNA template for PCR to identify *Salmonella* directly in food sample.

3.6.3. PCR reaction mixture

PCR was performed in 50 µl volumes containing 5 µl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3; 20 mM $MgCl_2$; 500 mM KCl and 0.1% gelatin), 200 mM concentrations of each deoxyribonucleotide triphosphate (dATP, dGTP, dCTP and dTTP), 50 pM of each primers, 1.25 units of *Taq* polymerase and 5 µl of DNA template. The PCR was run in thermocycler, DYAD Engine. The buffer for *Taq* polymerase, dNTPs and *Taq* polymerase were obtained from Bangalore Genei, Bangalore.

3.6.4. PCR primers and cyclic condition

Table 1 shows the primers used in this study. The primer pairs *hns* amplified a 152 bp fragment encoding a DNA binding protein, *inv*A amplied a 284 bp fragment of

*inv*A gene encoding the invasion associated protein. The primer pair *inv*E amplified a 166 bp fragment of *inv*E gene encoding another invasion associated protein. The thermocycling conditions are given in table 2.

Table. 1. Primers used for the detection of *Salmonella* species

Primer	Primer sequence	Product	Reference
hns	F 5′ TACCAAAGCTAAACGCGCAGCT 3′ R 5′ TGATCAGGAAATCTTCCAGTTGC 3′	152 bp	Jones *et al.* (1993)
invA	F 5′GTGAAATTATCGCCACGTTCGGGCAA 3′ R 5′ TCATCGCACCGTCAAAGGAACC 3′	284 bp	Rahn *et al.* (1992)
invE	F 5′ CAGGATACCTATAGTGCTGC 3′ R 5′ CACCAATATCGCCAGTACGA 3′	166 bp	Chen and Griffiths. (2001)

Table 2. PCR conditions for the various primers used in this study

Primer	Cycling conditions			Cycles
	Denaturation	Annealing	Extension	
invA	95 °C - 30 sec	64 °C - 30 sec	72 °C - 30 sec	35 – 38
invE	94 °C -2 min	55 °C - 1 min	72 °C - 1 min	30
hns	94 °C – 1 min 94 °C - 30 sec	60 °C - 1 min 60 °C - 30 sec	72 °C - 1 min 72 °C - 30 sec	5 30

3.6.5. Detection of PCR products by agarose gel electrophoresis

About 20 µl of the PCR product was mixed with 4 µl of sample loading buffer. The products were loaded onto 2% agarose gels, resolved at 125 volt in TAE buffer, stained with ethidium bromide (0.5 µg/ml), photographed and analysed using a gel documentation system (Herolab, Germany).

TAE Buffer (10X)

Tris base	48.4 g
Glacial acetic acid	11.42 g
0.5 M EDTA	20 ml

The solution was made up to a final volume of 1 liter using millipore water.

Sample loading buffer (6X)

Bromophenol blue	0.25 g
Sucrose	40.0 g
Distilled water	100 ml

The *Salmonella* isolates thus confirmed by PCR were further sent to Central Research Institute (CRI), Kasauli, UP, India, for serotyping.

3.7. Antibiogram analysis

The method described by Bauer *et al.* (1966) was followed for disc diffusion assay of antibiotics. *Salmonella* isolates were tested for sensitivity for the following antibiotics: ampicillin, nalidixic acid, co-trimoxazole, kanamycin, chloramphenicol, tetracycline, ciprofloxacin, erythromycin cephalexin, sulphafurazole, amikacin, gentamycin nitrofurantoin, amoxicillin and ceftriaxone (Hi-Media, Mumbai). The cultures were grown at 37 °C in Luria bertani broth for 16 -18 hours and a lawn was prepared on Mueller-Hinton agar (HiMedia, Mumbai). The antibiotic discs were placed on the culture lawn and incubated at 37 °C for 16 -18 hours. The inhibition zones were recorded and compared with the interpretive chart of Kirby-Bauer (NCCLS, 1993) and classified as resistant (R), intermediate (I) and sensitive (S).

Mueller-Hinton agar

Casein acid hydrolysate	17.5 g
Beef heart infusion	2.00 g
Starch soluble	1.5 g
Agar	17.00 g
Distilled water	1000 ml

About 38.0 grams of medium was suspended in 1000 ml of sterile water. It is then boiled to dissolve the ingredients completely and poured into sterile petriplates.

3.8. Random Amplification of Polymorphic DNA (RAPD)

To study the genetic variability among the serotype and PCR confirmed *Salmonella* spp., the isolates were subjected to random amplification of polymorphic DNA (RAPD) analysis.

3.8.1. Extraction of genomic DNA

DNA was extracted from the *Salmonella* spp. following the phenol chloroform isoamyl alcohol protocol as explained by Ausubel *et al.* (1992). The bacterial culture was grown overnight in 5 ml LB broth. Approximately, 1.5 ml of the grown culture was centrifuged at 10,000g for 10 minutes to pelletize the bacteria. The bacterial pellet was resuspended in 567 µl of TE buffer (1X) and 30 µl of SDS (10%) was added to the cell suspension. As much as 3 µl of 20 mg/ml Proteinase K (Bangalore Genie, Bangalore) was also added. The mixture was then incubated at 37 °C for 1 hour. To the incubated mixture 100 µl of the 5 M NaCl, 80 µl of CTAB-NaCl (10% CTAB / 0.7 M NaCl) was added and incubated at 65 °C for 10 minutes. After incubation, equal volume of choloroform: isoamyl alcohol (24:1) mixture was added and centrifuged at 10,000 g for 10 minutes. The supernatant was transferred to a fresh tube and equal volume of phenol: chloroform: isoamylalcohol (25: 24: 1) mixture was added and centrifuged at 10,000g for 10 minutes. The supernatant was transferred to a fresh tube, 0.6 volume of isopropanol was added and mixed gently to precipitate the DNA. The DNA was washed with 70% alcohol, dried briefly to evaporate the alcohol and dissolved in 100 µl of TE buffer. The solution containing genomic DNA was stored at -20°C for further use.

Tris-EDTA Buffer

Tris-Cl	10 mM (pH 8.0)
EDTA	1 mM (pH 8.0)

SDS (10%)

Sodium dodecyl sulphate	10.0 g
Double distilled deioned water	100 ml

NaCl (5 M)

NaCl	29.2 g
Double distilled deioned water	100 ml

CTAB - NaCl (10% CTAB / 0.7 M NaCl)

NaCl (4.1 g) was dissolved in 80 ml water and 10 g CTAB (hexadecytrimethyl ammonium bromide) was added while heating and stirring and final volume was adjusted to 100 ml.

Chloroform: Isoamyl alcohol (24: 1)

Chloroform	24 ml
Isoamyl alcohol	1 ml

Phenol: Chloroform: Isoamyl alcohol (25: 24: 1)

Phenol	25 ml
Chlororform	24 ml
Isoamylalcohol	1 ml

3.8.2. RAPD reaction

Two sets of primers, CRA 22 (5' CCGCAGCCAA 3') and CRA 23 (5' GCGATCCCCA 3') were used to determine the genetic fingerprints of confirmed *Salmonella* isolates. RAPD-PCR reaction was performed in a 30 μl reaction volume containing 3 μl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3; 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 200 μM concentrations of each deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), 30 μM of primer and 1.0 U of *Taq* polymerase (Bangalore Genei, Bangalore). The PCR conditions included an initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 45 °C for 30 seconds, extension at 72 °C for 30 seconds and a final delay at 72 °C for 5 minutes. PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide and the results were observed and analyzed using gel documentation system (Herolab, Germany).

Results

4. RESULTS

A total of 55 samples comprising 10 each of Nethravathy estuarine water, Nethravathy sediment, fish farm water and sediment and 5 each of shrimp, fish and oyster samples were analyzed for the incidence and prevalence of *Salmonella*. The results of the same are discussed hereunder.

4.1. Prevalence of *Salmonella* in aquatic environment

Out of 10 Nethravathy estuarine water samples, none of the samples turned positive, whereas, in the same estuary, 2 (20%) sediment samples showed positivity. In case of fish pond samples, no water sample gave positive results, whereas 1 sediment sample out of 10 (10%) samples was positive for *Salmonella.* In seafood samples, fish and shrimp showed 40% (2 out of 5 samples) and 20% (1 out of 5 samples) positivity respectively. However, the limited number of oysters (5) tested were negative for the presence of *Salmonella*.

4.2. Direct detection of *Salmonella* from enrichment lysate

Samples enriched in various enrichment broths were used for preparation of enrichment lysate and this was used for PCR detection of *Salmonella* using *hns* primer. In all samples and all cases, the results obtained in PCR from enrichment lysates were similar to the results of conventional method.

4.3. Detection of virulence genes in *Salmonella* isolates

From *Salmonella* positive samples, a total of 8 isolates were recovered and confirmed by PCR using *hns* primer. These isolates were also tested for the presence of virulence genes *inv*A and *inv*E. All the 8 isolates were found to carry *inv*A and *inv*E genes.

4.4. Serotypes of *Salmonella* isolates

Eight PCR confirmed *Salmonella* isolates were sent for serotyping to Central Research Institute (CRI), Kasauli, UP, India. All of them were *inv*A and *inv*E positive isolates. Serotyping classified 4 of them as serotype Bareily; two of which were isolated from estuary sediment and one each from fish and shrimp obtained from landing center.

