INTEGRON STUDY, MOLECULAR TYPING AND CHARACTERIZATION OF *SALMONELLA* SEROTYPES ISOLATED FROM SEAFOOD AND POULTRY

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by

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Integron study, molecular typing and characterization of *Salmonella* serotypes isolated from seafood and poultry**" is based on the original research work carried out by Mr. Siju M. Varghese under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

Dr. Sarita G. Bhat

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Integron study, molecular typing and characterization of *Salmonella* serotypes isolated from seafood and poultry**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin, under the guidance of Dr. Sarita G Bhat, Associate Professor and Head, and the thesis or no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles of recognition.

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
BLAST	Basic local alignment search tool
bp	Base pair
Bst	*Bacillus stearothermophilus*
CFU	Colony forming unit
cm	Centimeter
CTAB	Hexadecyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
g	Gram
h	Hour
H_2S	Hydrogen sulphide
l	Litre
KCl	Potassium chloride
LAMP	Loop mediated isothermal amplification
LIA	Lysine iron agar
MAR	Multiple antibiotic resistance
min	Minutes
mL	Millilitre
$MgCl_2$	Magnesium chloride
MTCC	Microbial type culture collection
NaCl	Sodium chloride
NCIM	National collection of industrial micro organisms
PCR	Polymerase chain reaction

QRDR	Quinolone resistance determining region
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Second
SGI	Salmonella genomic island
Subsp.	Subspecies
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TSI	Triple sugar iron
TTSS	Type three secretion system
U	Unit
UPGMA	Unweighted pair group method with arithmetic average
UV	Ultra violet
V	Volt
XLD	Xylose lysine desoxycholate
^{O}C	Degree celcius
µg	Microgram
µL	Microlitre
%	Percentage

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Chapter 1

INTRODUCTION

Salmonella is a highly adaptive Gram negative bacillus of the Enterobacteriaceae family. They are, with the exception of *Salmonella* enterica subspecies enterica serotype Gallinarum, motile, non-spore forming facultative anaerobes. The only two species of *Salmonella*, that includes more than 2600 serotypes, are pathogenic to a wide range of animals including humans.

Contaminated fish, poultry and pork are the major reservoirs of *Salmonella* and are also sources of cross contamination. Several serotypes of *Salmonella* cause disease in humans, poultry and pig, but fishes act only as carriers. Several potential pathogenic *Salmonella* serotypes have been isolated from fishes.

Salmonella infections are often food or water-borne, which may be a self limiting gastroenteritis or a life threatening enteric fever. Both *Salmonella enterica* subspecies *enterica* serotype Typhi and *Salmonella enterica* subspecies *enterica* serotype Paratyphi colonize only human beings and produce enteric fever. Some of these patients may also become chronic carriers. Its potential for food-borne zoonotic transmissions made it a subject for international, national and local surveillance programs. Epidemiological studies demand for the proper identification of the serotype and determination of the antimicrobial resistance/susceptibility profile.

The vast majority of the remaining serotypes are capable of colonizing the alimentary tract of a wide variety of animals including humans. Even though non-typhoidal salmonellosis is self-limiting, systemic infection needs antimicrobials for treatment. But the indiscriminate use of antibiotics in the

field of medicine, animal feeds and other agricultural practices has witnessed the rise of antibiotic resistant bacteria, which has great impact on human health (Witte, 1998; Singer *et al.*, 2003).

Bacteria effectively combat antibiotics by several mechanisms: mutation in the antibiotic targeted genes is one such mode. This is very well demonstrated in nalidixic acid resistance, where point mutations in the A subunit of gyrase gene (Griggs *et al.*, 1996) make the bacteria invulnerable for the antibiotic.

Several bacteria are reported to show resistance against multiple antibiotics. *Salmonella enterica* subspecies *enterica* serotype Typhimurium DT104 harbours a 43kb *Salmonella* Genomic Island 1 (SGI1) holding an array of antibiotic resistant genes (Threlfall *et al.*, 1994). These islands are reported to harbour class 1 integrons in multiple copies. A number of studies have reported their presence in other serotypes also. The antibiotic resistance gene array isolated from numerous other serotypes shows marked deviation in its composition from the DT104 strains.

The presence of integron, a natural gene capture and expression system in bacteria, augments the bacterial defense potential in combating the antibiotic stress (Stokes and Hall, 1989). Integrons are site-specific recombination systems consisting of an integrase gene, a recombination site and a promoter. The integrase helps in the integration of various gene cassettes at the recombination site. The gene cassette consists of a single ORF which is usually an antibiotic resistance gene and devoid of a promoter. These genes are expressed using the integron promoter. Based on the type of integrase gene, several integrons have been identified, five of which are associated with antibiotic resistance (Rowe-Magnus and Mazel, 2002). The most frequently observed is the class 1 integron which is associated with Tn 21 transposon

family (Hall, 1997). Integrons play a key role in the dissemination of antibiotic resistance genes among the members of bacterial world.

Salmonellosis, which involves infection, survival and pathogenesis, is a multifactorial process, in which the products of a large number of virulence determinants are required. Analysis of the genetic structure reveal that many of these virulence genes are clustered as islands in the *Salmonella* genome called the *Salmonella* Pathogenicity Islands (SPIs). Several SPIs have been reported so far (Mills *et al.*, 1995; Shea *et al.*, 1996; Blanc-Potard and Groisman, 1997; Wong *et al.*, 1998; Wood *et al.*, 1998). Some of these SPIs are conserved throughout the genus *Salmonella*, while a subset is specific for certain serotypes. SPIs appear to have been acquired by horizontal gene transfer (Groisman *et al.*, 1993). The acquisition of various SPIs along with its virulence genes had a crucial role in the evolution of *Salmonella* into a successful pathogen.

Bacteriophages have contributed greatly to the pathogenicity of bacteria. *Salmonellae* are well known for the presence of different phages in their genome (Figueroa-Bossi *et al.*, 1997; Miao and Miller, 1999; Mirold *et al.*, 1999). These phages strengthen the virulence potential of the bacteria and help in the spread of several virulence genes among different bacteria through transduction, resulting in the emergence of new virulent strains.

The genus *Salmonella* comprises more than 2600 serotypes and hence the first step is serotyping. Typing is an important step in the epidemiological study of bacterial pathogens and is an antigen-based method. The presence or absence of the various somatic (O) and flagellar (H) antigens is the basis of serotyping. In addition to serotyping, several nucleic acid based typing methods are also widely used which include PCR-ribotyping, RAPD, AFLP, ERIC-PCR, PFGE, to name a few. The efficiency and discriminative power or

these typing methods vary with the samples and hence the selection of the appropriate method is very important for a successful epidemiological study. Molecular fingerprinting will provide information about the origin of the infection and the probable route through which the pathogen has reached its destination. The selection and combination of the various fingerprinting techniques is very important for the epidemiological analysis.

Conventional detection system for *Salmonella* relies on the culture method which requires a minimum of 4 continuous days (Andrews *et al.*, 1995). Application of PCR technique has revolutionized *Salmonella* detection procedure such that, the detection time has been reduced to a few hours (Kumar *et al.*, 2008). Another DNA amplification method, Loop Mediated Isothermal Amplification (LAMP), has been developed which can be performed under isothermal conditions (Notomi *et al.*, 2000). It relies on autocycling strand displacement DNA synthesis catalysed by *Bst* polymerase. This method has been widely utilized for the detection of *Salmonella* from different sources (Okamura *et al.*, 2008; Techathuvanan *et al.*, 2010; Varghese *et al.*, 2012). LAMP has been preferred due to its rapidity, sensitivity and cost effectiveness over PCR in detection of microbial pathogens. Direct visualization of the positive result is also possible with this method.

The primary habitat of *Salmonella* is the gastrointestinal tract of animals and they are discharged into the water bodies through the feces. Aquatic animals act as asymptomatic reservoirs of a wide range of *Salmonella* serotypes. The inevitable delay in the detection of *Salmonella* contamination and the low sensitivity of the conventional methods is a serious issue in the seafood industry. Due to the indiscriminate use, the antibiotics are finally accumulated in the aquatic environment which provides the required antibiotic stress for the emergence of more and more antibiotic resistant phenotypes of

Salmonella. Several genetic determinants like integrons, genomic islands etc. play their role in acquisition and reshuffling of antibiotic resistance genes. A large number of virulence determinants are required for *Salmonella* pathogenicity. The virulence potential of *Salmonella* is determined, to some extent, by the presence of phages or phage mediated genes in the bacterial genome. There is much intra-serotype polymorphism in *Salmonella* and epidemiological studies rely on genetic resemblance of the isolated strains. Proper identification of the strain employing the traditional and molecular techniques is a prerequisite for accurate epidemiological studies (Soto *et al.*, 2000).

In this context, a study was undertaken to determine the prevalence of different *Salmonella* serotypes in seafood and to characterize them.

Objectives of the study

1. Determination of the prevalence of *Salmonella* in seafood.
2. Antibiotic resistance profiling and to study the involvement of special genomic determinants augmenting the emergence of resistance phenotypes.
3. Virulotyping to assess and compare the inter and intra-serotype variation in virulence gene profile of the isolated *Salmonella.*
4. Molecular fingerprinting of the isolated *Salmonella* strains.
5. Protocol standardization for rapid detection of *Salmonella* using Loop-mediated isothermal amplification.

Chapter 2

REVIEW OF LITERATURE

2.1. Discovery of *Salmonella*

The genus *Salmonella*, a member of the family Enterobacteriaceae, is named after Daniel Elmer Salmon, who with Theobald Smith is credited with the discovery of the organism that caused hog cholera (Salmon and Smith, 1886), now known as *Salmonella enterica* subspecies *enterica* serotype Choleraesuis.

2.2. Evolution of *Salmonella* nomenclature

Salmonella nomenclature is still a matter of controversy as researchers all over the world use different systems to communicate about this genus. The nomenclature system for *Salmonella* has evolved from the one serotype-one species concept proposed by Kauffmann (Kauffmann, 1966) on the basis of the serological identification of the O (somatic) and H (flagellar) antigens.

Each serotype was considered as a species. These "species" were named after the host or the place from which they were originally isolated. This resulted in large number of "species". DNA-DNA hybridization works of Crosa *et al.* (1973) showed that all of them except *Salmonella bongori*, were related at the species level and therefore belonged to a single species. Even though *Salmonella* Choleraesuis, being the approved type species (Skerman *et al.*, 1980) had priority as the species name, it did not gain much support. It was biochemically different from the major serotypes like *Salmonella* Typhi and *Salmonella* Enterica, being arabinose and trehalose negative (Kauffmann and Edwards, 1952).

In 1986, the taxonomic subcommittee on Enterobacteriaceae of International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology, Manchester, U.K., (Penner, 1988) unanimously supported the suggestion of accepting *Salmonella enterica* as the type species. Le Minor and Popoff (1987) made a request for an opinion, designating *Salmonella enterica* as type and only species of its genus and LT2 as the type strain of that species, to the Judicial Commission of the International Committee of Systematic Bacteriology. The commission had denied the request on safety grounds. The commission was reluctant to reduce the highly pathogenic *Salmonella typhi* to only a serovar (*Salmonella enterica* subsp. *enterica* serovar Typhi) (Wayne, 1991) as this can lead to neglect by the clinicians . However, the commission decided to reopen the original request for an opinion with alternative proposals. Le Minor and Popoff (1987) pointed out that *Salmonella bongori,* previously known as subspecies V, is considered as a separate species (Reeves *et al.*, 1989).

The antigenic formula of *Salmonella* serotypes are defined and maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (WHO Collaborating Centre). Based on somatic (O) and flagellar (H) antigen there are more than 2600 serotypes of *Salmonella* which are included under the two species.

The nomenclature system used at the Centers for Disease Control and Prevention (CDC) (Brenner *et al.*, 2000) is now widely accepted for communicating research findings in scientific publications. The CDC system divides the genus *Salmonella* into two species; *Salmonella enterica*, the type species and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies which are referred to by a Roman numeral and a name

(I, *Salmonella enterica* subsp. *enterica*; II, *Salmonella enterica* subsp. *salamae*; IIIa, *Salmonella enterica* subsp. *arizonae*; IIIb, *Salmonella enterica* subsp. *diarizonae*; IV, *Salmonella enterica* subsp. *houtenae*; and VI, *Salmonella enterica* subsp. *indica*). Subspecies include serotypes which are differentiated by antigenic properties. The name usually refers to the geographical area from which it was first isolated. To differentiate it from the species names, the serotype names begin with capital letters and are not italicized.

Even though a unified system has been approved by competent authorities in *Salmonella* nomenclature, it will take some more time for it filter down to reach the bottom and become the norm.

2.3. Serotyping

Serotyping is the common method of differentiating strains of *Salmonella*. Serotyping separates strains based on their somatic (O) and flagellar (H) antigens. The O antigen, designated by numbers, is a polysaccharide present on the cell surface lipopolysaccharide. Flagellar antigen is diphasic with different set of H antigens. Phase 1 antigens are represented by letters and the first discovered phase 2 antigens are represented by numbers. Only one of the H antigens will be expressed at a time. A slide agglutination test is commonly used in serotyping which normally requires over 250 antisera.

A protein microarray method for serotyping the 20 common serotypes has also been reported (Cai *et al.*, 2005).

2.4. Epidemiology

There is a close relationship between *Salmonella* serotypes in food of animal origin and public health problems. Sakai and Chalermchaikit (1996) associated the increase in human infections by *Salmonella enterica* subspecies *enterica* serotype Enteritidis with the increased prevalence of the same serotype in chicken. Reports from Northern Thailand showed the presence of *Salmonella* in all levels of food production, from farm to market (Padungtod and Kaneene, 2006).

2.5. Reservoirs of *Salmonella*

Salmonella is a food borne pathogen. They are transmitted through the oral-fecal route. There are several host adapted species. Nevertheless, almost all of them enjoy a wide host range without producing any symptoms in the hosts.

2.5.1. *Salmonella* and humans

Along with a very few host adapted serotypes, human beings proved to be successful hosts for several *Salmonella* serotypes. Even though typhoidal and non-typhoidal salmonellosis of humans owes its origin to food-borne *Salmonella*, transfer among humans is not uncommon.

2.5.2. *Salmonella* and pork

Contaminated pork is an important source of *Salmonella* infections (Baggesen *et al.*, 1996; Mead *et al.*, 1999). Reports regarding pork as a reservoir for *Salmonella* were present since the first isolation of *Salmonella* (Salmon and Smith, 1886). Along with the host adapted serotypes like *Salmonella enterica* subspecies *enterica* ser Typhisuis and *Salmonella enterica* subspecies *enterica* serotype Choleraesuis, several other serotypes with broad

host range are reported from swine. Many of the hosts behave as asymptomatic carriers of these dreaded pathogens in their tonsils, intestine and the gut-associated lymphoid tissue (Wood *et al.*, 1991; Fedorka-Cray *et al.*, 1995). They can excrete about 10^5 CFU/g *Salmonella* serotypes over a period and there are certain factors that may increase their potential to excrete the bacteria (Henry *et al.*, 1983). The survival potential of *Salmonella* in feces is also very high (Plym and Ekesbo, 1993). *Salmonella* Typhimurium is the most common serotype associated with pigs (Letellier *et al.*, 1999; Vieira-Pinto *et al.*, 2006; Rostagno *et al.*, 2007).