4.5. Antibiotic resistance in *Salmonella*

The PCR confirmed isolates were screened for their antibiotic resistance pattern. All the 8 isolates tested showed resistance to erythromycin. For antibiotics like nitrofurantoin, tetracycline, kanamycin, cephaexin and citraxone intermediate resistance was shown by all 8 isolates. For ampicillin 4 isolates showed intermediate resistance and others were sensitive. However, for amikacin, chloramphenicol, co-trimoxaozole, sulphafurazole, gentamicin, nalidixic acid, ciprofloxacin and amoxicillin, all 8 isolates were sensitive. No multiple drug resistant isolates could be found in this study. The detailed results are tabulated in table 5.

4.6. Genetic variability in *Salmonella*

To study the genetic variability of *Salmonella* isolates, random amplified polymorphic DNA (RAPD) analysis of the isolates were carried out using two different RAPD primers; CRA 22 and CRA 23. The amplified DNA fragment banding pattern for first 4 isolates were grouping into one cluster by both CRA 22 and CRA 23 primers (plate 3 and 4). Incidently, all the first 4 isolates were serotype Bareily as confirmed by serotyping. Interestingly these *S.* Bareily isolates came from different samples, two from estuarine sediment, one from fish and another from shrimp from landing center. Further, two other isolates from fish from landing center and another isolate from estuarine sediment showed a different RAPD pattern. These isolates were differentiated from other 4 isolates by giving discriminatory fingerprinting pattern. The remaining 4 isolates clustered together showing slight difference in their banding pattern to the former 4 isolates. In case of CRA 23, as in CRA 22 primer, two distinct groups could be seen from the gel picture (plate 4). First four Bareily serotype isolates showed similar band pattern while the rest 4 isolates showed similar fingerprint that grouped into another cluster. They had an additional band in between 250 and 500 bp marker fragment (plate 4). Both the primers differentiated the isolates into two groups; Bareily serotypes into one cluster and rest into another.

Table 3. Prevalence of *Salmonella* spp. in aquatic environment using conventional and molecular methods

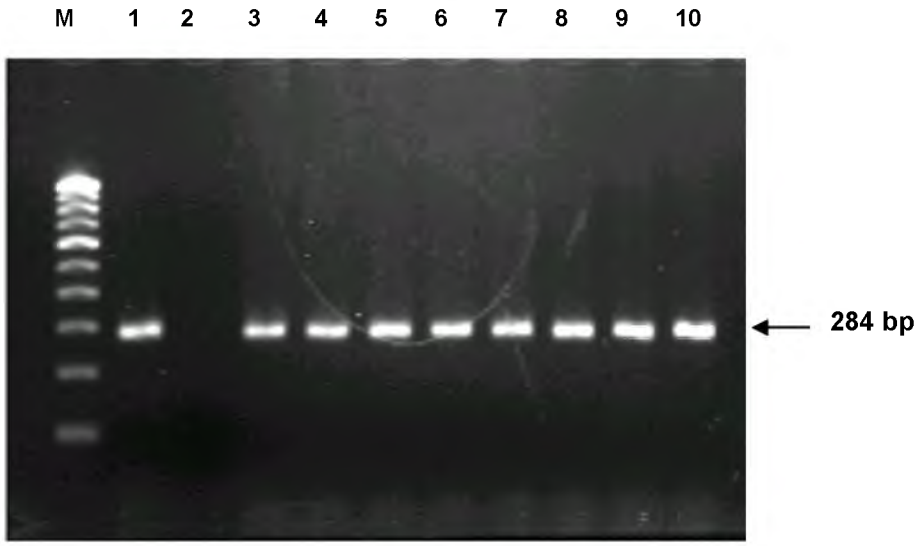
Sample type	Samples analysed	Samples positive by conventional test	Percentage positivity	Samples Positive by PCR	Percentage positivity	Isolates recovered
Estuary water	10	-	-	-	-	-
Estuary sediment	10	2	20	2	20	3
Fish farm water	10	-	-	-	-	-
Fish farm sediment	10	1	10	1	10	1
Fish (landing centre)	5	2	40	2	40	3
Shrimp (landing centre)	5	1	20	1	20	1
Oyster (oyster bed)	5	-	-	-	-	-
Total	**55**	**6**	**-**	**6**	**-**	**8**

Table 4. Results of PCR using various primers on *Salmonella* isolates

Sl. No.	Sample code	Enrichment medium	Selective plating	*hns*	*inv*A	*inv*E	Serotypes
1	LS-2	RV	BSA	+	+	+	*S.* Bareily
2	LF-1	RV	HEA	+	+	+	*S.* Bareily
3	NS-6	RV	XLD	+	+	+	*S.* Bareily
4	NS-6	RV	HEA	+	+	+	*S.* Bareily
5	LF-1	RV	BSA	+	+	+	ND
6	LF-4	RV	HEA	+	+	+	ND
7	NS-1	RV	BSA	+	+	+	ND
8	PS-2	RV	XLD	+	+	+	ND

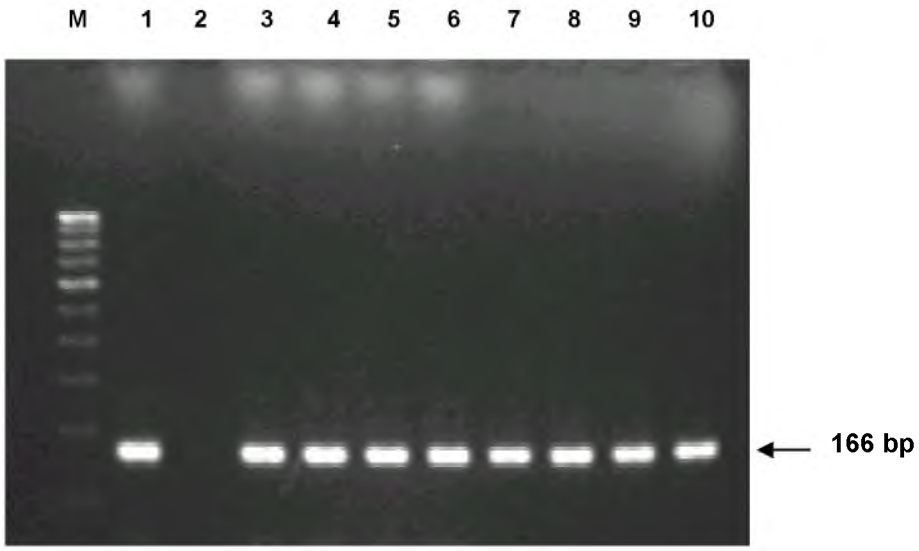
LS, landing centre shrimp; LF, landing centre fish; NS, netrhavathy sediment; PS, Pond sediment; RV, rappaport-vassiliadis medium; BSA, bismuth sulphite agar; HEA, hekton enteric agar; XLD, xylose lysine deoxycholate agar; ND, Not detected.

Plate 1. *inv*A PCR for detection of virulence genes in *Salmonella* spp.



M, 100 bp DNA marker; Lane 1, positive control; lane 2, negative control; Lane 3-10, *hns* positive *Salmonella* isolates

Plate 2. *inv*E PCR for detection of virulence genes in *Salmonella* spp.



M, 100 bp DNA marker; Lane 1, positive control; lane 2, negative control; Lane 3-10, *hns* positive *Salmonella* isolates

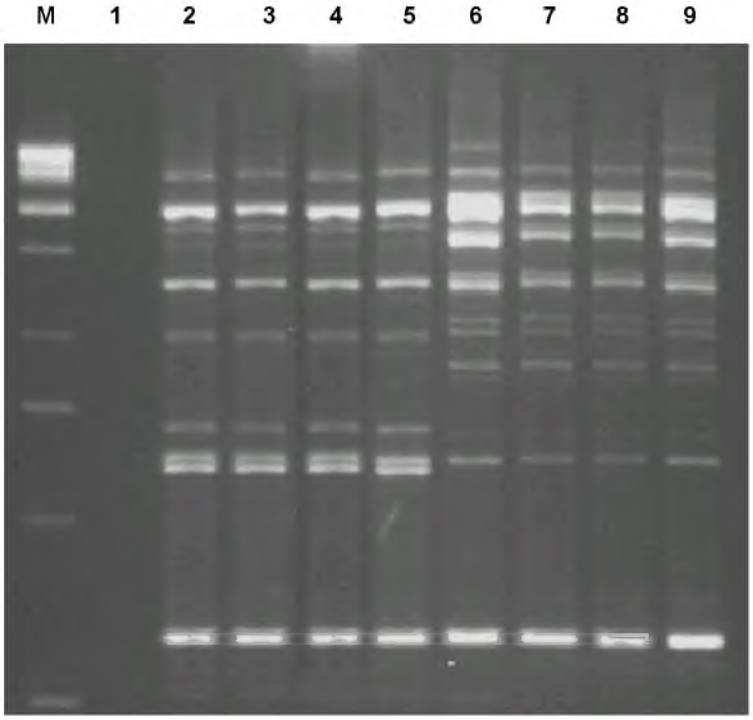
Table 5. Antibiotic resistance profile of *Salmonella* spp. isolated from various aquatic environment

Sl. No.	E	Nf	T	Ci	K	Cp	A	Ak	C	Co	Sf	G	Na	Cf	Am
1	R	I	I	I	I	I	S	S	S	S	S	S	S	S	S
2	R	I	I	I	I	I	S	S	S	S	S	S	S	S	S
3	R	I	I	I	I	I	S	S	S	S	S	S	S	S	S
4	R	I	I	I	I	I	S	S	S	S	S	S	S	S	S
5	R	I	I	I	I	I	I	S	S	S	S	S	S	S	S
6	R	I	I	I	I	I	I	S	S	S	S	S	S	S	S
7	R	I	I	I	I	I	I	S	S	S	S	S	S	S	S
8	R	I	I	I	I	I	I	S	S	S	S	S	S	S	S

E, erythromycin; Nf, nitrofurantoin; T, tetracycline; Ci, cetriaxone, K, kanamycin; Cp, cephalexin; A, ampicilin; Ak, amikacin; C, chloromphenicol; Co, co-trimoxaozole; Sf, suphafurazole; G, gentamicin; Na, nalidixic acid; Cf, ciprofloxacin; Am, amoxicillin.