2.5.3. *Salmonella* and poultry

Poultry and poultry products, including meat and eggs have long been recognized as an important source of food-borne infections caused by *Salmonella*. High prevalence of *Salmonella* has been reported from chicken slaughterhouses (Carraminana *et al.*, 2004). These slaughterhouses serve as potential sources of cross contamination as well. *Salmonella* Typhimurium and *Salmonella* Enteritidis are among the most frequently isolated serotypes from poultry (Henzler *et al.*, 1998; Krishnamoorthy *et al.*, 2003; Suresh *et al.*, 2006; Messens *et al.*, 2007). The global increase in human infections with *Salmonella* Enteritidis observed in the late 1980's and early 1990's was due to the presence of this organism within the poultry production industry worldwide (Rodrigue *et al.*, 1990).

Salmonella Enteritidis associated with egg and egg products is cause of significant public health concern in many countries (Cowden *et al.*, 1989; Stevens *et al.*, 1989; Luby and Jones, 1993). *Salmonella* mainly colonizes the intestinal tract of poultry. Egg contamination occurs either by horizontal transfer through egg shell penetration, which takes place at the cloacal region during or after laying. Several serotypes of *Salmonella* are capable of

penetrating the egg shells (Schoeni *et al.*, 1995). Secondly, through trans-ovarian infection, *Salmonella* colonizes the pre ovulary follicles of an infected bird, thereby contaminating the eggs and the next progeny (Snoeyenbos *et al.*, 1969; Perales and Audicana, 1989; Barnhart *et al.*, 1991; Thiagarajan *et al.*, 1994). *Salmonella* can grow in eggs even at 4^{O}C (Kim *et al.*, 1989; Schoeni *et al.*, 1995). Outbreaks of salmonellosis by *Salmonella* Enteritidis have been reported from several part of the world (Perales and Audicana, 1989; Rodrigue *et al.*, 1990; Wong *et al.*, 1994; Sakai and Chalermchaikit, 1996).

Salmonella enterica subspecies *enterica* serotype Infantis has also been reported as a prevalent serotype in the poultry industry (Raevuori *et al.*, 1978; Poppe *et al.*, 1991; Hinz *et al.*, 1996; Shahada *et al.*, 2010) and has been responsible for several outbreaks in humans (Barrell, 1987; Hatakka, 1992; Meehan *et al.*, 1992).

Other reports have shown that human salmonellosis caused by *Salmonella* serotypes like, Berta, Typhimurium and Livingstone were acquired from poultry (Olsen *et al.*, 1992; Millemann *et al.*, 1995; Crichton *et al.*, 1996).

2.5.4. *Salmonella* and fish

Even though *Salmonella* serotypes do not produce any disease in fish and other aquatic organisms, they are potential carriers of these pathogens. Aquatic environments act as a major reservoir of *Salmonella*, aiding its transmission between hosts (Cherry *et al.*, 1972). *Salmonella* contamination is a serious problem in seafood exporting industry. Heinitz *et al.*(2000) reported an overall prevalence of 7.4% and 1.3% for imported and domestic seafood respectively from 1990 to 1998 in United States. Contamination in raw seafood was 10% and 2.8% respectively for imported and domestic. In India

several studies have indicated high prevalence of *Salmonella* in seafood. Hatha and Lakshmanaperumalsamy (1997) reported that 14.25% of fish samples and 17.39% of crustacean samples collected from Coimbatore, South India were contaminated with *Salmonella*. Another report (Iyer and Shrivastava, 1989) showed that 12% of peeled and deveined shrimp, 10% of headless shell of shrimp, 14% of peeled undeveined shrimp, 25% of catfish and 20% of seer fish harbored *Salmonella*. Studies have demonstrated the ubiquitous presence of *Salmonella* in fishes and a close relationship between multidrug resistant *Salmonella* Paratyphi isolated from patients with gastroenteritidis and those isolated from their home aquarium (Levings *et al.*, 2006).

The actual picture of the prevalence of *Salmonella* in seafood is not furnished by these reports as detection was based on conventional culture method, which is less sensitive than the molecular methods. Nucleic acid-based methods have estimated that the real prevalence is significantly higher. PCR assay detected *Salmonella* in 70% fish, 59% of shrimps and 30% of oysters (Sanath Kumar *et al.*, 2003).

2.5.5. Other sources

Human salmonellosis associated with pets is a major public health concern (Woodward *et al.*, 1997; Austin and Wilkins, 1998; Jafari *et al.*, 2002). A study involving the analysis of the fecal samples of several reptiles showed dominance of several exotic *Salmonella* serotypes (Ebani *et al.*, 2005). However, reptiles usually do not show any sign or symptoms of *Salmonella* infection.

Reptiles are a major reservoir of *Salmonella* (Greenberg and Sechter, 1992; Monzon Moreno *et al.*, 1995; Geue and Loschner, 2002) with reptile-associated salmonellosis becoming a major public health issue. A high

prevalence of *Salmonella* with forty four different serotypes has been reported from samples collected from captive lizards (Pasmans *et al.*, 2005). However, *Salmonella* strains isolated from lizards are not usually found in mammals and birds (Baulmer *et al.*, 1998). The increased popularity of these pets is correlated with the increase in reptile-associated salmonellosis in humans (Woodward *et al.*, 1997; Mermin *et al.*, 2004).

Turtles are also considered a potential source of human salmonellosis (Williams and Helsdon, 1965; Cohen *et al.*, 1980) as they carry *Salmonella* as part of their normal intestinal flora and shed the bacteria in their feces. Transmission of *Salmonella* occurs mainly through reptilian eggs. One hour exposure of turtle eggs to contaminated internal contents can result in effective penetration (Feeley and Treger, 1969). Transovarian infection is also reported in reptiles (Austin and Wilkins, 1998).

Wild birds also proved to be a suitable reservoir for *Salmonella*. (Wilson and MacDonald, 1967; Refsum *et al.*, 2002; Pennycott *et al.*, 2006).

Fruits and vegetables too are sources for *Salmonella*. Contaminated alfalfa sprouts were responsible for outbreaks of *Salmonella* Enterica in Finland and Sweden (Ponka *et al.*, 1995). A study from the slums of Bangladesh showed that contaminated papaya was associated with enteric fever (Ram *et al.*, 2007). Another study reported that eating lettuce salad and cig kofte (a traditional raw food) was significantly associated with the development of typhoid fever in Turkey (Hosoglu *et al.*, 2006). Outbreak of infection by *Salmonella enterica* subspecies *enterica* serotype Muenchen has been reported to be caused by consumption of orange juice (Center for Disease Control and Prevention, 1999).

2.6. Host adapted serotypes of *Salmonella*

Majority of the *Salmonella* serotypes are ubiquitous, with a few host adapted ones. *Salmonella enterica* subspecies *enterica* serotypes Dublin, Choleraesuis and Pullorum are host-adapted serovars that cause disease primarily in cattle, swine and poultry, respectively. *Salmonella* Typhi and *Salmonella* Paratyphi are human-adapted causing enteric fever. Some of the host adapted serotypes infrequently cause diseases in other hosts.

2.7. Important serotypes

Salmonella Typhi is a host adapted serotype responsible for enteric fever in humans, most frequently isolated from humans (Kumar *et al.*, 2009). Infections with *Salmonella* Typhi continue to be a major health problem in many developing countries as this serotype naturally infects only humans, but is well adapted to establish a chronic carrier state with persistent excretion which can last for months or years (White and Parry, 1996). Multilocus sequence typing of housekeeping genes has showed that this serotype has evolved 50000 years ago (Kidgell *et al.*, 2002).

Unlike most of the other serotypes, *Salmonella* Typhi strains show a high degree of homogeneity among themselves, a single profile in multilocus enzyme electrophoresis indicating the clonal relatedness of *Salmonella* Typhi strains from different sources (Reeves *et al.*, 1989). A similar study also showed high degree of relatedness among the strains even though they showed two electrophoretic profiles with multilocus enzyme electrophoresis (Selander *et al.*, 1990)

Salmonella enterica subspecies *enterica* serotype Paratyphi is another host adapted serotype of humans, and is the second causative agent of enteric fever prevalent in many regions of Asia (Hafiz *et al.*, 1993; Sood *et al.*, 1999).

Salmonella enterica subspecies *enterica* serotype Typhimurium, a ubiquitous serotype responsible for the majority of non-typhoidal salmonellosis in humans, has been isolated from a wide range of sources which includes poultry (Singh *et al.,* 2010), seafood (Hatha and Lakshmanaperumalsamy, 1997) pork (Wondwossen *et al.,* 2000) calves (Ahmed *et al.,* 2009) and wild birds (Refsum *et al.,* 2002).

Salmonella Enteritidis is considered the main serotype infecting humans and poultry worldwide (Rodrigue *et al.,* 1990; Altekruse *et al.,* 1993; Roberts and Sockett, 1994) and its association with egg and egg products plays an important role in human salmonellosis (Luby and Jones, 1993; Suresh *et al.,* 2006). Even though most *Salmonella* Enteritidis infections in humans are sporadic, few outbreaks (Levine *et al.,* 1991; Taylor *et al.,* 1993) stress the importance of *Salmonella* Enteritidis in public health.

Salmonella enterica subspecies *enterica* serotype Weltevreden is another prevalent serotypes isolated from fishes and crustaceans (Hatha and Lakshmanaperumalsamy, 1997; Shabarinath *et al.,* 2007), reportedly associated with human infections in Malasia and Thailand (Thong *et al.,* 2002; Bangtrakulnonth *et al.,* 2004; Padungtod and Kaneene, 2006). An outbreak of salmonellosis in Singapore has been attributed to vegetables and fruits contaminated with *Salmonella* Weltevreden (Ooi *et al.,* 1997). It is a prominent serotype in the South East Asia and Western pacific region causing non-typhoidal salmonellosis (World Health Organisation, 2005).

Salmonella enterica subspecies *enterica* serotype Gallinarum has two biotypes; Gallinarum and Pullorum. They are non-motile pathogens which are host restricted to fowls causing distinct diseases. *Salmonella* Gallinarum causes fowl typhoid and *Salmonella* Pullorum causes dysentery (fowl typhoid).

Even though, considered as host adapted, infections in primates are also reported (Ocholi *et al.*, 1987).

Salmonella enterica subspecies *enterica* serotype Infantis is another serotype which is prevalent word wide in the poultry industry (Poppe *et al.*, 1991; Crichton *et al.*, 1996). This serotype was involved in a large broiler chicken-associated epidemic in Finland (Raevuori *et al.*, 1978). It is a pathogen of both animals and humans, and frequently isolated from swine also (Wondwossen *et al.*, 2000).

2.8. Salmonellosis

Salmonellosis is one among the major food-borne diseases the world over. The clinical manifestations of salmonellosis can vary from life threatening enteric fever to self limiting gastroenteritis.

2.8.1. Enteric fever

Enteric fever, which includes typhoid fever and paratyphoid fever, is a systemic disease caused by *Salmonella* Typhi and *Salmonella* Paratyphi respectively. Sarnighausen *et al.* (1999) reported that *Salmonella* Kapemba is also capable of producing enteric fever. Typhoid fever is a serious threat to public health all over the world, especially in the developing countries (Crump *et al.*, 2004). They are transmitted through the fecal-oral route and hence the infections are more prevalent where sanitary conditions remain poor and water supplies are untreated. Human beings are the only true reservoir and transmission occurs through contaminated food materials and water, and carriers.

Ingestion of *Salmonella* is followed by an asymptomatic period extending up to 60 days, followed by fever and malaise, signaling bacteraemia.

Chills, head ache and gastro intestinal disorders are the most common symptoms associated with enteric fever. Gastrointestinal bleeding and intestinal perforation are seen in severe cases of the disease. Hepatomegaly and splenomegaly is common. Rose spots usually occur on the abdomen and chest. Chronic biliary carriage may occur in a few cases.

2.8.2. Non-typhoidal salmonellosis

Salmonellosis caused by serotypes other than *Salmonella* Typhi and *Salmonella* Paratyphi is designated as non-typhoidal salmonellosis. Majority of them are non-host- specific serotypes. Human acquisition of these serotypes occurs mainly by consumption of contaminated food like poultry, pork, egg, seafood etc. Several factors like the serotype, host, inoculum to name a few, determine the severity of the disease produced by Salmonellae: the serotype determines the clinical syndrome produced.

The main symptoms are gastroenteritis with nausea, vomiting, and diarrhea with or without fever. A small proportion of these patients develop invasive infections which may result in extra gastrointestinal infections including bacteremia, and localized infections. Many serotypes are capable of producing invasive infections which begin by adhesion and penetration of epithelial cells (Groisman and Mouslim, 2000). Several bacterial fimbriae are involved in the adhesion process (Darwin and Miller, 1999). *Salmonella* is capable of gaining entry into non-phagocytic cells and producing gastroenteritis by multiplying in the Peyer's patches. The ability of *Salmonella* to gain entry into the non-phagocytic cells makes them a successful pathogen.

2.9. Detection of *Salmonella*

Salmonella being one of the major bacterial contaminants in food and food products, rapid and sensitive detection is of prime importance in food-

industry and public health sector. Traditional method relies on isolation of bacteria and subsequent identification by biochemical and serological methods (Andrews *et al.*, 1998) requiring a minimum of 6 days.

Several alternative methods were proposed for the easy detection of *Salmonella*. An immuno-magnetic monoclonal antibody-based assay was developed by Luk and Lindberg (1991) claiming rapidity and sensitivity. Enzyme linked immunosorbant assays were widely used for *Salmonella* detection and identification (Lee *et al.*, 1990; Keller *et al.*, 1993). Hybridization methods proved to be another effective method for *Salmonella* detection (Fitts *et al.*, 1983; Fitts, 1985; Gopo *et al.*, 1988). Hanes *et al.*, (1995) reported an allele-specific DNA probe (SE-probe) that targets the *spvA* gene in the virulence plasmid of *Salmonella* Enteritidis that utilized a single base difference between *Salmonella* Enteritidis, *Salmonella* Dublin and *Salmonella* Typhimurium. Under stringent conditions this probe will hybridize with all *Salmonella* Enteritidis strains which harbor the virulence plasmid, regardless of the phage type and geographical location.

Nucleic acid based methods by polymerase chain reaction proved to be the best known, fast and sensitive assay, compared to the culture method (Nissen and Sloots, 2002; Shabarinath *et al.*, 2007; Kumar *et al.*, 2008) . Detection by nucleic acid-based assays began when Widjojoatmodjo *et al*. (1991) reported the suitability of *oriC* as a molecular marker for *Salmonella*. Later *Salmonella* invasion gene, *invA*, became the first choice of researchers (1992). PCR assays targeting other *Salmonella* specific genes were also developed for specific detection of *Salmonella.* PCR amplification of *agfA* gene has been used for *Salmonella* detection by Doran *et al.* (1993). A nested PCR based on *viaB* gene was developed by Hashimoto *et al*. (1995) to detect *Salmonella* Typhi. A PCR method using a primer to amplify a 199bp

Salmonella-specific DNA fragment derived from a repetitive DNA of *Salmonella* Weltevreden was reported by Jitrapakdee *et al.* (1995). Cheng-Hsun and Ou (1996) reported a multiplex PCR based on *invA* and *spvC* genes which could detect the presence of *Salmonella* along with the virulence plasmid. Shabarinath *et al.,* (2007) reported an increased efficiency for *hns* primer compared to *invA* and *invE* primers in detecting *Salmonella* from seafood.

PCR assay based on *hilA* was successfully used for the detection of *Salmonella* in tomatoes (Guo *et al.*, 2000) and is suggested to be a suitable candidate for *Salmonella* detection in feces (Pathmanathan *et al.*, 2003). PCR primers targeting *tyv, prt, viaB,* and *fliC* genes were used in combination for accurate detection and identification of *Salmonella* Typhi and *Salmonella* Paratyphi A (Hirose *et al.*, 2002).

Even though rapid and sensitive detection of *Salmonella* is possible with molecular methods, the culture method is inevitable for the isolation and further research including epidemiological studies.

2.10. Loop Mediated Isothermal Amplification (LAMP)

LAMP is a novel nucleic acid amplification method performed under isothermal conditions (Notomi *et al.*, 2000) thereby doing away with the precision equipments for thermal cycling. It can produce about 10^9 copies of DNA in less than an hour from a few templates, requiring only a water bath as equipment.

LAMP is based on the auto-cycling strand displacement DNA synthesis property of *Bst* polymerase (Notomi *et al.*, 2000). It relies on 4 oligonucleotide primers; two inner and two outer primers, that targets 6 specific regions in the template DNA. The inner primers are designated as

forward inner primer (FIP) and backward inner primer (BIP). Various steps of LAMP reaction is illustrated in Fig 2.1.

Fig.2.1 Schematic representation of LAMP reaction mechanism

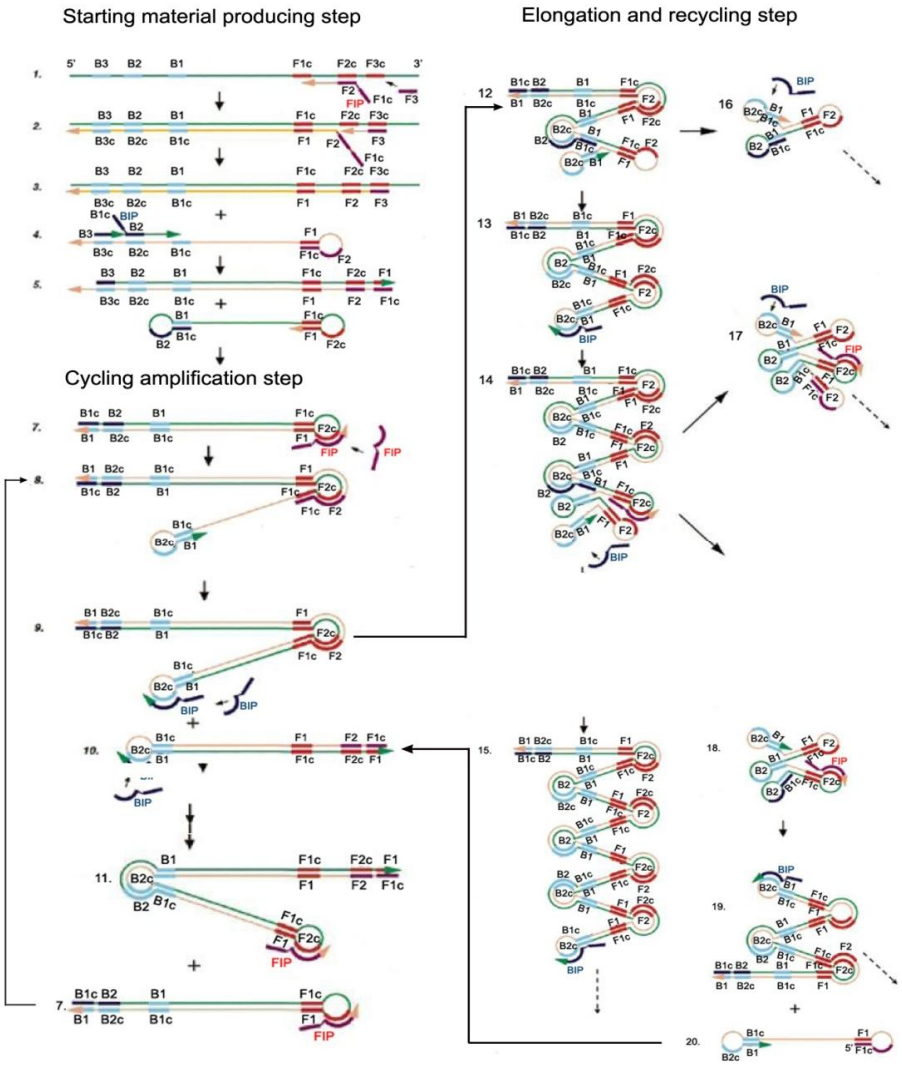


Figure courtesy (Notomi *et al.*, 2000)

The regions inside both ends of the template DNA to be amplified are designated as F2c and B2. F1c and B1 are two sequences present internal to F2c and B2 respectively. Outside the F2c and B2 are two sequences designated as F3c and B3 respectively. The FIP primer consists of 3 distinct sequences: F1c, a TTTT spacer and F2. Similarly BIP is composed of sequences B1c, TTTT spacer and B2. Sequences F2 is complementary to F2c and B2 is complementary to B2c.

FIP primer anneals to template DNA at the F2c region and begins complementary strand synthesis. F3 primer provided in a comparatively lower concentration hybridizes slowly to F3c in the template DNA and initiates strand displacement DNA synthesis. Due to the strand displacement activity of *Bst* polymerase, the FIP linked complementary strand is released and forms a loop at one end (structure 4). BIP primer will now get attached to this strand and DNA synthesis is initiated. Annealing of B3 primer initiates strand displacement DNA synthesis resulting in the production of a dumb-bell shaped strand (structure 6). Self primed DNA synthesis converts the dumb-bell shaped strand to a stem-loop structure which serves as the starting material for LAMP cycling in the next step.

FIP then hybridizes to the loop and initiates strand displacement DNA synthesis forming a gapped stem-loop structure having an additional inverted copy of the target sequence in the stem portion and a loop at the opposite end. Further strand displacement DNA synthesis produces a stem-loop structure which is twice as long as the target DNA (structure 9) and a complementary strand of the original stem-loop structure (structure10), which was produced at structure 7. These two products functions as templates for the subsequent reactions.

Subsequent amplification reactions result in the production of stem-loop structures with varying stem length. Multiple loops are also formed by annealing of alternately inverted repeats of sequences in the same strand. The use of four primers in the amplification process increases the specificity of the reaction.

Since the development, several improvements and modifications have been reported, increasing the ease of its use. Nagamine *et al.* (2001) reported the possibility of performing LAMP without the initial denaturation of the template DNA. The use of loop primer along with the other primers has accelerated the LAMP reaction (Nagamine *et al.*, 2002). Moreover, it reduced the template requirement from more than 10^4 to 10^3. The reaction will produce magnesium pyrophosphate as a bye product in excess amounts and hence, visualization of the positive result as a white precipitate is possible by naked eye (Mori *et al.*, 2001). Increase in turbidity, due to continuous production of this precipitate, is proportional to the amount of DNA synthesized. Hence, a real time monitoring of the reaction is possible by real time measurement of the turbidity.

Detection of microbes has been an important application of LAMP. Aoi *et al.* (2006) reported the use of LAMP assay for detection of ammonia-oxidising bacteria. LAMP assay has been used as a rapid and sensitive diagnostic tool for several microbial pathogens like *E. coli* (Hara-Kudo *et al.*, 2007 ; Hill *et al.*, 2008), *Vibrio spp.* (Yamazaki *et al.*, 2008a; Srisuk *et al.*, 2010 ; Cai *et al.*, 2010 ; Han and Ge, 2010), *Yersinia spp.* (Horisaka *et al.*, 2004; Saleh *et al.*, 2008), *Mycobacterium tuberculosis* (Pandey *et al.*, 2008), *Edwardsiella ictaluri* (Yeh *et al.*, 2005), *Campylobacter* spp. (Yamazaki *et al.*, 2008b), *Bacillus anthracis* (Jain *et al.*, 2011), *Pseudomonas aeruginosa* (Zhao *et al.*, 2011), *Listeria monocytogenes* (Tang *et al.*, 2011) *Salmonella*

(Hara-Kudo *et al.*, 2005; Wang *et al.*, 2008; Ueda and Kuwabara, 2009) among others.

LAMP has also been useful in virus surveillance by detecting Monkeypox Virus (Iizuka *et al.*, 2009), foot-and-mouth disease virus (Dukes *et al.*, 2006), Epstein–Barr virus (Iwata *et al.*, 2006), human herpesvirus 8 (Kuhara and Yoshikawa, 2007) to name a few.

Salmonella has been detected by LAMP assay from a variety of source: from pork (Techathuvanan *et al.*, 2010), poultry (Ohtsuka *et al.*, 2005; Okamura *et al.*, 2008) and artificially contaminated water (Varghese *et al.*, 2012). Several target genes like *fimY* (Zhang *et al.*, 2011) and *invA* (Wang *et al.*, 2008) have been used for detection of *Salmonella* by LAMP assay. Due to its cost effectiveness and high sensitivity it is widely used as an alternative to PCR for amplification and detection of specific genes. Lu *et al.* (2009) reported an Ethidium Monoazide-Loop Mediated Isothermal Amplification method, which can distinguish viable cells from dead cells. In order to increase the ease of observation, calcein and manganese ions are added to produce a characteristic color indicating a positive result (Tomita *et al.*, 2008; Tang *et al.*, 2011). *Mori et al*, (2006) reported a visual detection protocol using oligonucleotide probes labeled with fluorescent dyes.

2.11. *Salmonella* Pathogenicity Islands (SPIs)

Pathogenicity islands (PAI) are distinct regions on chromosomes of pathogenic bacteria harboring clusters of virulence genes and acquisition of PAIs is considered as ‘quantum leaps’ in the process of bacterial evolution (Groisman and Ochman, 1996).

In *Salmonella* too, majority of the virulence genes are clustered in the PAIs, referred to as ‘*Salmonella* pathogenicity islands’ (SPI) (Marcus *et al.*,

2000). *Salmonella* has evolved as a successful pathogen, after its deviation from *E.coli,* by the acquisition of pathogenicity islands containing virulence determinants. These SPIs are acquired from other species through horizontal gene transfer, a fact which is well proved by the presence of significant differences in the G+C content of these islands and the remaining genome along with the presence of insertion sequences which flank them (Groisman and Ochman, 1996).

Several SPIs have been reported since the identification of the first one (SPI-1) by Mills *et al.* (1995), a 40kb fragment in *Salmonella* Typhimurium chromosome, which was absent from the corresponding region of *E.coli* K-12 chromosome. This region, located at centisome 63 of *Salmonella* chromosome, contains genetic information for a large number of proteins involved in the formation of a type III secretion system (TTSS), which is a specialized protein secreting system involved in the translocation of effector proteins into eukaryotic cells (Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000). Some of the effector proteins are involved in the modification of actin cytoskeleton thereby, helping in the uptake of bacteria by the host cells (Hayward and Koronakis, 2002). Another subset of the effector proteins are involved in enteropathogenesis and intestinal epithelial inflammation (Wallis and Galyov, 2000). SPI enjoys a wide distribution in almost all serotypes (Ochman and Groisman, 1996; Hensel *et al.*, 1997a)

A study by Choi *et al.* (2007) showed that the expression of genes in SPI-1 requires the product of *luxS* gene. They showed that the cell-density-dependent induction of the *invF* gene is abolished if *luxS* is deleted. LuxS protein is involved in the synthesis of an auto inducer which activates the quorum sensing system (Meijler *et al.*, 2004).

SPI-2 is also a 40kb locus maping downstream of a $tRNA^{Val}$ locus at 31 min, encoding a second TTSS, enabling the bacteria to survive in epithelial cells and macrophages (Shea *et al.*, 1996; Ochman *et al.*, 1996; Hensel *et al.*, 1997a) . Hensel *et al.*, (1999b) reported a mosaic nature in the structure of SPI-2, supporting multiple evolutionary events in the evolution of this island. Heterogeneity is observed in SPI-2 indicated by a marked difference in base composition and codon usage between the 25 kb portion, encoding a second TTSS and the 15 kb portion, with genes for tetrathionate reductase and other functions. SPI-2 is involved in anaerobic respiration by encoding tetrathionate reductase (Hensel *et al.*, 1999a).

Even though both SPI-1 and SPI-2 encode various proteins for TTSS, SPI-2 genes show less similarity to homologues in SPI-1 than to those of other species (Hensel *et al.*, 1997b). This is a clear indication of independent horizontal gene transfer rather than duplication of homologous gene clusters in SPI-1 (Hensel, 2000).

SPI-3 is an insertion of 17kb downstream of *selC* gene (Blanc-Potard and Groisman, 1997). This island maps at 82 centisome. SPI-3 harbors at least 10 ORFs, organized in six transcriptional units (Blanc-Potard *et al.*, 1999) and it includes the *mgtCB* operon encoding a high affinity Mg^{2+} uptake system required for survival in the nutritional limiting intra-phagosomal environment (Snavely *et al.*, 1991; Blanc-Potard and Groisman, 1997; Blanc-Potard *et al.*, 1999). Variation in G+C content among the members along with the presence of functionally unrelated genes in the island suggest multistep horizontal gene transfer events (Blanc-Potard *et al.*, 1999)

SPI-4, a 27kb virulence region located at 93 min on the chromosome map, flanked by the *ssb* and *soxSR* loci, is needed for intra-macrophage

survival (Wong *et al.*, 1998). It encodes 18 proteins; three of which shows homology with toxin secreting proteins.

In 1998 another SPI designated as SPI-5 was identified (Wood *et al.*). This locus maps at approximately 20 centisome of *Salmonella* Typhimurium chromosome. This island is flanked by $tRNA_1^{Ser}$ and *copS/copR*. It harbors at least six genes involved in enteric but not systemic salmonellosis (Wood *et al.*, 1998).