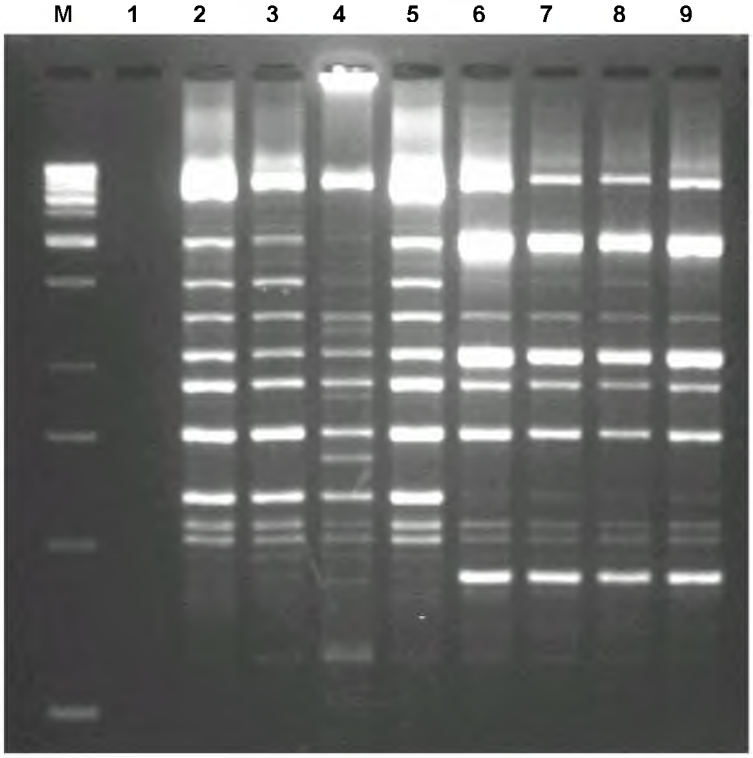
R, resistant; **I**, intermediate resistant; **S**, sensitive;

Plate 3. RAPD analysis of *Salmonella* spp. using CRA 22 primer



M: 1kb DNA marker; Lane 1, negative control; Lane 2-5, Serovar Bareilly; Lane 6-9, serovar unknown.

Plate 4. RAPD analysis of *Salmonella* spp. using CRA 23 primer



M: 1kb DNA marker; Lane 1, negative control; Lane 2-5, Serovar Bareilly; Lane 6-9, serovar unknown.

Discussion

5. DISCUSSION

Salmonella infection of humans and animals is of great public health concern worldwide. The incidence of human *Salmonella* infection over the years have markedly increased in many countries (Chiu *et al.* 2004). Many serovars of *Salmonella* have been isolated in natural environments contaminated by human and animal feces, particularly in rivers, estuarine waters and seawaters and associated organisms (Touron *et al.,* 2005). Use of such aquatic environments for commercial, domestic or human activities pose threat of transmission of the pathogen to humans. Seafood harvested from such contaminated water bodies become the important and direct route of infection of *Salmonella. Salmonella* spp. isolated in this study from various aquatic environments and their implications are discussed hereunder.

5.1. Prevalence of *Salmonella* in aquatic environment

5.1.1. Incidence of *Salmonella* in estuarine samples

Many serovars of *Salmonella* have earlier been isolated in natural environments such as rivers, estuaries and seawater (Roszak, *et al* 1984; Baudart *et al*., 2000; Winfield and Groisman, 2003; Touron *et al.,* 2005). In the present study, *Salmonella* could not be isolated from estuarine water samples. However, estuarine sediment sample showed prevalence of 20%. The findings are in line with that of Hendricks (1971) who recorded higher percentage of *Salmonella* incidence in sediment samples than overlying waters. In aquatic environment, *Salmonella* are found to survive for over four weeks (Roszak *et al* 1984). The constant sedimentation process in aquatic bodies deposit organic matters along with biological pollutants like bacteria on the sediment. Therefore, there is possibility that Salmonella survive in sediment longer as it carries high organic load for bacterial nourishment. Thus sediment could be an important source for contamination of coastal fish.

5.1.2. Incidence of *Salmonella* in fish farm samples

As far as fish pond water and sediments are concerned, in the present study, no water samples were positive for *Salmonella* spp. As in case of estuarine samples, fish farm sediment also showed the presence of *Salmonella* spp. with a prevalence rate of 10%. It is a widespread practice among fish farmers to fertilize the farm with manure containing animal faeces for enhancement of fish food organisms. Therefore, it is not uncommon to encounter pathogenic organisms originating from animal faeces in such

fish ponds. The possibility of fish pond water and sediment contamination through artificial fish feed is also possible as *Salmonella* have also been found in fishmeal (Hauge, 1969, Veldman *et al*., 1995) and in fish feed factories (Nesse *et al.,* 2003). Fishmeal is an important ingredient in the pelleted artificial formulated fish feed. The aquatic birds also could contribute to this. The present finding of isolation of *Salmonella* from fish pond sediment is in agreement with that of earlier workers who isolated *Salmonella* from fish pond (Cho and Kim, 1999) and shrimp pond (Dalsgaard *et al.,*1995).

5.1.3. Incidence of *Salmonella* in seafood

Seafood like fish and shrimp tested in this study also showed a significant percentage of positivity for *Salmonella* spp. Fish samples collected from landing centre recorded prevalence at 40%. Many workers have reported the presence of *Salmonella* in fish. In a study conducted in an integrated fish farm in a South East Asian country, *Salmonella* spp. were present in 28% fish samples (Twiddy and Reilly, 1995). In India, Hatha and Lakshmanaperumalsamy (1997) reported *Salmonella* from west coast fish samples. Further, Kumar *et al.* (2003) and Shabarinath *et al.* (2007) recorded higher prevalences of *Salmonella* in finfish samples of west coast waters. Though the fish harvested from open sea waters seems to be free from human pathogens, the fishes collected from polluted coastal waters or washed in contaminated harbour waters are more likely to carry *Salmonella*. Unhygienic post harvest practices by the personnel may also lead to contamination of fish.

Another important component of seafood, shrimp were also taken in this study for screening the pathogen. As much as 20% of the shrimp samples recorded positive for *Salmonella*. Though shrimps are the inhabitants of offshore waters, their benthic mode of life could expose them to pathogens associated with sediments more often than surface water dwellers. One report discusses that *Salmonella* could also be isolated from the gut of marine shrimp (Nayyerahmed *et al.,* 1995). This suggests that *Salmonella* could survive in coastal sediments and could be present in shrimp before any pre-process handling. Further, contamination on board the vessel and at fishing harbor due to unhygienic handling might also contribute to the presence of *Salmonella* in shrimp.

Molluscan shellfishes, as they are sedentary filter feeders, can be good indicators of the microbiological quality of water in which they live. In this study, oysters were sampled from their natural beds to detect *Salmonella* in them but no sample showed positivity. This could be because of the small number of samples (5) tested in this study. Heinitz *et al*. (2000) tested seafood and shellfish around the world for the presence of *Salmonella* spp. and found that US shellfish, particularly oysters had a 1.2% prevalence of *Salmonella* in domestic shellfish. In the waters of North West Spain, the overall incidence of *Salmonella* was recorded at 1.8% for the samples of shellfish including mussels, oysters, clams and cockles (Martinez-Urtaza *et al.,* 2003), all filter feeder molluscans. More study is required on the presence of *Salmonella* in molluscan shellfish from India

5.2. Direct detection of *Salmonella* by PCR from enrichment lysate

Polymerase chain reaction (PCR) techniques have earlier been widely employed by many workers to detect *Salmonella* spp. in environmental samples including seafood using *hns, inv*A and *inv*E gene specific primer pairs (Kumar *et al.,* 2003; Shabarinath *et al.,* 2007). Polymerase chain reaction (PCR) was run on enrichment broth lysates targeting *hns* gene, the amplification of which indicates the presence of *Salmonella* spp. in the samples. In the present study all the samples which gave positive for *Salmonella* spp. by conventional methods also gave positive by PCR. Since the conventional method of detection of *Salmonella* spp. involves higher labour and time, the rapid and specific PCR based techniques have better application in screening seafood for the presence of the pathogen.

5.3. Detection of *Salmonella* virulence genes

The biochemically positive isolates were confirmed by PCR using *hns* primer The primer *hns* had earlier been recommended by Kumar *et al.,* 2003 for the detection and confirmation of *Salmonella* spp. in seafood and aquatic environment. A total of 8 hns positive isolates were recovered. Further, the 8 isolates showed the presence of invasive protein genes, *inv*A and *inv*E, which are considered pathogenic markers in *Salmonella* spp. Oliveira *et al.* (2003) amplified *inv*A in *Salmonella* Enteritidis isolated from poultry, pigs, humans and food samples. The authors claimed that *inv*A is a good target for detecting pathogenic *Salmonella* in such samples. Salehi *et al.* (2005), Kumar *et al.* (2003) and Shabarinath *et al.* (2007) also detected *Salmonella* using *inv*A primers.

The presence of *inv*A and *inv*E positive virulent *Salmonella* strains in seafood like fish and shrimp and aquatic environments may get transmitted to human through use of such water and seafood. Seafood from the vicinity of polluted waters have risk of contamination with *Salmonella*.

5.4. Serotypes of *Salmonella* isolates

Four out of eight isolates of PCR confirmed *Salmonella* spp. sent for serotyping were classified as serotype Bareily. All of them were *inv*A and *inv*E positive isolates; two from sediment samples and one each from fish and shrimp samples. Bareily serotypes were earlier also isolated from seafood by Khan *et al.* (2006). However, in west coast of India Shabarinath *et al.* (2007) isolated serotypes Weltevreden and Newport from fish and shellfish samples. This study further adds to the range of as many serotypes of *Salmonella* may be prevalent in west coast waters.

5.5. Antibiotic resistance in *Salmonella* isolates

All the eight cultures tested were resistant to antibiotic erythromycin. Erythromycin resistant *Salmonella* were earlier isolated from fish, shrimp and oyster samples of west coast of India by Shabarinath *et al.* (2007). Hsu *et al.,* (2006) also isolated 74% of erythromycin resistant *Salmonella* from swine samples. All the isolates showed intermediate resistance to nitrofurantoin, tetracycline, cetriaxone, kanamycin and cephalexin. However, Shabarinath *et al,* (2007) could isolate *Salmonella* from seafood in west coast waters that are resistant to tetracycline, ampicillin and kanamycin. In case of ampicillin four isolates showed intermediate response and rest were sensitive. For rest of the antibiotics like, amikacin, chloromphenicol, co-trimoxaozole, suphafurazole, gentamicin, ncalidixic acid, ciprofloxacin and amoxicillin, all the cultures were sensitive. No multidrug resistant *Salmonella* could be found in this study.

5.6. Genetic variability among *Salmonella* isolates

RAPD profiling has been widely used for genetic differentiation of *Salmonella* (Williams *et al.*, 1990; Lin *et al.,* 1996; Hilton and Penn, 1998; Guerra *et al.,* 2000; Khoodoo *et al.,* 2002). In the present study, both RAPD primers, CRA22 and CRA23 grouped all Bareily serotypes into one cluster and rest four isolates into another. This indicates that, there was no genetic variability among the Bareily serotypes isolated in this study and are clonal. In another cluster all four isolates showed similar band pattern

indicating their homogeneity in genetic make up. This is interesting in the context of genetic variability among isolates belong to *S.* Weltevreden (Shabarinath *et al.,* 2007). The homogeneity of *S.* Bareily is interesting since two of them were isolated from estuarine sediment, one from fish and another from shrimp. It is also interesting to note that four isolates of *Salmonella* coming from different sources (fish pond sediment, fish from landing center, estuarine sediment) showed identical RAPD profile suggesting that they may be derived from a single source. This study indicates that RAPD is a useful technique for studying genetic heterogeneity in salmonella isolates from seafood.

Summary

6. SUMMARY

Samples including estuarine water and sediment, fish farm water and sediment, fish, shrimp and oysters were screened for the presence of *Salmonella* using conventional microbiological method and using enrichment lysate PCR. Estuarine and fish farm water samples were found to be free of *Salmonella* spp. However, sediment samples showed 10% - 20% prevalence. In case of seafood, fish and shrimp showed 40% and 20% prevalence respectively, whereas the small number of oyster samples were negative for the pathogen.

Polymerase chain reaction could be employed for the detection of *Salmonella* using *hns* primer. Further, the use of PCR in screening *Salmonella* isolates for the presence of virulence genes like *inv*A and *inv*E was found more helpful in detecting pathogenic strains. Using PCR, 8 isolates of pathogenic *Salmonella* carrying *inv*A and *inv*E genes were confirmed and recovered; out of which 4 were Bareily serotypes.

None of the isolates showed resistance to multiple antibiotics. Using RAPD, it was seen that 8 isolates were grouped into two clusters of different clonal origin. Four isolates were identified as *S.* Bareily by serotyping. Though these four isolates came from different sources such as estuarine sediment, fish and shrimp from fish landing center, they all belonged to S. Bareily and showed identical antibiogram and RAPD profile. Other four isolates from fish pond sediment, estuarine sediment, fish from landing center showed identical antibiogram and RAPD profile.

References

7. REFERENCES

ACHARD, C. and BENSAUDE, R. 1896. Infections paratyphoidiques. Bull. Mem. Soc. Med. Hop. de Paris. 13: 820-833 (not referred to orginal).

ADESIYUN, A., OYAH, N., SEEPERSADSINGH, N., RODRIGO, S., LASHLEY, V. and MUSAI, L. 2007. Antimicrobial resistance of *Salmonella* spp. and *Escherichia coli* isolated from table eggs. *Food Control,* 18: 306-311.

AGRON, P. G., WALKER, R. L., KINDE, H., SAWYER, S. J., HAYES, D. C., WOLLARD, J. and ANDERSEN, G. L. 2001. Identification by subtractive hybridization of sequences specific for *Salmonella enterica* serovar Enteritidis. *Appl. Environ. Microbiol.,* 67 (11): 4984-4991.

ALPUCHE-ARANDA, C. M., RACOOSIN, E. L., SWANSON, J. A. and MILLER, S. I., 1994. *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. *J. Exp. Med.,* 179: 601-608.

AUSUBEL, F., BRENT, R., KINGSTON, R. E., MOORE, D. D., SEIDMAN, J. G., SMITH, J. A. and STRUHL, K. (Eds.) 1992. *Current protocols in Molecular Biology*. 2rd Ed. Unit. 2.4. Green Publications Associations, New York.

BAHNSON, P. B., FEDORKA-CRAY, P. J., LADELY, S. R. and MATEUS-PINILLA, N. E. 2006. Herd-level risk factors for *Salmonella enterica* subsp. enterica in U.S. market pigs. *Prev. Vet. Med.,* 76: 249–262.

BAUDART, J. K., LEMARCHAND, K., BRISABOIS, A. and LEBARON, P. 2000. Diversity of *Salmonella* strains isolated from the aquatic environment as determined by serotyping and amplification of the ribosomal DNA spacer regions. *Appl. Environ. Microbiol.,* 66: 1544-1552.

BHAN, M. K., BAHL, R. and BHATNAGAR, S. 2005. Typhoid and paratyphoid fever. Lancet*, 366 (9497): 1603-1603.*

BHASKAR, N., SETTY, T. M. R., REDDY, G. V. S., MANOJ, Y. B., ANANTHA, C. S., RAGHUNATH, B. S. and ANTONY, J. M. 1995. Incidence of *Salmonella* in cultured shrimp *Penaeus monodon. Aquaculture,* 138: 257-266.

BLANC-POTARD, A. B. and GROISMAN, E. A. 1997. The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. *EMBO J.,* 16: 5376-5385.

BLANC-POTARD, A. B., SOLOMON, F., KAYSER, J. and GROISMAN, E. A. 1999. The SPI-3 pathogenicity island of *Salmonella enterica*. *J. Bacteriol.*, 181: 998-1004.

BRANDS, D. A., INMAN, A. E., GERBA, C. P., MARE, C. J., BILLINGTON, S. J., SAIF, L. A., LEVINE, J. F. and JOENS, L. A. 2005. Prevalence of *Salmonella* spp. in oysters in the United States. *Appl. Environ. Microbiol.,* 71: 893-897.

BAUER, A. W., KIRBY, W. M. M., SHERRIS, J. C. and TURCK, M. 1966. Antibiotic susceptibility testing by standardized single disc method. *Am. J. Clin. Path.,* 45: 493-496.

BRYAN, 1973. Microbial safetly of fishery products. Eds., Chichester, C. O. and Graham, H. D., Academic press, New York, pp. 273.

BURR, M. D., JOSEPHSON, K. L. and PEPPER, I. L. 1998. An evaluation of ERIC PCR and AP PCR fingerprinting for discriminating *Salmonella* fingerprinting. *Lett. Appl. Microbiol.*, 27: 24-30.

CASIN, I., BREUIL, J., BRISABOIS, A., MOURY, F., GRIMONT, F. and COLLATZ, E. 1999. Multidrug resistant human and animal *Salmonella typhimurium* isolates in France belong predominantly to a DT104 clone with the chromosome and integron – encoded B lactamase PSE-1. *J. Infect. Dis.,* 179: 1173-82.

CASTELLANI, A. 1902. Die agglutination bei gemischter infection und die Diagnose der letzteren. *Z. Hyg.* 40: 1-20 (not refered to original).