Analysis of the complete genome of *Salmonella* Typhi CT18 reveals the presence of five more minor SPIs (Parkhill *et al.*, 2001; Asten and Dijk, 2005). Islands with few virulence genes were also identified, based on the variability in G+C content. They were designated as "pathogenicity islets" (Groisman and Ochman, 1997)

There are several regulatory genes which are present in and outside these SPIs which controls the expression of various virulence genes. Gene *hilA*, present in SPI1 is one among them (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996). It is a transcriptional regulator of several invasion genes, whose transcription in turn is activated by *sirA* (Johnston *et al.*, 1996).

Choi *et al.* (2007) reported that *luxS* gene product is necessary for the expression of the virulence genes present in the pathogenicity islands.

2.12. Horizontal gene transfer

Bacterial genome is highly dynamic in nature. Horizontal gene transfer (HGT) has been attributed as the major cause of abrupt emergence of variants of bacterial strains. Transformation, conjugation and transduction are the three mechanisms by which microbes share their genomes (Jain *et al.*, 2002). HGT takes place between species, genus or even kingdoms of organisms: bacteria to

archaea (Nelson *et al.*, 1999), bacteria to eukaryotes (Doolittle, 1998), animals to bacteria (Wolf *et al.*, 1999) and so on.

2.13. Virulence plasmids

Several *Salmonella* serotypes possess plasmids which provide virulence determinants involved in the process of pathogenesis (Gulig, 1990; Chiu *et al.*, 1999; Chu *et al.*, 1999; Chu *et al.*, 2001). The size of the plasmid varies with the serotype and may range from 50 to 285kb (Ou *et al.*, 1990). All of them share a common 7.8kb *spv* (*Salmonella* plasmid virulence) region which confers the virulence determinants. Other loci may play a role in the other stages of infection process (Gulig *et al.*, 1993). Several loci of unknown function is also reported from these plasmids (Koski *et al.*, 1992). *Salmonella* virulence plasmids were considered non-conjugative, until a self-transmissible plasmid was reported from *Salmonella* Typhimurium (Ahmer *et al.*, 1999) and another one giving multiple antibiotic resistance (Guerra *et al.*, 2002).

The virulence property is sometimes strengthened by the formation of resistance and virulence combined plasmids, where the virulence plasmids harbour the antibiotic resistance genes, spreading the antibiotic resistance genes among the bacterial community (Fluit, 2005). Several studies emphasizes the role of plasmids in the resistance phenotypes (Tosini *et al.*, 1998; Guerra *et al.*, 2001).

2.14. Phages

Salmonellae harbour several temperate bacteriophages, which help in lateral gene transfer by transduction. The incorporation of lysogenic phages into the bacterial gene can result in the lyosogenic conversion of non-pathogenic bacteria to pathogenic forms by the addition of virulence genes

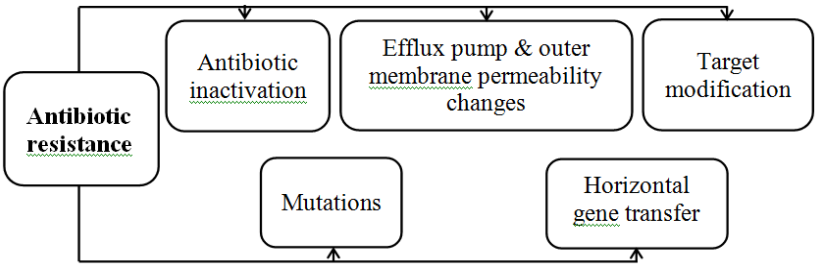
(Krylov, 2003; Canchaya *et al.*, 2003). Lysogenic conversion can result in the conversion of one phage type into another (Mmolawa *et al.*, 2002). Mirold *et al.*, (2001) reported the possibility of transfer of virulence determinants present in one phage to other unrelated phages.

S. Typhimurium is well known for the presence of different phages like Gifsy-1, Gifsy -2 and Gifsy -3, Fels-2 and a P2-like phage, SopE that encodes the *sopE* gene (Figueroa-Bossi *et al.*, 1997; Hardt *et al.*, 1998b; Figueroa-Bossi and Bossi, 1999; Miao and Miller, 1999; Mirold *et al.*, 1999). Prophage-like elements Gifsy-1 and Gifsy-2 are present at 57 and 24 units of *Salmonella* genome and their sequence is identical over a portion (Figueroa-Bossi *et al.*, 1997). They contribute to the virulence of their bacterial hosts (Stanley *et al.*, 2000; Ho and Slauch, 2001). Association of virulence gene with phage genes or to non functional phage attachment sites suggests the role of phages in the spread of virulence genes (Blanc-Potard and Groisman, 1997; Hensel *et al.*, 1997a; Hardt *et al.*, 1998b; Gunn *et al.*, 1998; Wood *et al.*, 1998).

2.15. Antibiotic resistance

Acquisition of resistance phenotype in microbes is by some common mechanisms (Fig.2.2). Increasing antibiotic resistance gains a global attention (Su *et al.*, 2004; Alcaine *et al.*, 2007).

Fig 2.2. Chemical and genetic mechanisms in antibiotic resistance phenotype



Salmonellae resistant to antimicrobials have become a serious health care issue

(Butaye *et al.*, 2006; Parry and Threlfall, 2008). Since the early 1990s the increase in antibiotic resistance in non-typhoidal. Salmonellae is cause for concern (Su *et al.*, 2004; Alcaine *et al.*, 2007).

2.15.1. Multiple antibiotic resistance in *Salmonella*

Multidrug resistant *S.*Typhimurium increased more than double between 1981 and 1990 in Great Britain (Threlfall *et al.*, 1993). Threlfall *et al.* (1996) showed that *S.* Typhimurium resistant to ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole increased from 1% to 25%, 1.5% to 25%, and 0% to 25% respectively, from 1986 to 1993 in UK. Emergence of *S.* Typhimurium DT104 with its multiple antibiotic resistance has become the source of deep concern (Threlfall *et al.*, 1994) as they harbour multiple antibiotic resistance determinants giving resistance to at least ampicillin, chloramphenicol-florfenicol, streptomycin-spectinomycin, sulfonamides, and tetracycline (ACSSuT phenotype) (Sandvang *et al.*, 1997; Glynn *et al.*, 1998). This strain was first isolated from cattle in UK but has now been isolated from a wide range of sources (Besser *et al.*, 1997; Low *et al.*, 1997).

Gebreyes and Altier (2002) reported a new phage type of *Salmonella* Typhimurium with AKSSuT type penta resistance giving resistance against ampicillin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline. The antibiotic resistance genes were found on a plasmid. A study on antibiotic susceptibility profiling of one hundred and eighty seven isolates representing eighty seven *Salmonella* serotypes isolated from imported seafood collected from various field laboratories of U.S. Food and Drug Administration, showed that 8% of the isolates were resistant to at least one antibiotic and 2.7% were resistant to three or more antibiotics (Zhaoa *et al.*, 2003). Isolates which are resistant to two or more antibiotics originates from high-risk sources of

contamination. Reports from a study conducted in Spain showed that out of the 133 Salmonellae isolated from chicken slaughterhouse, 65.4% were resistant to multiple antibiotics (Carraminana *et al.*, 2004).

Quinolones have been successfully used for the treatment of salmonellosis, caused especially by multiple drug resistant strains (Barnass *et al.*, 1990). It has been very effectively used in the treatment of enteric fever (Tran *et al.*, 1995; Vinh *et al.*, 1996). But the increasing emergence of quinolone resistant Salmonellae poses a major public health concern (Piddock *et al.*, 1993; Molbak *et al.*, 1999; Threlfall and Ward, 2001; Ling *et al.*, 2003; Ahmed *et al.*, 2009). Quinolone resistance is conferred by point mutations in the *gyrA* gene which encodes the GyrA subunit (Belland *et al.*, 1994; Heisig *et al.*, 1995; Griggs *et al.*, 1996). These mutations are clustered in a region of the gene product between amino acids 67 and 106, termed quinolone resistance-determining region (QRDR) (Yoshida *et al.*, 1990). In nalidixic resistant bacteria, amino acid serine at position 83 is changed to phenylalanine, tyrosine or alanine or aspartic acid at 87^{th} position is changed to glycine Asn or tyrosine.Yoshida *et al.* (1991) reported another QRDR region from *gyrB* gene of *E.coli* which results in reduced quinolone susceptibility compared to *gyrA* mutations.

Topoisomerase IV is reported as a secondary target for quinolone resistance in many Gram negative bacteria. The subunits, which are homologous to the GyrA and GyrB subunits respectively, are sites for quinolone action (Khodursky *et al.*, 1995). Mutations in the genes of ParC and ParE at the corresponding positions of GyrA and GyrB are responsible for high-level quinolone resistance (Heisig, 1996; Vila *et al.*, 1996; Breines *et al.*, 1997).

The prevalence of *Salmonella* strains resistant to at least one antibiotic is increasing day by day (MacDonald *et al.*, 1987). An increasing trend of multiple antibiotic resistance has been reported from several serotypes (Threlfall *et al.*, 1994; Wondwossen *et al.*, 2000; White *et al.*, 2001). It is quite alarming that even the third generation cephalosporins are not spared by Salmonellae (Rossi *et al.*, 1995; Fey *et al.*, 2000; White *et al.*, 2001). Ciprofloxacin is also becoming useless in their treatment (Threlfall and Ward, 2001; Adhikari and Baliga, 2002).

The indiscriminate use of antimicrobials in the production of food has been attributed to the emergence of antimicrobial resistance (Aarestrup, 1995). The use of single antibiotic can also give resistance to other antibiotics whose genes are present in the same mobile genetic element (Aarestrup *et al.*, 2001).

2.16. *Salmonella* Genomic Island 1

In the 1990s, emergence of a new multidrug resistant *Salmonella* Typhimurium strain, *Salmonella* Typhimurium definitive phage type 104 (DT104), was reported from the United Kingdom (Threlfall *et al.*, 1994). Since then, this phage type has been reported from several countries like Canada (Poppe *et al.*, 1996), the United States (Besser *et al.*, 1997), Israel (Metzer *et al.*, 1998), Denmark (Baggesen and Aarestrup, 1998) etc. The emergence of epidemic strain is a matter of great concern in the clinical field (Glynn *et al.*, 1998) as they harbor multiple antibiotic resistance determinants (Ridley and Threlfall., 1998; Briggs and Fratamico, 1999). All these resistance genes are packed in a 43kb genomic island designated as *Salmonella* Genomic Island 1 (SGI1) (Boyd *et al.*, 2001) (Fig 2.2).

Fig 2.3. Map of *Salmonella* Genomic Island 1

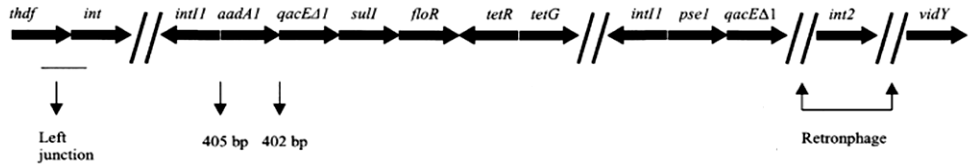


Figure courtesy (Ebner *et al.*, 2004)

In *Salmonella* Typhimurium SGI1 is located between *thdf* and *int2* genes. The *int2* gene is a part of the retron sequence which is not present along with the genomic islands in other serotypes. In them SGI1 is located between *thdf* and *yidY* genes (Boyd *et al.*, 2001; Mulvey *et al.*, 2006). SGI1 is flanked by direct repeats at the boundaries supporting the site specific recombination events which might have resulted in the transfer of it to the *Salmonella* genome. This island harbours two class 1 integrons; one has an aminoglycoside resistance gene (*aadA1)* and the other had the β-lactamase gene (*pse-1*) (Briggs and Fratamico, 1999). Genes conferring resistance to florfenicol-chloramphenicol (*floR*) and tetracycline (*tetR and tetA* [class G]) are present in between the two integrons (Briggs and Fratamico, 1999; Boyd *et al.*, 2000; Boyd *et al.*, 2001). Majority of the SGI1 has the above mentioned ACSSuT type of resistance pattern even though variants are also present (Threlfall *et al.*, 1998; Ng *et al.*, 1999; Daly and Fanning, 2000; Frana *et al.*, 2001).

It has been shown that the antibiotic resistance genes of *S.* Typhimurium DT104 can be efficiently transduced by ES18, a P22-like phage and by phage PDT17, which is released by DT104 isolates (Schmieger and Schicklmaier, 1999).

2.17. Integron

Integrons are site specific recombination systems which help in the acquisition and dissemination of genes (Stokes and Hall, 1989), common in gram negative bacterial pathogens in general and Enterobacteriaceae in particular (Sallen *et al.*, 1995; Jones *et al.*, 1997; Martinez-Freijo *et al.*, 1998).

Integrons usually harbour antibiotic resistance genes and hence play a vital role in the emergence of new multidrug resistant bacteria (Hall and Stokes, 1993). Several classes of integrons have been identified so far based on the integrase gene they harbor (Hall and Collis, 1995). Five distinct integron classes have been found associated with cassettes that contain antibiotic resistance genes.

Class 1 integrons are the most frequently found ones. They have a 5´ conserved segment (5´CS), a 3´conserved segment (3´CS) and an internal variable region. The 5´conserved region has an integrase gene (*intI*) belonging to the tyrosine recombinase family (Nunes-Duby *et al.*, 1998), a site for recombination (attI) and a promoter (Stokes and Hall, 1989). The 3´ region is defined by a truncated version of quaternary ammonium compound resistance gene, *quacEΔ1*, a sulphonamide resistance gene, *sul1*, and an open reading frame *orfC* of unknown function. The 3´ conserved region can have a varied structure in isolates from different locations (Hall *et al.*, 1994) or may be absent altogether (Recchia and Hall, 1995). Two unusual class 1 integrons In6 and In7, having two copies of *sul1* have been reported (Stokes *et al.*, 1993).

The target for the integrase protein are mobile gene cassettes which normally carries a single open reading frame (Recchia and Hall, 1995) and a recombination sequence termed as attC or 59-base element (Stokes *et al.*, 1997). The 59-base element comprise a family of diverse sequences differing in their sequence and length but has a consensus sequence at their boundaries

which correspond to the inverse core site (RYYYAAC) and the core site (GTTRRRY; R - purine, Y - pyrimidine).

The cassettes are devoid of any promoter and are expressed from a strong promoter located in the 5´ conserved region of the integron. Due to the presence of the promoter, integrons can act as a natural expression vector for the antibiotic resistance genes which are inserted in the correct orientation (Stokes and Hall, 1989). The same integrase can excise the gene cassette from the integron which will be integrated in another integron by site-specific recombination (Collis and Hall, 1992b). They are excised as covalently closed circular molecules (Collis and Hall, 1992a). Fluit and Schmitz (1999) reported about 60 antibiotic resistance gene cassettes. Stockpiling of antibiotic resistance gene cassettes in the integron can lead to the emergence of multidrug resistant bacteria which may pose a potential risk to public health (Rowe-Magnus *et al.*, 2002). Integrons harbouring up to eight antibiotic resistance gene cassettes has been reported previously (Naas *et al.*, 1999).