CHANDRA, M., SINGH, B. R., SHANKAR, H., AGARWAL, M., AGRAWAL, R. K., SHARMA, G. and BABU, N. 2006. Study on prevalence of *Salmonella* infection in goats. *Small Ruminant Research,* 65: 24–30.

CHANDRAN, A. and HATHA, A. M. 2005. Relative survival of *Escherichia coli* and *Salmonella typhimurium* in a tropical estuary. *Water Res.,* 39: 1397–1403.

CHEN, J. and GRIFFITHS, M. W. 2001. Detection of *Salmonella* and simultaneous detection of *Salmonella* and Shiga like toxin producing *E. coli* using magenetic capture hybridization polymerase chain reaction. *Lett. Appl. Microbiol.,* 32: 7-11.

CHIU, C., SU, L. and CHU, C. 2004. Salmonella enterica *Serotype Choleraesuis: epidemiology, pathogenesis, clinical disease, and treatment.* Clin. Microbiol. Rev., *17:311-322.*

CHO, J. and KIM, S. 1999. Viable, but non-culturable, state of a green fluorescence protein-tagged environmental isolate of *Salmonella typhi* in groundwater and pond water. *FEMS Microbiol. Letters,* 170: 257-264.

COLAK, H., HAMPIKYAN, H., BINGOL, E. B. and ULUSOY, B. 2007. Prevalence of *L. monocytogenes* and *Salmonella* spp. in Tulum cheese. *Food Control,* 18: 576–579.

COLLAZO, C. M. and GALAN, J. E. 1997. The invasion – associated type III protein secretion system in *Salmonella* - a review. *Gene.,* 192: 51-59.

CONNOR, B. A. and SCHWARTZ, E. 2005. Typhoid and paratyphoid fever in travellers. *Lancet Infect. Dis.,* 5(10): 623-628.

CROSS, J. E., BRENNER, D. J., EWING, W. H., and FALKOW, S., 1973. Molecular relationships among *Salmonellae. J. Bacteriol.*, 115: 307-315.

CRUMP, J. A., LUBY, S. P. and MINTZ, E. D. 2004. The global burden of typhoid fever. *Bull. World Health Organ.*, 82: 346-353.

DALSGAARD, A. and OLSEN, J. E. 1995. Prevalence of *Salmonella* in dry pelleted chicken manure samples obtained from shrimp farms in a major shrimp production area in Thailand. *Aquaculture*, 136: 291-295.

DOUBLET, B., WEILL, F. X., FABRE, L., CHASLUS-DANCLA, E. And CLOECKAERT, A., 2004. Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster containing a novel 30-N-aminoglycoside acetyltransferase gene cassette, aac(3)-Id, in *Salmonella enterica* serovar Newport. *Antimicrob. Agents Chemother.*, 48: 3806- 3812.

EBERTH. C. J., 1880. Die organismen in den organinen bie typhus abdominalis. *Archiv für pathologische Anatomie und physiologie und für kliniseche medicin* 81: 58-74 (not referred to orginal).

EBNER, P. D. and MATHEW, A. G. 2001. Three molecular methods to identify *Salmonella enterica* serotype Typhimurium DT104: PCR fingerprinting, multiplex PCR and rapid PFGE. *FEMS Microbiol. Lett.,* 205: 25-29.

EDWARDS, P. R. and EWING, W. H., 1972. Identification of enterobacteriaceae. 3rd Ed., Burgess Publishing Company, Minneapolis. pp. 452-467.

ESPIGARES, E., BUENO, A., ESPIGARES, M. and GALVEZ, R. 2006. Isolation of *Salmonella* serotypes in wastewater and effluent: Effect of treatment and potential risk. *Int. J. Hyg. Environ. Health,* 209: 103–107.

FARZAN, A., FRIENDSHIP, R. M., DEWEY, C. E., WARRINER, K., POPPE, C. and KLOTINS, K. 2006. Prevalence of *Salmonella* spp. on Canadian pig farms using liquid or dry-feeding. *Prev. Vet. Med.,* 73: 241-254.

FDA, 1992. *Salmonella*. In: *Bacteriological Analytical Manual*, Food and Drug Administration, AOAC International, Arlington. pp. 53-64.

FLUIT, A. C., 2005. Towards more virulent and antibiotic-resistant *Salmonella*. *FEMS Immunol. Med. Microbiol.,* 43: 1-11.

FONE, D. L., BARKER, R. M., 1994. Association between human and farm animal infections with *Salmonella* Typhimurium DT104 in Herefordshire. *Commun. Dis. Rep. CDR Rev.,* 4: R136-R140.

FRANCIS, C. L., STARNBACH, M. N. and FALKOW, S. 1992. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low oxygen condition. *Mol. Microbiol*., 6: 3077-87.

FRANKEL, G. 1989. Unique sequences in region VI of the flagellin gene of *Salmonella typhi. Mol. Microbiol.,* 3:1379-1383.

FRICKER, C. R., 1987. The isolation of Salmonellas and Campylobacters. *J. Appl. Bacteriol.,* 63: 99-116.

FRIEDRICH, M. J., KINSEY, N. E., VILA, J. and WOHLHIETER, J. A. 1993. Nucleotide sequence of a 13.9 kb segment of the 90 kb virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. *Mol. Microbiol*., 8: 543-558.

GALYOV, E., WOOD. M., ROSQVIST, R., MULLAN, P., WATSON, P., HEDGES, S. and WALLIS, T. 1997. A secreted effector protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. *Mol. Microbiol.* 25**:** 903-912.

GAUTHIER, M. and BLAIS, B.W. 2004. Cloth-based hybridization array system for the detection of multiple antibiotic resistance genes in *Salmonella enterica* subsp. enterica serotype Typhimurium DT104. *Lett. Appl. Microbiol.,* 2004, 38: 265–270.

GEBREYES, W. A.., ALTIER, C. and THAKUR, S. 2006. Molecular epidemiology and diversity of *Salmonella* serovar Typhimurium in pigs using phenotypic and genotypic approaches. *Epidemiol. Infect.*, 134: 187-198.

GLYNN, M. K., BOPP, C., DEWITT, W., DABNEY, P., MOKHTAR, M. and ANGULO, F. J. 1998. Emergence of multidrug resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. *N. Engl. J. Med.* 338: 1333–1338.

GOMEZ, T. M., MOTARJEMI, Y., MIYAGAWA, S., KAFERSTEIN, F. K. and STOHR. K. 1997. Foodborne Salmonellosis. World health stat. Q. 50: 80-81.

GROISMAN, E. A., ANNE, B. B. P., and KEIICHI, V., 1999. Pathogenicity islands and the evolution of *Salmonella* virulence. In: KAPER, J.B. and HACKER, J. (Eds.), *Pathogenicity islands and other mobile virulence elements.* ASM Washington D. C., pp. 127-150.

GUERRA, G., LACONCHA, I., SOTO, S. M., GONZA¨LEZ-HEVIA, M. A. and MENDOZA, M. C. 2000. Molecular characterisation of emergent multiresistant *Salmonella enterica* serotype organisms causing human salmonellosis. *FEMS Microbiol. Lett.,* 190: 341-347.

GUINEY, D. G., FANG, F. C., KRAUSE, M., LIBBY, S. 1994 Plasmid mediated virulence genes in nontyphoid *Salmonella* serovars. *FEMS microbiol. Lett.,* 124:1-9.

GULIG, P. A., 1990. Virulence plasmids of *Salmonella typhimurium* and other *Salmonella. Microb. Pathog.*, 8: 3-11.

HALL, R. M. and STOKES, H. W. 1993. Integrons: novel DNA elements which capture genes by site-specific recombination. *Genetica*, 90:115–32.

HARAKEH, S., YASSINE, H. and EL-FADEL, M. 2006. Antimicrobial-resistant patterns of *Escherichia coli* and *Salmonella* strains in the Lebanese aquatic environments *Environ. Pollution,* 143: 269-277.

HARVEY, R. W. S. and PRICE, T. H., 1982. *Salmonella* isolation and identification techniques. Alternative to standard method. In: CORRY, J. E. L., ROBERTS, D. and SKINNER, F. A., (Eds.,), *Isolation and identification methods for food poisoning organisms.* Academic Press: London, New York. pp. 35 – 42.

HATHA, A. A. M. and LAKSHMANAPERUMALSAMY, P. 1997. Prevalence of *Salmonella* in fish and crustaceans from markets in Coimbatore, South India. *Food Microbiol.,* 14: 111-116.

HAUGE, S. 1969. Fish meal and bacteriological problems in that product. *Nord. Vet. Med.*, 21: 46–48.

HEIN, I., FLEKNA, G., KRASSNIG, M. and WAGNER, M. 2006. Real-time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project . *J. Microbiol. Methods.* 66: 538-547.

HEINITZ, M. L., RUBLR, R. D., WAGNER, D. E. and TATINI, S. R. 2000. Incidence of *Salmonella* in fish and seafood. *J. Food Prot.*, 63: 579-592.

HENDRICKS, W., 1971. Increased recovery rate of *Salmonella* from stream bottom sediment versus surface waters. *Appl. Microbiol.,* 21: 379-380.

HENSEL, M. 2004. Evolution of pathogenicity islands of *Salmonella enterica*. *Int. J. Med. Microbiol.* 294: 95-102.

HENSEL, M., HENSLEY, A. P., NIKOLAUS, T., SAWERS, G. and BERKS, B. C. 1999b. The genetic basis of tetrathionate respiration in *Salmonella typhimurium*. *Mol. Microbiol.*, 32: 275-288.

HENSEL, M., NIKOLAUS, T. and EGELSEER, C. 1999a. Molecular and functional analysis indicates a mosaic structure of *Salmonella* pathogenicity island 2. *Mol. Microbial.*, 31: 489-498.

HILTON, A. C. and PENN, C. W. 1998. Restriction enzyme analysis of randomly amplified polymorphic DNA amplicons of *Salmonella enterica* ser. Enteritidis PT4 and Typhimurium DT104. *Lett. Appl. Microbiol.,* 27: 158-162.

HOHMANN, E. L. 2001. Nontyphoidal salmonellosis. *Clin. Infect. Dis.*, 32: 263–269.

HSU, S., CHIU, T., PANG, J., HSUAN-YUAN, C., CHANG, G. and TSEN, H. 2006. Characterisation of antimicrobial resistance patterns and class 1 integrons among *Escherichia coli* and *Salmonella enterica* serovar Choleraesuis strains isolated from humans and swine in Taiwan. *Int. J. Antimicrob. Agents,* 27: 383-391.

HUMPHREY, T. J., THRELFALL, E. J. and CRUICKSHANK, J. G. 1998. Salmonellosis. In: Palmer, S.R., Lord Soulsby and D.I.H. Simpson (eds); Zoonoses. Oxford University Press, Oxford, pp. 207-215.

ICMSF, 1996. Salmonella In: ROBERTS, T. A., BAIRD-PARKER, A. C. and TOMPKIN, R. B. (Eds.), *Microorganisms in food 5- Microbiolgical specification of food pathogens. II MSF.* 1ST ed, Blackie Academic and Professional, London. Pp. 217-275.

JITRAPAKDEE, S., TASSANAKAJON, A., BOONSAENG, V., PIANKIJAGUM, S. and PANYIRN, S. 1995. A simple, rapid and sensitive detection of *Salmonella* in food by polymerase chain reaction. *Mol. Cell. Probes,* 9: 375-382.

JONES, D. D., LAW, R. and BEJ, A. K. 1993. Detection of *Salmonella* spp. in oysters using polymerase chain reaction (PCR) and gene probes. *J. Food Sci.,* 58: 1191-1197.

JOOSTEN, H., BIDLAS, E. and GAROFALO, N. 2006. *Salmonella* detection in probiotic products. *Int. J. Food Microbiol.,* 110: 104–107.

JORDAN, E., EGAN, J., DULLEA, C., WARD, J., MCGILLICUDDY, K., MURRAY, G., MURPHY, A., BRADSHAW, B., LEONARD, N., RAFTER, P. and MCDOWELL, S. 2006. *Salmonella* surveillance in raw and cooked meat and meat products in the Republic of Ireland from 2002 to 2004. *Int. J. Food Microbiol.,* 112: 66–70.

KARIUKI, S., REVATHI, G., KIIRU, J., LOWED, B., BERKLEY, J. A. and HART, C. A. 2006. Decreasing prevalence of antimicrobial resistance in non-typhoidal *Salmonella* isolated from children with bacteraemia in a rural district hospital, Kenya. *Int. J. Antimicrob. Agents* 28: 166-171.

KAUFFMANN, F., 1935. Weitere erfahruson mit dem kombinierten anreicherungvergaheen fur *Salmonella* bazillen. *Leitsch. Hyg. Infect.*, 117: 26-32. (not referred to orginals).

KAUFFMANN, F., 1941. Die bacteriology der *Salmonella*–gruppe. Munksgaard, Copenhagen (not referred to original).

KAUFFMANN, F., 1963. Zur Tialdiagnose der *Salmonella*-subgenera I, II and III. *Acta. Path. Microbiol. Scand.*, 58: 109-113 (not referred to original).

KAUFFMANN, F., 1966. Zur klassifizierung und nomenkatur der sub genera I – IV. *Zbl. Bakt. Hyg. Ref.,* 202: 482-483 (not referred to original).

KAUFFMANN, F., 1978. Das fundament munksgard. Copenhagen. (not referred to orginal).

KHAN, A. A., CHENG, C. M., VAN, K. T., WEST, C. S., NAWAZ, M. S. and KHAN, S. A. (2006). Characterisation of class I integron resistance gene cassettes in *Salmonella enterica* serovars Oslo and Bareily from imported seafood. *J. Antimicrob. Chemotherapy,* 58: 1308-1310.

KHOODOO, M. H. R., ISSACK, M. I. and JAUFEERALLY-FAKIM, Y. 2002. Serotyping and RAPD profiles of *Salmonella enterica* isolates from Mauritius. *Lett. Appl. Microbiol.,* 35: 146-152.

KING, S. and METZGER, W. I. 1968. A new plating medium for the isolation of enteric pathogens Hektoen enteric agar. *Appl. Microbiol.*, 16: 577-578.

KLERKS, M. M., ZIJLSTRA, C. and VAN BRUGGEN, A. H. C. 2004. Comparison of real-time PCR methods for detection of *Salmonella enterica* and *Escherichia coli* O157:H7, and introduction of a general internal amplification control. *J. Microbiol. Methods,* 59: 337-349.

KUDAKA, J., ITOKAZU, K., TAIRA, K., IWAI, A., KONDO, M., SUSA, T. and IWANAGA, M. 2006. Characterisation of *Salmonella* isolated from Okinawa, Japan. *Jap. J. Infect. Dis.,* 59: 15-19.

KUMAR, H. S., SUNIL, R., VENUGOPAL, M. N., KARUNASAGAR, I. and KARUNASAGAR, I., 2003. Detection of *Salmonella* spp in tropical seafood by polymerase chain reaction. *Int. J. Food Microbiol.,* 2787: 1-5.

KUMAR, S., BALAKRISHNA, K. and BATRA, H. V. 2006. Detection of *Salmonella enterica* serovar Typhi (*S. Typhi*) by selective amplification of *inv*A, *via*B, *fli*C-d and *prt* genes by polymerase chain reaction in mutiplex format. *Lett. Appl. Microbiol.,* 42: 149-154.

LAILLER, R., SANAA, M., CHADOEUF, J., FONTEZ, B., BRISABOIS, A., COLMIN, C. and MILLEMANN, Y. 2005. Prevalence of multidrug resistant (MDR) *Salmonella* in bovine dairy herds in western France. *Prev. Vet. Med.,* 70 : 177–189.

LAUDERDALE, T. AARESTRUP, F. M., CHENA, P., LAIA, J., WANGA, H., SHIAUA, Y., HUANGA, I., and HUNGA, C. 2006. Multidrug resistance among different serotypes of clinical *Salmonella* isolates in Taiwan. *Diag. Microbiol. Infect. Dis.,* 55: 149–155.

LAWSON, A. J., DESAI, M., O'BRIEN, S., DAVIES, R. H., WARD, L. R. and THRELFALL, E. J. 2004. Molecular characterization of an outbreak strain of multiresistant *Salmonella enterica* serovar Typhimurium DT104 in the UK . *Clin. Microbiol. Infect.,* 10: 143-7.

LE MINOR, L. and POPOFF M., 1988. *Antigenic Formulae of Salmonella serovars.* In: 5th revision WHO collaborating center for reference and research on *Salmonella.* Institute Pasteur, Paris. pp.45-56.

LE MINOR, L. and POPOFF, M. 1987. Request for an opinion Designation of *Salmonella enterica* sp. Nov. nom. Rev as the type and only species of the genus Salmonella. *Int. J. Syst. Bacteriol.* 37: 465-468.

LE MINOR, L., 1981. The genus '*Salmonella*'. In: MORTIMER, P. STARR, HEINZ, STOLP., HANS, G. TRUPER., ALBERT, BALOWS., HANS and SCHLEGEL, G. (Eds.), *Prokaryotes.* pp. 2760-2774.

LE MINOR, L., POPOFF, M. Y., LAURENT, B. and HERMANT, D. 1986. Individualisation d'une septième sous-espèce de *Salmonella*: *S. choleraesuis* subsp. *indica* subsp. nov. *Ann. Inst. Pasteur. Microbiol.*, 137 B: 211-217 (not referred to original).

LE MINOR, L., VE'RON, M. and POPOFF, M., 1982. Taxonomie Des *Salmonella. Ann . Microbiol. Cinst. Pasteur.,* 133: 223-243 (not referred to original).

LEUSDEN-VAN, F. M., SCHOTHORST-VAN, M. and BECKERS, H. J., 1982. The standard *Salmonella* isolation method. In: Corry, J.E.L., Roberts, T.A. And Skinner, F.A., (Eds.), *Isolation and identification methods for food poisoning organisms.* Academic Press, London. pp. 67-83.

LEVERSTEIN-VAN, H. M. A., BLOK, H., DONDERS, A., PAAUW, A., FLUIT, A. C. and VERHOEF, J. 2003. Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin. *J. Infect. Dis.,* 187: 251–259.

LIM, H., LEE, K. H., HONG, C., BAHK, G. and CHOI, W. S. 2005. Comparison of four molecular typing methods for the differentiation of *Salmonella* spp. *Int. J. Food Microbiol.,* 105: 411-418.