Class 2 integrons are similar in organization with that of class 1 but it is found associated with Tn7 transposon. They carry three conserved resistance genes, *dfrA1*, *sat1* and *aadA1*, which confer resistance to trimethoprim, streptothricin and streptomycin/spectinomycin, respectively (Hansson *et al.*, 2002). Biskri and Mazel (2003) reported a plasmid borne integron 2 with unusual gene cassettes. It has an erythromycin esterase gene inserted in between *sat1* and *aadA1* at the expense of the *dfrA1* gene. A similar work by Ahmed *et al.*, (2005) showed the presence of another unusual class 2 integron from *Salmonella* Enteritidis. This integron has another *sat* gene which replaced the *dfrA1* gene of the classical class 2 integron.

Class 3 integrons are not found so frequently as the other two. The configuration of the three distinctive features of the integron, integrase gene, recombination site and the promoter, is similar to that found in the corresponding 5' region of class 1 integron (Collis *et al.*, 2002). Integron 3 was first reported from a carbapenem-resistant strain of *Serratia marcescens* (Arakawa *et al.*, 1995). Correia *et al.*, (2003) reported the presence of a class 3 integron from a small plasmid in *Klebsiella pneumonia*.

Mazel *et al.*, (1998) reported a new integrase gene, *intI4*, from *Vibrio cholerae* which codes for a previously unknown integrase similar to that of the other well characterized integrons. Later, presence of a part of class 5 integron was reported from *Vibrio mimicus* (Clark *et al.*, 2000).

Integrons equip bacteria to scavenge foreign genes, especially antibiotic genes. As the antibiotic resistance gene cassettes are subjected to episodic selection, unnecessary genes are removed through excision events catalysed by integrase (Collis and Hall, 1992b), which will result in the reduction of genetic burden.

Integrons by themselves are not mobile (Brown *et al.*, 1996; Rowe-Magnus *et al.*, 1999), but they may be part of mobile elements like transposons, plasmids etc (Heikkila *et al.*, 1993; Correia *et al.*, 2003) which further enhance the spread of antibiotic resistance genes. Large conjugative plasmids have been reported from *Salmonella* previously which harbours both class 1 and class 2 integrons (Rodriguez *et al.*, 2006).

2.18. Typing

Typing is an important step in the surveillance program of any food-borne pathogen. Epidemiologically, it is increasingly important to be able to

type *Salmonella* isolates, because it helps in tracing the source of an outbreak and monitoring trends in antimicrobial resistance associated with a particular type.

2.18.1. Phage typing

Several bacteriophages are known to infect *Salmonella*. The infection is a selective process due to compatibility of phage and phage receptors present on the surface of the hosts. A strain is assigned a phage type based on the array of the typing phage which is able to infect the bacterium and form plaques (Hickman-Brenner *et al.*, 1991).

Phage typing has been used for epidemiological study of *Salmonella* (Hickman-Brenner *et al.*, 1983; Fernandez *et al.*, 2003). It has been successfully used for the subtyping of *Salmonella* Enteritidis (Hickman-Brenner *et al.*, 1991; Katouli *et al.*, 1993). Phage typing has limitations as it could be performed only by reference laboratories and the epidemiological implications derived from this method are limited as the discriminative index is low. Several methods have been standardized for analyzing genetic variability, which is useful for epidemiological studies. The application of molecular techniques has revolutionized epidemiological studies. Intraserovar typing is being performed for epidemiological studies.

Molecular typing plays a key role in understanding disease transmission and tracking and has been used in identification of clinical strains isolated from different sources (Gudmundsdottir *et al.*, 2003; Kubota *et al.*, 2005; Yong *et al.*, 2005).

2.18.2. Ribotyping

Ribotyping has successfully been applied for molecular typing of several *Salmonella* serotypes (Altwegg *et al.*, 1989; Martinetti and Altwegg, 1990; Pignato *et al.*, 1992; Esteban *et al.*, 1993; Usera *et al.*, 1994). In spite of the technical difficulties the results of ribotyping is comparable even with PFGE (Navarro *et al.*, 1996). This method involves the analysis of restriction fragment length polymorphisms in rRNA gene, a complex technique which is beyond the technical resource of many laboratories.

2.18.3. PCR-Ribotyping

Kostman *et al.* (1992) developed a new technique, called PCR-Ribotyping, based on the amplification of the internal transcribed spacer (ITS) sequences between the 16S and 23S genes in the rRNA transcriptional units. The spacer regions hold enough variation with respect to their sequence and length, which can be effectively utilized for characterization of bacteria at the genus (Jensen *et al.*, 1993), species (Dolzani *et al.*, 1995) and subspecies (Kostman *et al.*, 1992; Dolzani *et al.*, 1994) level. The ease of its use made it a choice of researchers engaged in surveillance and epidemiological studies. Even though it was used for differentiating *Salmonella* at the serotype level and intraserovar level (Lagatolla *et al.*, 1996; Christensen *et al.*, 2000), the D value is low compared with other typing methods (Lim *et al.*, 2005). Sequencing of the PCR-Ribotype amplicons could reveal the clonal relationship of *Salmonella* serotypes isolated from different source and time (Oliveira *et al.*, 2009).

2.18.4. Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995) proved to be a promising molecular fingerprinting technique, having

application in epidemiological studies of *Salmonella*. It has been used in the surveillance of *Salmonella* Typhimurium (Tamada *et al.*, 2001; Hu *et al.*, 2002; Lawson *et al.*, 2004; Mikasova *et al.*, 2005), *Salmonella* Abortusequi (Akiba *et al.*, 2003), *Salmonella* Enteritidis (Scott *et al.*, 2001), *Salmonella* Havana (Reche *et al.*, 2003) etc. Nair *et al.* (2000), reported a high discriminative index for AFLP, which was comparable with PFGE.

2.18.5. Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) is a typing method based on allelic differences in the nucleotides of housekeeping genes (Maiden *et al.*, 1998). It has been widely used as a typing method for several bacterial pathogens including *Salmonella* (Kotetishvili *et al.*, 2002; Noller *et al.*, 2003; Nemoy *et al.*, 2005; Torpdahl *et al.*, 2005). Several studies show that MLST shows a mixed type of discriminative power. Kotetishvili *et al.*, (2002) showed that MLST has a better discriminatory ability than serotyping and PFGE typing with various *Salmonella* strains. Even though MLST shows good discriminative power among different *Salmonella* serotypes it is not considered suitable for distinguishing closely related strains in a particular serovar (Fakhr *et al.*, 2005; Sukhnanand *et al.*, 2005).

2.18.6. ERIC-PCR

Multiple copies of Enterobacterial repetitive intergenic consensus (ERIC) sequences are present in the genomes of *Escherichia coli*, *Salmonella* Typhimurium and other Enterobacteriaceae (Hulton *et al.*, 1991). These highly conserved elements are 126bp long and include a central core inverted repeat. Since the description of ERIC sequences (Versalovic *et al.*, 1991), polymorphism in ERIC patterns has been widely used for genotyping bacteria. Van Lith *et al.*, (1994) reported that ERIC-PCR can be used for exploring

variations between serotypes, as genotypes obtained are serotype-specific. But reports of ERIC profiles showing more than one genotype within a serotype (Urlings *et al.*, 1998) make it a valuable tool for assessing intra-serotype variations, which is a very useful in epidemiological studies. The usefulness of ERI-PCR for genotyping different *Salmonella* serotypes has been reported earlier (Millemann *et al.*, 1996; Chmielewski *et al.*, 2002; Lim *et al.*, 2005). The discriminative index of ERIC-PCR is comparatively very high (Chmielewski *et al.*, 2002).

2.18.7. Random Amplified Polymorphic DNA

RAPD technique (Williams *et al.*, 1990) relies on a single arbitrary primer, which binds to random segments of genomic DNA to reveal polymorphism. It detects polymorphism over the entire genome. Even though RAPD has strongly been criticized for lack of reproducibility, it is widely used for *Salmonella* strain differentiation (Hilton *et al.*, 1996; Hilton and Penn, 1998; Lim *et al.*, 2005; Shabarinath *et al.*, 2007; Albufera *et al.*, 2009).

2.18.8. Pulse field gel electrophoresis

Pulse field gel electrophoresis is considered as the “gold standard” in molecular typing of *Salmonella* (Olsen *et al.*, 1994; Murase *et al.*, 1995; Weide-Botjes *et al.*, 1998). This method is of great help in the investigation of clonal relatedness within and between serotypes. Ridley *et al.* (1998) reported a high discriminative value to PFGE than other genotypic methods for epidemiological studies of *Salmonella* Enteritidis.

2.19. *Salmonella* monitoring and surveillance

Many animals harbour *Salmonella* and act as sources for infections in humans. They enter the food chain through contamination of the carcass by animal feces at the time of processing (White *et al.,* 2001) or through those who handle food. Measures taken to prevent contamination by these routes are an effective way to prevent salmonellosis. Some serotypes are exclusively associated with particular hosts. Prevalence data of strains isolated from each source is an important component of any surveillance program. The changing antibiotic resistance profile is also one important aspect to be monitored routinely.

Contaminated food produced in one area or country can cause disease in another area or country demonstrating the importance of a proper surveillance program. Surveillance programs at various level including international, national, regional and local, levels are being established to track *Salmonella* outbreaks, their epidemiology, and antimicrobial resistance patterns (Marano *et al.,* 2000; Threlfall *et al.,* 2003). FoodNet and PulseNet are other important surveillance programs for food-borne pathogens and/or susceptibility.

Even though many countries have succeeded in minimizing the incidence of *Salmonella* in water and food materials to a greater extent by adopting better sanitary measures, it is still endemic in several countries. *Salmonella* spreads mainly through the oral-fecal route and hence it is a major health concern to the entire human population. An integrated robust approach in surveillance is the need of the time.

MATERIALS AND METHODS

3.1. Isolation of *Salmonella*

3.1.1. Sources of *Salmonella*

Salmonellae were isolated from different seafood samples (Table 1.1) according to the FDA protocol given in the Bacteriological Analytical Manual (Andrews *et al.*, 1995).

Table 1.1. Table showing the sample source and number

Sl.No.	Source	**No. of Samples**
1	Mackerel	5
2	Sardine	24
3	Squid	5
4	Anchovies	8
5	Total	42

Thirteen *Salmonella* Typhimurium and fourteen *Salmonella* Enteritidis strains isolated from poultry (kindly provided by Dr. A. A. Mohammed Hatha) were included in the study to compare the virulence potential. Identity of these strains was once again confirmed by serotyping.

3.1.2. Sample collection

Fish samples were collected from various markets in and around Kochi. They were brought to the laboratory on ice as soon as possible in sterile polythene bags and processed immediately.

3.1.3. Enrichment

Enrichment helps in multiplying the number of bacteria present in the sample, thereby increasing the chance of isolation of the desired organism, even if their initial load is very low. For proper multiplication and isolation of Salmonellae a two-step enrichment protocol was utilized.

3.1.3.1. Pre enrichment

Salmonellae can be sub-lethally injured by heating, drying, irradiation, or other processing. It is necessary that samples are pre-enriched in a non-selective medium for efficient recovery. Lactose broth (Himedia, Mumbai, India) was utilized for pre-enrichment of sample, wherein 25g sample was thoroughly homogenized with minimum amount of lactose broth using a sterile mortar and pestle, volume made up to 225mL with the same medium and incubated at 37^{o}C for 24h.

3.1.3.2. Selective enrichment

This step inhibits or minimizes the growth of bacteria other than Salmonellae. Selective-enrichment was done in two enrichment media.

3.1.3.2.1. Tetrathionate broth

One millilitre culture from the pre-enrichment lactose broth was inoculated into 9mL tetrathionate broth (Himedia, Mumbai, India) and incubated for 24h at 37^{o}C. Oxgall present in the medium inhibits Gram-positive organisms, while tetrathionate formed in the medium inhibits normal intestinal flora from fecal specimens. Calcium carbonate neutralizes and absorbs toxic chemicals formed in the medium.

3.1.3.2.2. Rappaport-Vassiliadis broth

Ten millilitre Rappaport-Vassiliadis broth (Himedia) was inoculated with 0.1mL culture from the pre-enrichment broth and incubated at 42^{o}C for 24h. Malachite green in the medium provides selectivity to the medium, while the low pH (5.2±0.2) and high osmotic pressure of the medium favors the preferential growth of Salmonellae.

3.1.4. Selective plating

Bismuth sulphite agar (Himedia), xylose lysine desoxycholate agar (Difco laboratories, Detroit, Michigan) and hektoen enteric agar (Difco) were used for the selective plating. A loopful culture from Tetrathionate broth and Rappaport-Vassilliadis broth was streak inoculated on to the selective plates and incubated at 37^{0}C for 24-48 h.

3.1.4.1. Bismuth sulphite agar

This is a highly selective medium. Bismuth sulfite indicator and brilliant green present in the media inhibits Gram-positive bacteria and coliforms. Ferrous sulphate gives the characteristic brown to black colour to the colonies in the presence of H_2S. Production of metallic bismuth will produce a metallic sheen in the medium around the colonies. *Salmonella* colonies are black with or without metallic sheen or black to green (sometimes the surrounding medium also becomes black).

3.1.4.2. Xylose Lysine desoxycholate agar

Fermentation of xylose in XLD medium exhibited by majority of the enterics except *Shigella*, makes the medium acidic. Salmonellae rapidly ferment xylose and being lysine decarboxylase positive, reverts the medium pH to alkaline by the decarboxylation of lysine. Similar reversion by other

lysine decarboxylase positive coliforms is prevented by the presence of excess lactose and sucrose in the medium. The H_2S indicator system consisting of sodium thiosulfate and ferric ammonium citrate provides a black colour to the H_2S positive colonies. Characteristic colony colour of Salmonellae is pink with or without black centre or completely black.

3.1.4.3. Hektoen enteric agar

The presence of bile salts in the Hektoen enteric agar medium inhibits Gram-positives making it selective. The presence of lactose, sucrose and salicin aids in the optimal differentiation of enteric pathogens by the color of the colonies and of the medium adjacent to the colonies. Ferric ammonium citrate and sodium thiosulfate in the medium enables the detection of hydrogen sulfide production. Salmonellae are bluish–green with or without black centre or completely black.

3.2. Identification of *Salmonella*

Isolated colonies showing characteristic morphology were picked after 24h of incubation and purified by streaking on MacConkey plates (Himedia). If no typical colonies were formed within 24 h, the plates were incubated for another 24h.

The purified cultures were then maintained as stock cultures by paraffin overlay method. Nutrient agar was prepared in vials and a single colony was streak inoculated on that. The culture vials were incubated overnight at 37^0C. Sterilized liquid paraffin was overlaid and the vials were kept in dark for further studies.