LIN, A. W., USERA, M. A., BARRETT, T. J. and GOLDSBY, R. A. 1996. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella enteritidis. J. Clin. Microbiol.,* 34: 870-876.

LOGUE, C. M., SHERWOOD, J. S., OLAH, P. A., ELIJAH, L. M. and DOCKTER, M. R. 2003. The incidence of antimicrobial-resistant *Salmonella* spp. on freshly processed poultry from US Midwestern processing plants. *J. Appl. Microbiol.*, 94: 16-24.

LOUIS, P. C. A., 1829. Fievre typhoide. bailliere, Paris. (not referred to original).

MALORNY, B., HOORFAR, J., BUNGE, G. and HELMUTH, R., 2003. Multicenter validation of the analytical accuracy of S*almonella* PCR: Towards an international standard. *Appl. Environ. Microbiol.,* 69: 290-296.

MARMUR, J., FALKOW, S. and MANDEL, M., 1963. New approaches in bacterial taxonomy. *Ann. Rev. Microbiol.*, 17: 329-379.

MARTINEZ-URTAZA, J. and LIEBANA, E. 2005. Use of pulsed-field gel electrophoresis to characterize the genetic diversity and clonal persistence of *Salmonella senftenberg* in mussel processing facilities. *Int. J. Food Microbiol.,* 52 (2): 255-263.

MARTINEZ-URTAZA, J., SACO, M., HERNANDEZ-CORDOVA, G., LOZANO, A., GARCIA-MARTIN, O. and ESPINOSA, J., 2003. Identification of *Salmonella* serovars isolated from live molluscan shellfish and their significance in the marine environment. *J. Food Prot.,* 66: 226-232.

MCEVOY, J. M., DOHERTY, A.M., SHERIDAN, J.J., BLAIR, I.S. and McDOWELL, D.A. 2003. The prevalence of *Salmonella* spp. in bovine faecal, rumen and carcass samples at a commercial abattoir. *J.App.Microbiol.,* 94: 693-700.

MELLOUL, A. HASSANI, L. and RAFOUK, L. 2001. *Salmonella* contamination of vegetables irrigated with untreated wastewater. *World J. Microbiol. Biotechnol.,* 17: 207-209.

MICHAEL, E., O. and SAMUEL, I. M. 2001. *Salmonella*: A model for bacterial pathogenesis. *Ann. Rev. Med.,* 52: 259-74.

MICHAEL, G. B., CARDOSO, M. and SCHWARZ, S. 2006. Molecular analysis of *Salmonella enterica* subsp. Enterica serovar Agona isolated from slaughter pigs. *Vet. Microbiol.* 112: 43–52.

MILLER, S. I. and PEGUES, D. A. 2000. *Salmonella* species, including *Salmonella typhi*. In principles and practice of infectious diseases ed. MANDELL, G. L., BENNETT, J. E. and DOLIN, R. 2: 2344-2363. Philadelphia; Churchill livingstone, Vol 2 5th ed).

MORETRO, T., MIDTGAARD, E. S., NESSE, L. L. and LANGSRUD, S. 2003. Susceptibility of *Salmonella* isolated from fish feed factories to disinfectants and air-drying at surfaces. *Vet. Microbiol.,* 94: 207–217.

MREMA, N., MPUCHANE, S. and GASHE, B. A. 2006. Prevalence of *Salmonella* in raw minced meat, raw fresh sausages and raw burger patties from retail outlets in Gaborone, Botswana. *Food Control*, 17: 207-212.

NAGAL, K. B., MANDIAL, R. K., KATOCH, R. C., and CHAHOTA, R. 2006. Occurrence of *Salmonella enterica*, subspecies *enterica* serovar Berta (*Salmonella* Berta) in bovine calves in Himachal Pradesh, India. *Veterinarski Archiv.,* 76 (2): 153-157.

NCCLS, National Committee for Clinical Laboratory Standards. 1993. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A5. National Committee for Clinical Laboratory Standards, Villanova, Pa.

NESSE, L. L., NORDBY, K., HEIR, E., BERGSJOE, B., VARDUND, T., NYGAARD, H., HOLSTAD, G. 2003. Molecular analysis of *Salmonella enterica* isolates from fish feed factories and fish feed ingredients. *Appl. Environ. Microbiol*. 69, 1075–1081.

NIELSEN, C. 1995. Animal Evolution: Interrelationships of the living phyla. Oxford University Press, Oxford.

NORTH, W. R. and BARTRAM, M. T., 1953. The efficiency of selenite broth of different compositions in the isolation of *Salmonella. Appl. Microbiol.*, 1: 130-134.

NAYYERAHMED., I. KARUNASAGAR and I. KARUNASAGAR. 1995. Microbiology of cultured shrimp in India. FAO Fish Rep, 514 (suppl.): 13-22.

O'SHEA, M. L. and FIELD, R. 1991. Detection and disinfection of pathogens in storm generated flows. *Can. J. microbiol.* 38:267-276

OCHMAN, H. and GROISMAN, E. A. 1996. Distribution of pathogenicity islands in *Salmonella* spp. *Infect. Immun*. 64: 5410-5412.

OELSCHLAEGER, T. A., ZHANG, D., SCHUBERT, S., CARNIEL, E., RABSCH, W., KARCH, H. AND HACKER, J. 2003. The high-pathogenicity island is absent in human pathogens of Salmonella enterica *subspecies I but present in isolates of subspecies III and VI.* J. Bacteriol., *185:*

OLIVEIRA, S. D., RODENBUSCH, C. R., MICHAEL, G. B., CARDOSO, M. I. R., CANAL, C. W. and BRANDELII, A. 2003. Detection of virulence genes in *Salmonella enteritidis* isolated from different sources, *Braz. J. Microbiol.,* 34 (Suppl.1):123-124.

OLSEN, J. E., BROWN, D. J. MADSEN, M. and BISGAARD, M. 2003. Cross-contamination with *Salmonella* on a broiler slaughterhouse line demonstrated by use of epidemiological markers. *J. Appl. Microbiol.,* 94: 826–835.

PANG, T. 1998. Genetic dynamics of *Salmonella typhi* - diversity in clonality. *Trends Microbiol.,* 6: 339-342.

PARRY, C. M. 2003. Antimicrobial drug resistance in *Salmonella enterica*. *Curr. Opin. Infect. Dis.,* 16: 467– 472.

PATEL, J. R., BHAGWAT, A. A., SANGLAY, G. C. and SOLOMON, M. B. 2006. Rapid detection of *Salmonella* from hydrodynamic pressure-treated poultry using molecular beacon real-time PCR. *Food Microbiol.*, 23: 39-46.

PELZER, K. D., 1989. Salmonellosis . *J. Vet. Med. Assoc.,* 195: 456-463.

PIDDOCK, L. V. J., 2002. Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. *FEMS Microbiol. Rev.*, 26: 3-16.

POKHAREL, B. M., KOIRALA, J., DAHAL, R. K., MISHRA, S. K., KHADGA, P. K. and TULADHAR, N. R. 2006. Multidrug-resistant and extended-spectrum beta-lactamase (ESBL)-producing *Salmonella enterica* (serotypes Typhi and Paratyphi A) from blood isolates in Nepal: surveillance of resistance and a search for newer alternatives. *Int. J. Infect. Dis.,* 10: 434-438.

RAHN, K., DeGRANDIS, S. A., CLARKE, R. C., McEWEN, S. A., GALAN, J. E., GINOCCH, O. C., CURTIS, R. and GYLES, C. L. 1992. Amplification of an *inv*A gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes,* 6: 271-279.

REEVES, M. W., EVINS, G. M., HEIBA, A. A., PLIKAYTIS, B. D. and FARMER III, J. J., 1989. Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by mutilocus enzyme electrophoresis and proposal of *Salmonella* bongori comb. *J. Clin. Microbiol.,* 27: 313-320.

REILLY, P. J. A. and TWIDDY, D. R. 1992. *Salmonella* and *Vibrio cholerae* in brackish water cultured tropical prawns. *Int. J. Food Microbiol.,* 16: 293-301.

RIYAZ-UL-HASSAN, S., VERMA, V. and QAZI, G. N. 2004. Rapid detection of *Salmonella* by polymerase chain reaction. *Mol. Cell. Probes*, 18: 333–339.

RODRIGUEZ-LAZARO, D., HERNA´NDEZ, M, ESTEVE, T., HOORFAR, J. and PLA, M. 2003. A rapid and direct real time PCR-based method for identification of *Salmonella* spp. *J. Microbiol. Methods*, 54: 381-390.

ROSZAK, D. B., GRIMES, D. J. and COLWELL, R. R. 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. microbiol.,* 30: 334-338.

ROTGER, R. and CASADESUS, J. 1999. The virulence plasmids of *Salmonella*. *Internatl. Microbiol.,* 2: 177-184.

SAHEKI, K., KOBAYASHI, S. and KAWANISHI, T. 1989. *Salmonella* contamination of eel culture ponds. *Nippon Suisan Gakkaishi*, 55, 675-679 (In Japanese).

SALEHI, T. Z., MAHZOUNIEH, M. and SAEEDZADEH, A. 2005. Detection of *InvA* gene in isolated *Salmonella* from broilers by PCR method. *Int. J. Poultry Sci.,* 4 (8): 557-559.

SALMON, D. E. 1885. Report of the chief of the bureau. p 228. In: First annual report of the bureau of animal industry for the year 1884. Government printing office. Washington D.C. (not referred to original).

SHABARINATH, S., KUMAR, H. S., KHUSHIRAMANI, R., KARUNASAGAR, I. and KARUNASAGAR, I. 2007. Molecular characterisation of *Salmonella* associated with tropical seafood. *Int. J. Food Microbiol.,* (EPUB ahead of print).

SHAHADA, F., CHUMA, T., TOBATA, T., OKAMOTO, K., SUEYOSHI, M. and TAKASE, K. 2006. Molecular epidemiology of antimicrobial resistance among *Salmonella enterica* serovar Infantis from poultry in Kagoshima, Japan. *Int. J. Antimicrob. Agents,* 28: 302-307.

SHANAHAN, P. M. A., JESUDASON, M. V., THOMSON, C. J. and AMYES, S. G. B. 1998. Molecular analysis of an identification of antibiotic resistance genes in clinical isolates of *Salmonella typhi* from India. *J. Clin. Microbiol.,* 36: 1595-1600.

SHARPE, N. S., HOLT, J. G. 1989. (eds) Bergey's manual of systematic bacteriology, Williams & Wilkins, Baltimore, Vol 3 and 4: pp 661-662.

SHEA, J. E., BEUZON, C. R., GLEESON, C., MUNDY, R. and HOLDEN, D. W., 1999. Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. *Infect. Immun.,* 67: 213-219.

SHEWAN, J. M., 1962. *Salmonella.* In: BORGSTROM, G. (Ed.), Fish as food. Vol.2. Academic Press, New York. pp. 443

SINHA, A., SAZAWAL, S., KUMAR, R., SOOD, S., REDDAIAH, V. P., SINGH, B., RAO, M., NAFICY, A., CLEMENS, J. D. and BHAN, M. K. 1999. Typhoid fever in children aged less than 5 years. *Lancet*, 354(9180): 734-737.

SOUMET, C., ERMEL, G., ROSE, N., ROSE, V., DROUIN, P., SALVAT, G. and COLIN, P., 1999. Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella* Enteritidis and *Salmonella* Typhimurium from environmental swabs of poultry houses. *Lett. Appl. Microbiol.,* 28: 113-117.

STEVENS, A. KABORÉ, Y., PERRIER-GROS-CLAUDE, J., MILLEMANN, Y., BRISABOIS, A., CATTEAU, M., CAVIN, J., and DUFOUR, B. 2006. Prevalence and antibiotic-resistance of *Salmonella* isolated from beef sampled from the slaughterhouse and from retailers in Dakar (Senegal). *Int. J. Food Microbiol.,* 110: 178-186.

STOKES, H. W and HALL, R. M. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol.*, 3:1669–83.

SURESH, T., HATHA, A. M. M., SREENIVASAN, D., SANGEETHA, N. and LAKSHMANAPERUMALSAMY, P. 2006. Prevalence and antimicrobial resistance of *Salmonella enteritidis* and other *Salmonellae* in the eggs and egg-storing trays from retails markets of Coimbatore, South India. *Food Microbiol.,* 23: 294-299.

TAYLOR, W. I., 1965. Isolation of shigellae. I. Xylose-lysine agars: New media for the isolation of enteric pathogens. *Am. J. Clin. Pathol.,* 44: 471-475.

THRELFALL, E. J, FROST, J. A., WARD, L. R. and ROWE, B. 1994. Epidemic in cattle and humans of *Salmonella typhimurium* DT104 with chromosomally integrated multiple drug resistance. *Vet. Rec.,* 134: 577.

TINDALL, B. J., GRIMONT, P. A. D., GARRITY, G. M. and EUZEBY, J. P. Nomenclature and taxonomy of the genus *Salmonella*. *Int. J. Syst. Evol. Microbiol.*, 55: 521-524.

TORPDAHL, M., SKOV, M. N., SANDVANG, D. and BAGGESEN, D. L. 2005. Genotypic characterization of *Salmonella* by multilocus sequence typing, pulsed-field gel electrophoresis and amplified fragment length polymorphism. *J. Microbiol. Methods,* 63: 173-184.

TOURON, A., BERTHE, T., PAWLAK, B. and PETIT, F. 2005. Detection of *Salmonella* in environmental water and sediment by a nested-multiplex polymerase chain reaction assay. *Res. Microbiol.,* 156: 541–553.

TOWNSEND, S. M., KRAMER, N. E., EDWARDS, R., BAKER, S., HAMLIN, N., SIMMONDS, M., STEVENS, K., MALOY, S., PARKHILL, J., DOUGAN, G. and BAUMLER, A. J., 2001. *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. *Infect. Immun.*, 69: 2894–2901.

TWIDDY, D. R. and REILLY, P. J. A. 1995. Occurrence of antibiotic-resistant human-pathogens in integrated fish farms. In: FAO Fisheries Report. No. 514, pp 23-37. Rome.

URFER, E., ROSSIER, P., MEAN, F., KRENDING, M. J., BURNENS, A., BILLE, J., FRANCIOLI, P. and ZWAHLEN, A. 2000. Outbreak of *Salmonella* Braenderup gastroenteritis due to contaminated meat pies: clinical and molecular epidemiology. *Clin. Microbiol. Infect.,* 6: 536-542.

VARNAM, A. H. and EVANS, M. G., 1991. *Salmonella* In: VARNAM, A. H. and EVANS, M. G. (Eds), *Food borne pathogens-An illustrated text*. Wolfe Publishing Ltd, Aylesbury. pp. 51-86.

VAZQUEZ-NOVELLE, M. D., PAZOS, A. J., ABAD, M., SANCHEZ, J. L. and PEREZ-PARALLE, M. L. 2005. Eight-hour PCR-based procedure for the detection of *Salmonella* in raw oysters. *FEMS Microbiol. Lett.,* 243: 279-283.

VELDMAN, A., VAHL, H. A., BORGGREVE, G. J. and FULLER, D. C. 1995. A survey of the incidence of *Salmonella* species and enterobacteriaceae in poultry feeds and feed components. *Vet. Rec.*, 136: 169–172.

VO, A. T. T., DUIJKEREN, E., FLUIT, A. C., HECK, M. E. O. C., VERBRUGGEN, A., MAAS, H. M. E. and GAASTRA, W. 2006. Distribution of *Salmonella enterica* Serovars from humans, livestock and meat in Vietnam and the dominance of *Salmonella* Typhimurium Phage Type 90. *Vet.Microbiol.,* 113: 153–158.

WALLIS, T. S. and GALYOV, E. E. 2000. Molecular basis of *Salmonella* induced enteritis. *Mol. Microbiol.*, 36: 997-1005.

WALLIS, T. S., PAULIN, S. M., PLESTED, J. S., WATSON, P. R. and JONES, P. W. 1995. The *Salmonella* Dublin virulence plasmid mediates systemic but not enteric phases of salmonellosis in cattle. *Infect. Immun.*, 63: 2755-2761.

WHITE, P. B., 1926. Further studies on *Salmonella* species. In: *Great Britain Medical Research Special report No 10,* Her Majesty's Stationary Office, London, pp. 24-32. (not referred to original).

WILL, L. A., DIESCH, S. L. and POMEROY, P. S. 1973. Survival of *Salmonella typhimurium* in animal manure disposal in a model oxidation ditch. *Am. J. Publ. Health,* 63: 322-326.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. and TINGEY, S. V., 1990. DNA polymorphism amplified arbitary primers are useful as genetic markers. *Nucl. Acids Res.,* 18: 6531-6535.

WILSON, W. J. and BLAIR, E. M. M., 1927. Use of glucose bismuth sulphite iron medium for the isolation of *Bacillus typhosus* and *Bacillus proteus*. *J. Hyg.*, 26: 374-391. (not referred to original).

WINFIELD, D. M. and GROISMAN, E. A., 2003. Role of non-host environments in the life style of *Salmonella* and *Escherichia coli. Appl. Environ. Microbiol.*, 69: 3687-3694.

WONG, K. K., McCLELLAND, M., STILLWELL, L. C., SISK, E. C., THURSTON, S. J. and SAFFER, J. D. 1998. Identification and sequence analysis of a 27-kb chromosomal fragment containing a *Salmonella* pathogenicity island located at 92 minutes on the chromosomal map of *Salmonella enterica* serovar typhimurium . *Infect. Immun.,* 66: 3365-3371.

WOOD, M. W., JONES, M. A., WATSON, P. R., HEDGES, S., WALLIS, T. S. and GALVOY, E. E. 1998. Identification of pathogenicity island required for *Salmonella* enteropathogenicity. *Mol. Microbiol.,* 29: 883-891.

WYATT, L. E., NICKELSON R. and VANDERZANT, C. 1979. Occurrence and control of *Salmonella* in freshwater catfish. *J. Food Sci.,* 44: 1067-1069.

YUK, H. G. and SCHNEIDER, K. R. 2006. Adaptation of *Salmonella* spp. in juice stored under refrigerated and room temperature enhances acid resistance to simulated gastric fluid. *Food Microbiol.,* 23: 694–700.

ZHAO, S., DATTA , A. R., AYERS, S., FRIEDMAN, S., WALKER, R. D. and WHITE, D. G. 2003. Antimicrobial resistant *Salmonella* serovars isolated from imported foods.