3.2.1. Biochemical tests

The identity of the isolated cultures was confirmed by biochemical tests (Andrews *et al.*, 1998) and serotyping.

3.2.1.1. Triple sugar iron (TSI) agar

This medium is used to check the ability of bacteria to utilize glucose (0.1%), sucrose (1%) and lactose (1%). Acid production from fermentation of these sugars is indicated by yellow colour. Those organisms utilizing only glucose produce comparatively less amount of acid and will complete glucose utilization and fermentation in the first few hours of incubation. The butt remains acidic (yellow) due to the presence of acid, whereas the slant reverts to alkaline (red) condition due to oxidation of fermentation products under aerobic condition. When lactose and/or sucrose is fermented along with glucose, large amount of acid is produced which will make the entire medium acidic (yellow colour). The formation of gas is indicated by the presence of bubbles or cracks in the medium. This medium contains sodium thiosulphate and hence can detect the formation of H_2S. It is indicated by blackening of the medium.

Inoculation is done by touching the centre of an isolated colony by a sterile needle and stabbing the butt and streaking the surface of the slant. The tubes were incubated at 37^{o}C for 24h.

Salmonellae with some exceptions utilize only glucose and produce H_2S and other gases.

3.2.1.2. Lysine iron agar (LIA)

This medium is used to differentiate members of Enterobacteriaeae on the basis of decaboxylation and deamination of lysine and formation of hydrogen sulphide. Dextrose is the carbohydrate source. Lysine

decarboxylation is indicated by an alkaline reaction (purple colour) in the butt and lysine deamination by the formation of a red slant. Ferric ammonium citrate and sodium thiosulphate are indicators of hydrogen sulphide production.

LIA was used in combination with TSI agar. The same needle, which was used for inoculating TSI agar slants, without flaming, was used to inoculate LIA slants. Inoculation was done by stabbing the butt and streaking the surface of slant. The culture tubes were incubated at 37^{o}C for 24h.

Salmonellae are lysine decarboxylase positive and lysine deaminase negative.

3.2.1.3 Urease test

This test is done to check the ability of the organism to produce urease enzyme. Christensen's urea agar was used. The utilization of urea by the microbes results in the formation of ammonia which makes the medium alkaline. The utilization of urea is indicated by phenol red, used as indicator dye, which turns to pink colour.

The slants were heavily inoculated with the culture and incubated for 24h at 37^{o}C.

Salmonellae are urease negative and hence will not show any colour change.

3.2.1.4 Indole test

Bacteria utilizing tryptophan produce indole, which upon reaction with p-dimethyl aminobenzaldehyde forms a red/pink coloured ring at the top of the liquid medium.

Culture media was inoculated with bacteria and incubated for 24 to 48 h. To each tube about 0.5mL Kovac's reagent was added.

Salmonellae cannot utilize tryptophan and hence there will not be any coloured ring formation.

3.2.1.5 Simmon's citrate

Metabolism of citrate by microbes results in an increase of pH which is indicated by a change in colour of the medium from green to deep blue.

Slants of media were inoculated with culture and incubated for 24h at 37^{o}C.

Salmonella utilize citrate which is indicated by a deep blue colour of the medium.

3.2.1.6 Carbohydrate fermentation

Utilization of glucose, lactose, dulcitol and salicin was tested using phenol red carbohydrate broth (Appendix). An inverted Durham's tube was placed in the culture tube. Culture was inoculated to the broth using a sterile loop. Fermentation of carbohydrate will produce acid and gas, which will be indicated as yellowish colour in the medium and gas in Durham's tube.

Salmonellae are glucose and dulcitol positive; lactose and salicin negative.

3.2.2 Serology

All cultures giving typical reactions for Salmonellae in the above biochemical tests were tested for agglutination with *Salmonella* O antiserum Poly A-I & Vi (Becton Dickinson, Maryland, USA).

A loopful of culture was emulsified in 0.85% NaCl. One drop of this suspension was placed on a clean glass slide. One drop of *Salmonella* polyvalent antiserum was added to it and thoroughly mixed using a sterile

loop. The slide was tilted back and forth and observed for agglutination. The same culture with added saline instead of antisera served as control.

A positive reaction will give agglutination in test mixture and no agglutination in the saline control.

3.3. Antibiotic susceptibility

Antibiotic susceptibility of the test cultures were determined by the disk diffusion method (Bauer *et al.*, 1966)

3.3.1. Antibiotic susceptibility test

Isolates were screened for susceptibility to a panel of 19 antibiotics coming under 10 antimicrobial classes (Table 3.2). The experiments were done in triplicates and the average values were taken. Susceptibility to the selected antibiotics was determined on Mueller-Hinton agar (HiMedia, Mumbai, India) by the disk diffusion method as described below.

- A single, isolated colony of the test strain was picked and transferred to 3mL physiological saline (Appendix).
- Turbidity of the cell suspension was adjusted to 0.5 McFarland standard, either by adding new inoculum or physiological saline.
- A uniform smear of the culture was made on Muller Hinton agar plate using a sterile cotton swab.
- Antibiotics discs were placed on to the plates and incubated for 24h at 37^0C.
- Results were interpreted based on the inhibition zone around the discs as provided by the manufacturer (HiMedia,) (Table 2)

Table 3.2 Antibiotic class, disc concentration and zone interpretation chart used in this study

Antimicrobial agent	**Class**	**Disc content** (μg/disc)	**Resistant** (mm or less)
Ampicillin	Penicillins	10	13
Carbenicillin	Penicillins	100	19
Azithromycin	Macrolide	15	13
Cefixime	Cephalasporins III	5	15
Ceftriaxone	Cephalasporins III	30	13
Cefuroxime	Cephalasporins II	30	14
Chloramphenicol	Phenicol	30	12
Ciprofloxacin	Fluroquinolone	5	15
Norfloxacin	Fluroquinolone	10	12
Nalidixic acid	Quinolone	30	13
Doxycycline	Tetracyclines	30	12
Tetracycline	Tetracyclines	30	14
Gentamicin	Aminoglycoside	10	12
Kanamycin	Aminoglycoside	30	13
Netilmicin	Aminoglycoside	30	12
Streptomycin	Aminoglycoside	10	11
Amikacin	Aminoglycoside	30	14
Sulphafurazole	Folate pathway inhibitor	300	12
Trimethoprim	Folate pathway inhibitor	5	10

This chart is adapted from the zone-size interpretative chart published by HiMedia Pvt. Laboratories Ltd., India (2005).

3.3.2. Multiple Antibiotic Resistance (MAR) Index

MAR index of bacterial isolates gives an indication of the antibiotic stress of the environment from which they were isolated.

MAR index was calculated using the formula a/b, where 'a' is the number of antibiotics to which the isolate was resistant, and 'b' is the number of antibiotics to which the isolate was exposed (Krumperman, 1983).

3.4. DNA extraction

The protocol used for isolation of DNA (Esteban *et al.*, 1993) is described below.

- A single colony was inoculated into Luria broth (HiMedia) and incubated at 37^{o}C overnight with constant shaking.
- 1.5 mL culture was taken in a microfuge tube and centrifuged at 8000 rpm (Sigma, Germany) for 5 min.
- The pellet was resuspended in 567µL Tris-EDTA (TE) buffer (pH 8).
- To the suspension, 30µL of 10% sodium dodecyl sulphate (SDS) and 3µL Proteinase K (20mg/mL) were added and mixed well.
- The culture was the incubated for 1h at 37^{o}C in a water bath.
- 100µL of 5M NaCl and 80µL Hexadecyltrimethyl ammonium bromide (CTAB) (10mg/mL) were added and incubated for 10min at 65^{o}C in a water bath.
- The tubes were allowed to cool to room temperature and an equal volume of chloroform-isoamyl alcohol (24:1) was added.
- The contents were mixed gently and centrifuged at 10000 rpm for 10min.

- The aqueous layer at the top containing the DNA was carefully transferred to a fresh microfuge tube using a sterile cut- tip.
- An equal volume of phenol-chloroform-isoamyl alcohol mixture (25:24:1) was added to the aqueous layer and centrifuged again at 10000 rpm for 10 min.
- The aqueous layer was collected in a new tube and 0.6 volume of isopropanol was added to it.
- Mixed gently and centrifuged at 12000 rpm for 10min.
- The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged as above.
- The supernatant was discarded and the pellet was dried at room temperature.
- The purified DNA was the dissolved in 100µL TE buffer (pH 8)
- The concentration of DNA was estimated using UV spectrophotomer (Shimadzu, Japan).
- DNA was stored at -20^{0}C until further use.

3.5. Polymerase Chain Reaction (PCR) assays

All PCR reaction mixtures were prepared in the following method unless otherwise stated. The reactions were performed in a final volume of 20µL containing 200µM of each deoxyribonucleotide triphosphate, 1.5mM $MgCl_2$, 1X *Taq* Buffer [10mM Tris-HCl, (pH 8.3), 50mM KCl] 0.5µM of each primer, 1U *Taq* DNA Polymerase, 1µL (100ng) template DNA and H_2O to a final volume of 20µL. PCR assays were performed in MJ Mini (BioRad) Thermal cycler. The thermal profile for PCR reactions may vary with different primers and the size of amplicons.

3.6. Agarose gel electrophoresis

- Agarose gels of different strength (depending upon the expected amplicon length) were prepared in Tris-Acetate-EDTA (TAE) buffer.
- Ethidium bromide was added at a concentration of 0.5mg/mL.
- Definite volume of PCR product was mixed with gel loading dye (Appendix I) and loaded into the wells. DNA markers were run along with the products for confirmation of amplicon size.
- Electrophoresis was performed at a constant volt (5V/cm) using Mini gel electrophoresis unit (Genei, Bangalore, India)
- Gel pictures were captured with gel documentation system (Syngene, UK)

3.7. Detection of mutations in Quinolone Resistance-Determining Region (QRDR) of gyrase A gene

QRDR represents a region in the *gyrA* gene coding for the A subunit of gyrase enzyme. Point mutations at specific locations in this region give quinolone resistance phenotype to bacteria. QRDR region of nalidixic acid resistant bacteria was amplified by PCR using the following primers.

Primer	Sequence (5´- 3´)	Amplicon	Reference
STGYRA1	tgt ccg aga tgg cct gaa gc	470bp	(Giraud *et al.*, 1999)
STGYRA12	cgt tga tga ctt ccg tca g		

PCR assay conditions

Annealing - 55^0C for 30 sec.

Extension - 72^0C for 30 sec.

The amplicons obtained were sequenced. It was then compared with the normal gyrase A gene sequence in the database for detecting mutations in the QRDR using CLUSTALW software (Thompson *et al.*, 1994).

3.8. Detection of *Salmonella* Genomic Island 1 (SGI1) by PCR

Strains with multi drug resistance were analyzed by PCR to determine the presence of *Salmonella* Genomic Island 1. SGI1 is a genomic island present in several multidrug resistant *Salmonella* serotypes. SGI1 harbors several antibiotic resistance genes and integrons. It is located between the *thdF* and *yidY* genes of *Salmonella* chromosome (Boyd *et al.*, 2001).

3.8.1. PCR detection of the left junction of SGI1

SGI1 is found next to *thdF* gene. The primer set used amplifies a portion of the *thdF* gene and the integrase gene, the left boundry of SGI1. The presence of the specific amplicon confirms the presence of SGI1.

Primer	Sequence (5´- 3´)	Amplicon	Reference
U7-L12	aca cct tga gca ggg caa g	500bp	(Boyd *et al.*, 2001)
LJ-R1	agt tct aaa ggt tcg tag tcg		

PCR assay conditions

Annealing - 55^0C for 30 sec.

Extension - 72^0C for 45 sec.

3.9. PCR screening of Integrons

Integrons are genetic platforms that help in the acquisition, expression and dissemination of antibiotic genes. Several classes of integrons have been identified based on the type of integrase they harbor. PCR method was used for the detection of class 1 integron and class 2 integron using primers targeting conserved regions.

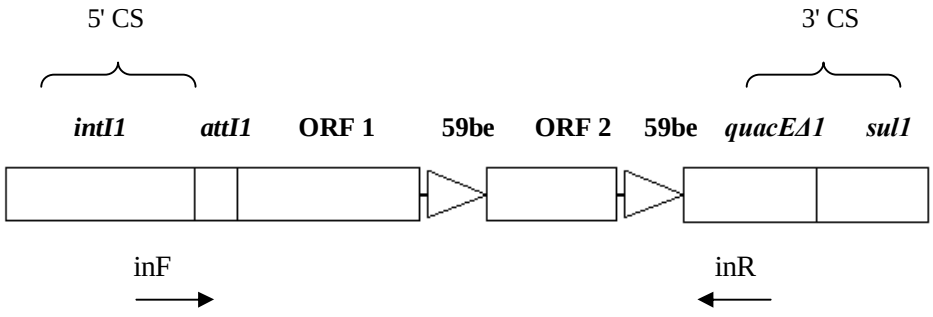
3.9.1. Class 1 integron

Class 1 integron is the most widely distributed member of the integron family. PCR method was used to confirm the presence of class 1 integron and the type of antibiotic resistance gene they harbor.

3.9.1.1. Amplification of class 1 integron associated gene cassette

Primers *in*F and *in*R were used for PCR. Fig.3.1. shows the organization of a typical class 1 integron, indicating the position of the primers used for PCR amplification.

Fig.3.1. Schematic diagram of Class 1 integron showing the position of primers.



Amplicons of variable size and number will be obtained based on the number of gene cassettes and number of integrons present. The amplicons were sequenced to identify the gene cassettes it harbors.

Primer	Sequence (5´- 3´)	Amplicon	Reference
inF	ggc atc caa gca gca agc	variable	(Dalsgaard *et al.*, 2000)
inR	aag cag act tga cct gat		

PCR assay conditions

Annealing - 55^0C for 30 sec.

Extension - 72^0C for 2.5 min.

3.9.2. PCR detection of class 2 integron

The class 2 integrons detection was done using the following oligonucleotide primer set by PCR. .

Primer	Sequence (5´- 3´)	Amplicon	Reference
int2F	cac gga tat gcg aca aaa agg t	789bp	(Mazel *et al.*, 2000)
in2R	gta gca aac gac tga cga aat g		

PCR assay conditions

Annealing - 62^0C for 30 sec.

Extension - 72^0C for 60 sec.

3.10. PCR screening of virulence genes

Virulence genes were screened employing PCR assay with specific primers. Multiplex and uniplex PCR assays were employed.

3.10.1. Multiplex PCR for *invE/A*, *mgtC* and *ttrC*

The primer pair invE/A, targets both *invE* and *invA* genes. They are invasion genes helping in the early stages of infection. Their ubiquitous presence in Salmonellae makes them markers. The other genes, *mgtC* and *ttrC*, are involved in high efficiency magnesium uptake and tetrathionate respiration respectively. The presence of three bands of the specified size confirms the presence of the four genes.

Primer	Sequence (5´- 3´)	Amplicon	Reference
invE/A 1	tgc cta caa gca tga aat gg	457bp	(Stone *et al.*, 1994)
invE/A 2	aaa ctg gac cac ggt gac aa		
ttrC 1	gtg ggc ggt aca ata ttt ctt tt	920bp	(Soto *et al.*, 2006)
ttrC 2	tca cga ata atc agt agc gc		
mgtC 1	tga cta tca atg ctc cag tga at	655bp	(Soto *et al.*, 2006)
mgtC 2	att tac tgg ccg cta tgc tgt tg		

PCR assay conditions

Annealing - 60^0C for 30 sec.

Extension - 72^0C for 60 sec.

3.10.2. PCR for *spaM*

This gene is a member of *inv-spa* invasion gene complex and is involved in the entry of the bacteria into host cells.

Primer	Sequence (5´- 3´)	Amplicon	Reference
SALFP2	cag cgg cgc tgt acg g	384bp	This work
SALRP2	ctg ctg tat ctc tcg ctg		

3.10.3. PCR for *spi4R*

spi4R is involved in intra macrophage survival and toxin secretion.

Primer	Sequence (5´- 3´)	Amplicon	Reference
spi4R 1	gaa tag aag aca aag cga tca tc	1269bp	(Soto *et al.*, 2006)
spi4R 2	gct ttg tcc acg cct ttc atc		

PCR assay conditions

Annealing - 60^0C for 30 sec.

Extension - 72^0C for 90 sec.

3.10.4. Multiplex PCR for *phoP/Q and slyA*

phoP/Q is a two-component transcriptional regulator of many virulence genes. *slyA* encodes a cytolytic toxin that is tentatively designated as salmolysin. It has a putative regulatory function as well.

Primer	Sequence (5´- 3´)	Amplicon	Reference
phoP/Q1	atg caa agc ccg acc atg acg	299bp	(Way *et al.*, 1993)
phoP/Q2	gta tcg acc acc acg atg gtt		
slyA1	gcc aaa act gaa gct aca ggt g	700bp	(Guerra *et al.*, 2000)
slyA2	cgg cag gtc agc gtg tcg tgc		

PCR assay conditions

Annealing - 60^0C for 30 sec.

Extension - 72^0C for 60 sec.

3.10.5. PCR for *pipA*

The *pipA* pathogenicity island encoded protein is involved in enteric salmonellosis.

Primer	Sequence (5´- 3´)	Amplicon	Reference
pip A 1	ctc ttg gat gat ttt ctt ctt ta	406bp	(Soto *et al.*, 2006)
pip A 2	ctt atc tca ggc gcg ggt gg		

PCR assay conditions

Annealing - 60^0C for 30 sec.

Extension - 72^0C for 30 sec.

3.10.6. PCR for *sodC1*

sodC1 is a Gifsy-2 mediated virulence gene encoding a periplasmic super oxide dismutase.

Primer	Sequence (5´- 3´)	Amplicon	Reference
SOD1	tat tgt cgc tgg tag ctg	468bp	(Bacciu *et al.*, 2004)
SOD2	cag gtt tat cgg agt aat		

3.10.7. PCR for *sopE*

Salmonella outer protein E (*sopE*) gene is a phage mediated virulence gene. It is a *Salmonella* pathogenicity island 1- dependent translocated effector protein.

Primer	Sequence (5´- 3´)	Amplicon	Reference
sopE 1	tca gtt gga att gct gtg ga	642bp	(Hopkins and Threlfall, 2004)
sopE 2	tcc aaa aac agg aaa cca cac		

PCR assay conditions

Annealing - 55^0C

Extension - 1 min

3.11. Screening of lysogenic phages

The presence of lysogenic phages in *Salmonella* is an indication of their inherent potential for horizontal gene transfer. Their presence may provide the host with additional virulence determinants.

3.11.1. Induction of lysogenic phages

Induction of lysogenic phages was done using mitomicin C using the protocol described by Yee *et al.* (1993) with modifications.

- Salmonellae were grown in nutrient broth to mid-logaritmic phase.
- 2mL culture was pelleted and the pellet was resuspended in 2mL fresh broth.
- Mitomycin C was added at a concentration of 1µg/mL and incubated overnight.
- The culture was filtered through 0.22µm membrane filters to obtain phage lysate as the filtrate.
- 1mL of the host culture and 1mL of serially diluted phage lysate was mixed and incubated at 37^0C for 1 h.
- To the mixture 3mL of 0.8% agarose was added and poured on to a nutrient agar base plate and incubated overnight for the development of plaques.

To enhance the visibility of the plaques, tetrazolium dye was used as previously described (Pattee, 1966). The plates were flooded with trypticase soy broth containing 2,3,5 -triphenyltetrazolium chloride (0.1%) and incubated at 37^oC for 20 min. After incubation the broth was poured off and the plates were examined for clear areas. The plaque areas remain colourless where as the surrounding bacterial lawn will retain the red colour due to reduction of the dye to insoluble formazan by the live bacterial cells.

3.11.2. PCR for *gogB*

Gifsy one gene B (*gogB*) is a putative virulence gene carried by Gifsy-1, a *Salmonella* lambdoid prophage.

Primer	Sequence (5´- 3´)	Amplicon	Reference
gogB1	gct cat cat gtt acc tct at	598bp	(Bacciu *et al.*, 2004)
gogB2	agg ttg gta ttt ccc atg ca		

PCR assay conditions

Annealing - $55^{O}C$

Extension - 45sec.

3.11.3. PCR for *gtgE*

Gifsy two gene E (*gtgE*) is another gene with putative virulence characteristics, encoded by a *Salmonella* lambdoid prophage, Gifsy-2.

Primer	Sequence (5´- 3´)	Amplicon	Reference
gtgE1	agg agg agt gta aag gt	1114bp	(Bacciu *et al.*, 2004)
gtgE2	gta gaa ctg gtt tat gac		

PCR assay conditions

Annealing - $55^{0}C$

Extension - 90 sec.

3.12. Molecular Typing

Molecular typing has been done with PCR using primers targeting specific sequences in the bacterial genome. The amplified products were separated on agarose gel, producing a banding profile depending upon the number and position of the repeated units. The gel pictures were captured and the banding patterns were analysed using popgene32 software. Clustering analysis was done using unweighted pair group method with arithmetic averages (UPGMA) (Michener and Sokal, 1957)

3.12.1. Enterobacterial repetitive intergenic consensus (ERIC) PCR

ERIC sequences are present in multiple copies in the genome of *Salmonella*. These 126bp long sequences are highly conserved at the nucleotide level (Hulton *et al.*, 1991).

PCR assay was performed using specific primers and the amplicons were resolved on agarose gel. Gel pictures were captured and the pattern was used for clustering the strains using popgene32 software based on unweighted pair group method with arithmetic averages (UPGMA).

Primer	Sequence (5´- 3´)	Amplicon	Reference
ERIC 1	atg taa gct cct ggg gat tca c	Variable	(Versalovic *et al.*, 1991)
ERIC 2	aag taa gtg act ggg gtg agc g		

Annealing - 52^0C for 30 sec.

Extension - 72^0C for 4 min.

3.12.2. PCR Ribotyping

This fingerprinting technique is based on amplification of the spacer regions between 16S and 23S genes in the ribosomal RNA transcriptional unit. rRNA genes are present in multiple copies. The spacer region shows sequence and length variations.

PCR was performed using specific primers and the amplicons were resolved on agarose gel. Gel images were captured using gel documentation system. The profiles obtained were compared and clustered based on unweighted pair group method with arithmetic averages (UPGMA).

Primer	Sequence (5´- 3´)	Amplicon	Reference
Pr F	ttg tac aca ccg ccc gtc a	Variable	(Kostman *et al.*, 1992)
Pr B	ggt act tag atg ttt cag ttc		

PCR assay conditions

Annealing - 60^0C for 30 sec.

Extension - 72^0C for 2min.

3.12.3. Discriminative Index

The discriminatory index was calculated for each typing method using the Simpson's index of diversity (Hunter and Gaston, 1988). The index was calculated using the formula

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

where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the j^{th} type. The probability that a single strain sampled at random will belong to the j^{th} group is n_j/N. The probability that two strains sampled consecutively will belong to that group is $n_j (n_j - 1)/N (N - 1)$.

3.13. Loop-Mediated Isothermal Amplification (LAMP)

It is a novel DNA amplification technique which can be performed under isothermal condition. It is based on auto-cycling strand displacement DNA synthesis property of *Bst* polymerase (Notomi *et al.*, 2000). It relies on 4 oligonucleotide primers; two inner and two outer primers, which targets 6 specific regions in the template DNA. The use of four primers in the amplification process increases the specificity of the reaction.

3.13.1. Optimization of LAMP assay for detection of low number of *Salmonella* in water

The minimum time required for detection of very low number of *Salmonella* and the effect of non-target DNA on the sensitivity of LAMP assay was determined. The results were then compared with that of conventional PCR assay.

3.13.1.1. Sensitivity of LAMP assay

Four different *Salmonella* serotypes (Table 3.3) were cultured overnight in nutrient broth (HiMedia) and 10-fold serial dilutions were prepared using physiological saline. 25mL sterile water was spiked with known number of CFUs ranging from $2X10^5$ to 2 and inoculated to 225mL lactose broth (HiMedia). The CFU was estimated by plating appropriate dilutions on XLD plates (Difco). The inoculated lactose broth was incubated

with shaking at 37^0C. Two millilitre cultures were retrieved from each flask at regular intervals. DNA from these samples were isolated and used as template for LAMP and PCR assays. All the experiments were conducted independently and repeated thrice.

Table 3.3. Cultures used in this study

No.	Serotype	Source[a]
1.	*Salmonella enterica* subsp. *enterica* ser. Abony	NCIM 2257
2.	*Salmonella enterica* subsp. *enterica* ser. Typhimurium	ATCC 23564
3.	*Salmonella enterica* subsp. *enterica* ser. Typhi	MTCC 734
4.	*Salmonella enterica* subsp. *enterica* ser. Paratyphi	MTCC 735
5.	*Escherichia coli*	ATCC 9961

[a] NCIM – National collection of industrial microorganisms, Pune, Maharastra India,
MTCC – Microbial type culture collection and gene bank, Chandigarh, India.

3.13.1.2. Specificity

The specificity of LAMP assay was determined using *E.coli* cultures (Table 3.3). They were grown in lactose broth. LAMP and PCR assays were carried out using DNA isolated from these bacterial cultures at regular intervals of enrichment in lactose broth.

3.13.1.3. Effect of non-target DNA on sensitivity of LAMP

10^4 CFUs of *E. coli* were co inoculated separately with 2 CFUs of each of the 4 *Salmonella* serotypes in lactose broth and incubated at 37^OC. LAMP and PCR assay were performed with DNA isolated from these cultures.

3.13.1.4. Template DNA preparation

Template DNA for LAMP and PCR assays was prepared by the boiling method as follows. 2mL culture suspension was centrifuged at 10000xg for 10 min. (Sigma, 2-16K, Germany). The harvested pellet was washed twice with sterile water and resuspended in 100µL Tris-EDTA buffer (pH 8). The tubes were kept in a boiling water bath for 10min. and chilled immediately on ice. It was then centrifuged at 10000 x g for 15 min. The supernatant containing DNA was directly used for LAMP and PCR assay.

3.13.1.5. LAMP assay

Four primers (Table 3.4) were used for the LAMP assay (Wang *et al.*, 2008). These primers target six distinct regions of the *Salmonella* specific invasion protein gene (*inv*A). The reaction was carried out in 25µL reaction mixture containing 1.4µM each of FIP and BIP primers, 0.2µM each of F3 and B3 primers, 1.6mM of dNTPs, 1M betaine (Sigma), 1X thermopol buffer [20mM Tris-HCl, 10mM $(NH_4)_2SO_4$, 10mM KCl, 2 mM $MgSO_4$ and 0.1% Triton X-100 (pH 8.8)] and 4.5µL of template DNA.

The tubes with the reaction mixture were kept at 95^{o}C for 5 min. to denature the template DNA. The tubes were then plunged into ice and 8 units of *Bst* DNA polymerase large fragment (New England Biolabs, UK) were added. The reaction mixture was then kept at 65^{0}C for 1h for amplification. The reaction was terminated by keeping at 80^{0}C for 10 min. A negative control was also kept, with all the ingredients except the template DNA. 5µL of the LAMP product was resolved in a 2% agarose gel. Ethidium bromide was used for staining. The image of gel was captured using gel documentation system (Syngene,UK)

Table 3.4. Primer sequences for LAMP assay.

Primer	Sequence (5´- 3´)
FIP	ccc aga tcc ccg cat tgt tga ttt ttc cgc ccc ata tta tcg cta t
BIP	gac cat cac caa tgg tca gca ttt tat tgg cgg tat ttc ggt ggg
F3	gtt caa cag ctg cgt cat ga
B3	cgc tat tgc cgg cat cat ta

3.13.1.6. PCR assay

PCR was done with the same template DNA that was used for LAMP assay. Primers F3 and B3 of LAMP assay (Table 3.4) were used as primers for the reaction (Wang *et al.*, 2008). Amplified products were resolved on 0.8% agarose gel and images were captured using gel documentation system.

PCR assay conditions

Annealing - 55^0C for 30 sec.

Extension - 72^0C for 30sec.

3.13.2. Detection of *Salmonella* in seafood

Based on the optimized protocol for detection of *Salmonella* in water, seafood samples were analyzed for the presence of *Salmonella* employing the LAMP assay. A comparison was also done with the PCR assay in order to ascertain the sensitivity.

3.13.2.1. LAMP assay

Five Sardine samples were collected from 5 different locations. They were processed separately. Twenty five grams of each sample was

homogenized using a sterile mortar and pestle. It was then added to 225mL lactose broth and incubated with shaking at 37^{O}C. Two millilitre samples were retrieved from each flask at regular intervals of 1h. DNA was isolated using boiling method (Agarwal *et al.*, 2002). LAMP assay was performed using this DNA. *Salmonella* Typhimurium (ATCC 23564) was used as a positive control. Amplified products were resolved on 2% agarose gel and images were captured using gel documentation system.

3.13.2.2. PCR assay

PCR assay also was carried out using the same template DNA. *Salmonella* Typhimurium (ATCC 23564) was used as a positive control. Amplified products were resolved on 0.8% agarose gel and images were captured using gel documentation system.

Chapter 4

RESULTS

4.1. Isolation and identification of *Salmonella*

Forty two seafood samples were screened for the presence of *Salmonella* employing the conventional culture techniques using different enrichment and selective media. The typical colonies on the different selective plates were identified biochemically to genus level. Table 4.1 shows the biochemical characters invariably exhibited by *Salmonella* in this study.

Table 4.1. Biochemical characters of *Salmonella*

Biochemical tests	Reactions
TSI	Alkaline slant and acid butt
LIA	Alkaline slant and butt
Indole	Negative
Urease	Negative
Citrate utilization	Positive
Glucose utilization	Positive
Dulcitol utilization	Positive
Lactose utilization	Negative
Salicin utilization	Negative

A total of 37 *Salmonellae* were isolated from different seafood samples. The serotype of these isolates and the poultry isolates was determined at the National *Salmonella* and *Escherichia* centre, Kasauli. The identity and source of the different *Salmonella* serotypes isolated is shown in table 4.2.

Table 4.2. Identity and source of the *Salmonella* strains.

Strain name	**Serotype**	**Antigenic Formula**	**Source**
TAB-4, TAC-7, TAC-22	Typhimurium	4,12:i:1,2	Anchovies
TSD-9, TSD-11, TSH-16, TSD-19, TSH-25, TSF-32, TSF-33,TSG-38, TSI-52, TSI-53, TSJ-58, TSJ-59, TSJ-60, TSK-72, TSK-75	Typhimurium	4,12:i:1,2	Sardine
TQE-14, TQE-15	Typhimurium	4,12:i:1,2	Squid
WAB-5, WAB-6	Weltevreden	3,10:r:z_6	Anchovies
WSH-17, WSD-18, WSD-28, WSH-29, WSF-31, WSG-37, WSG-39, WSI-51, WSI-54, WSI-55, WSJ-61, WSK-71, WSK-74	Weltevreden	3,10:r:z_6	Sardine
OSI-50, OSK-73	Oslo	6,7:a:enx	Sardine
ST-2, ST-32, ST-35, ST-41, ST-42, ST-44, ST-48, ST-49, ST-51, ST-72, ST-77, ST-126, ST-128	Typhimurium	4,12:i:1,2	Poultry
SE-24, SE-26, SE-29, SE-31, SE-33, SE-43, SE-45, SE-46, SE-47, SE-50, SE-52, SE-57, SE-98, SE-118	Enteritidis	9,12:g,m:-	Poultry

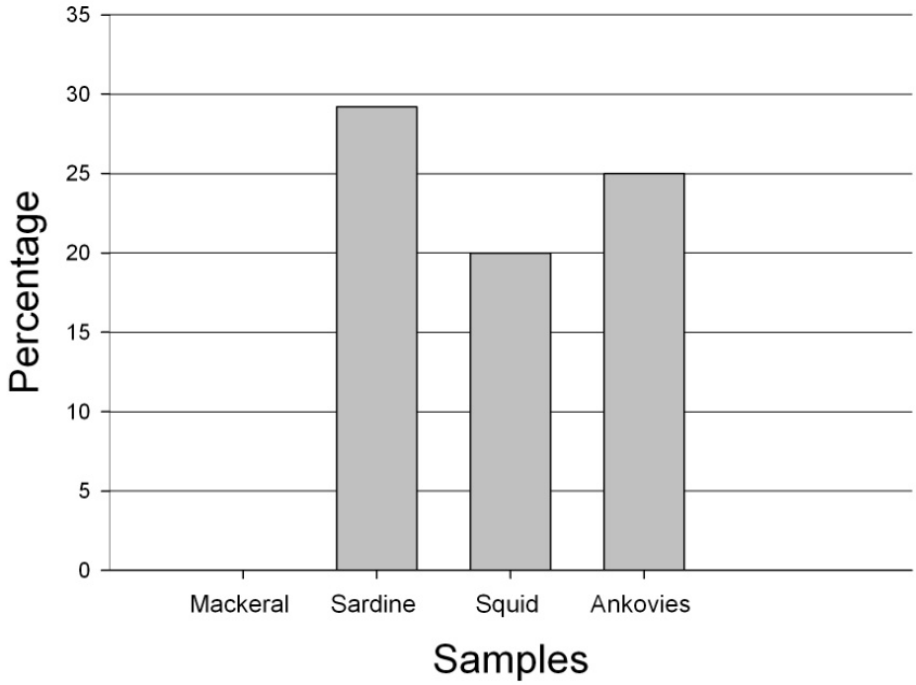
4.2. Prevalence of *Salmonella* in the samples

The seafood samples which were found positive for *Salmonella* are shown in Table 4.3. Screening of the samples showed that 23.8% were contaminated with different serotypes of *Salmonella.* Sardine showed high contamination followed by Anchovies and Squid (Fig.4.1). All the Mackerel samples were free from *Salmonella* contamination.

Table 4.3. Prevalence of *Salmonella* in tested seafood samples

	Source	**No. of samples tested**	**Positive**
1	Mackerel	5	0
2	Sardine	24	7
3	Squid	5	1
4	Anchovies	8	2
Total		**42**	**10**

Fig. 4.1. Percentage of samples positive for *Salmonella*



Three different serotypes were obtained from the seafood samples analyzed in this study. *Salmonella* Typhimurium (N=20) and *Salmonella Weltevreden* (N=15) were the most prevalent serotypes, but *Salmonella* Oslo (N=2) was also observed.

Samples collected from the same lot harboured more than one serotype of *Salmonella* (Table 4.4). All the 7 positive samples of Sardine showed the presence of *Salmonella* Typhimurium and *Salmonella* Weltevreden. Interestingly, two samples were contaminated with all the above mentioned three serotypes.

Table 4.4. Prevalence of different *Salmonella* serotypes in the seafood samples

Sl.No.	**Source**	**No. of samples Positive**	**No. of samples positive for *S.*Typhimurium**	**No. of samples positive for *S.*Weltevreden**	**No. of samples positive for *S.*Oslo**
1	Sardine	7	7	7	2
2	Anchovies	2	2	1	0
3	Squid	1	1	0	0

Salmonella Typhimurium was present in both positive samples of Anchovies, whereas, *Salmonella* Weltevreden was present only in one sample. Squid sample showed the presence of only *Salmonella* Typhimurium

4.3. Antibiotic susceptibility

The antibiotic susceptibility of the 37 *Salmonella* strains isolated from various seafood samples was determined using the disc diffusion method (Fig.4.2). 19 antibiotics coming under 10 classes of antibiotics were used for this study. The antibiotic susceptibility profile of each strain is presented in Table 4.5. (a) to 4.5 (e). *Salmonella* Oslo was sensitive to all antibiotics tested. *Salmonella* Typhimurium strains TSF-32, TSF-33 and TSG-38 and *Salmonella*

Weltevreden strains WSF-31, WSG-37 and WSG-39 were resistant to ampicillin, carbenicillin, doxycycline, kanamicin, nalidixic acid, sulfafurazole, tetracycline and trimethoprim. Three *Salmonella* Typhimurium strains (TSD-9, TSD-11 and TSD-19) and one *Salmonella* Weltevreden strain (WSD-28) were resistant to sulfafurazole and trimethoprim.

Fig. 4.2. Antibiotic resistance shown by strain WSG-39 against ampicillin, nalidixic acid, trimethoprim and tetracycline on Muller Hinton agar plates.

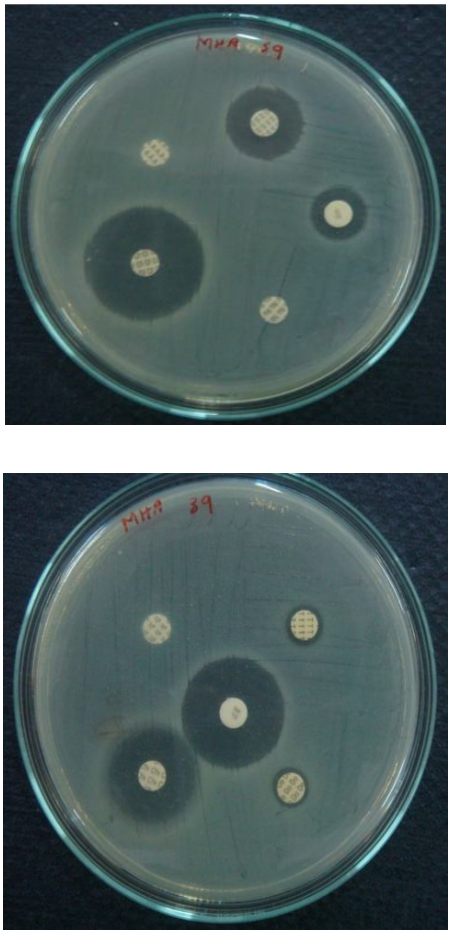


Table 4.5 (a) Antibiogram of *Salmonella* Typhimurium isolated from seafood

	A	Ak	At	Cb	Cfx	Ci	Cu	C	Cf	Do	G	K	Na	Nx	Nt	S	Su	T	Tr
TAB-4	17	22	19	20	23	24	17	23	31	14	22	19	20	27	23	16	22	16	24
TAC-7	18	21	20	21	18	23	19	23	27	14	20	19	19	23	22	17	22	18	23
TAC-22	16	22	25	22	23	27	18	25	38	14	25	21	23	35	28	18	25	16	24
TSD-9	16	20	23	20	19	24	18	22	32	14	20	18	18	29	24	17	**0**	16	**0**
TSD-11	14	19	23	21	18	22	17	22	30	14	20	18	19	26	21	17	**0**	17	**0**
TQE-14	17	20	18	21	20	25	17	27	30	15	20	18	18	26	22	17	22	15	23
TQE-15	17	21	18	22	18	23	20	23	28	15	18	19	18	26	21	14	25	17	25
TSH-16	20	21	18	21	19	25	18	27	27	16	20	16	17	28	24	17	24	15	24
TSH-25	17	21	21	21	19	23	18	21	30	14	20	18	20	24	24	17	22	17	25
TSD-19	16	18	18	21	19	22	17	25	30	14	20	16	18	25	20	17	**0**	18	**0**

A-Ampicillin, Ak-Amikacin, At-Azithromycin, Cb-Carbenicillin, Cfx-Cefixime, Ci-Ceftriaxone, Cu-Cefuroxim, C-Chloramphenicol, Cf-Ciprofloxacin, Do-Doxycycline, Fr-Furazolidone, G-Gentamycin, K-Kanamycin, Na-Nalidixic acid, Nx-Norfloxacin, Nt-Netilmicin, S-Streptomycin, Su- Sulfafurazole, T-Tetracycline, Tr-Trimethoprim
Inhibition zone values given in bold are resistant (Based on inhibition zone chart given in table 3.2)

Table 4.5 (b) Antibiogram of *Salmonella* Typhimurium isolated from seafood (contd…)

	A	Ak	At	Cb	Cfx	Ci	Cu	C	Cf	Do	G	K	Na	Nx	Nt	S	Su	T	Tr
TSF-32	**0**	20	20	**10**	19	25	17	21	24	**8**	14	**9**	**0**	20	19	15	**0**	**7**	**0**
TSF-33	**0**	21	20	**10**	20	24	19	22	25	**9**	14	**10**	**0**	24	21	15	**0**	**8**	**0**
TSG-38	**0**	20	20	**10**	19	24	15	21	24	**7**	14	**8**	**0**	18	21	15	**0**	**7**	**0**
TSI-52	18	20	19	21	23	27	17	21	29	15	20	18	20	25	21	16	23	17	25
TSI-53	18	21	18	20	22	24	20	24	26	14	20	19	19	25	22	17	25	17	23
TSJ-58	14	20	18	22	23	25	18	25	29	18	18	20	19	29	23	18	25	17	24
TSJ-59	16	20	18	22	20	28	20	24	32	18	17	19	19	28	21	17	24	18	23
TSJ-60	17	21	19	22	19	24	18	23	31	14	19	20	18	28	21	18	24	17	23
TSK-72	19	20	19	23	18	25	19	27	30	15	19	18	20	29	21	16	24	17	25
TSK-75	18	19	20	22	18	24	20	23	28	16	18	18	18	27	22	18	24	16	24

A-Ampicillin, Ak-Amikacin, At-Azithromycin, Cb-Carbenicillin, Cfx-Cefixime, Ci-Ceftriaxone, Cu-Cefuroxim, C-Chloramphenicol, Cf-Ciprofloxacin, Do-Doxycycline, Fr-Furazolidone, G-Gentamycin, K-Kanamycin, Na-Nalidixic acid, Nx-Norfloxacin, Nt-Netilmicin, S-Streptomycin, Su- Sulfafurazole, T-Tetracycline, Tr-Trimethoprim
Inhibition zone values given in bold are resistant (Based on inhibition zone chart given in table 3.2)

Table 4.5(c) Antibiogram of *Salmonella* Weltevreden isolated from seafood

	A	**Ak**	**At**	**Cb**	**Cfx**	**Ci**	**Cu**	**C**	**Cf**	**Do**	**G**	**K**	**Na**	**Nx**	**Nt**	**S**	**Su**	**T**	**Tr**
WAB-5	18	22	18	22	19	24	18	21	30	14	21	18	19	27	24	16	25	17	23
WAB-6	20	19	19	22	18	25	18	22	30	16	18	20	20	24	22	18	24	16	25
WSH-17	16	23	21	21	20	25	19	25	28	15	19	18	19	20	21	15	24	16	25
WSD-18	16	22	19	21	20	25	18	22	28	15	19	18	18	26	23	15	25	17	25
WSD-28	17	20	20	22	20	23	17	23	30	15	19	19	20	30	23	16	**0**	16	**0**
WSH-29	17	22	25	22	23	28	20	28	35	14	23	20	22	30	28	16	22	16	25
WSF-31	**0**	20	21	**9**	19	25	19	21	26	**8**	14	**8**	**0**	21	22	12	**0**	**8**	**0**
WSG-37	**0**	19	19	**11**	18	24	17	22	25	**8**	13	**9**	**0**	20	20	15	**0**	**8**	**0**
WSG-39	**0**	21	19	**11**	19	25	20	23	27	**8**	13	**10**	**0**	23	23	18	**0**	**8**	**0**
WSI-51	16	21	19	21	19	24	17	25	30	14	17	16	19	24	23	19	24	16	25

A-Ampicillin, Ak-Amikacin, At-Azithromycin, Cb-Carbenicillin, Cfx-Cefixime, Ci-Ceftriaxone, Cu-Cefuroxim, C-Chloramphenicol, Cf-Ciprofloxacin, Do-Doxycycline, Fr-Furazolidone, G-Gentamycin, K-Kanamycin, Na-Nalidixic acid, Nx-Norfloxacin, Nt-Netilmicin, S-Streptomycin, Su- Sulfafurazole, T-Tetracycline, Tr-Trimethoprim
Inhibition zone values given in bold are resistant (Based on inhibition zone chart given in table 3.2)

Table 4.5(d) Antibiogram of *Salmonella* Weltevreden isolated from seafood (contd...)

	A	Ak	At	Cb	Cfx	Ci	Cu	C	Cf	Do	G	K	Na	Nx	Nt	S	Su	T	Tr
WSI-54	18	19	19	21	19	27	17	23	29	14	20	19	19	25	22	18	24	17	25
WSI-55	17	19	23	22	23	25	18	24	28	14	18	16	18	24	23	17	25	17	23
WSJ-61	18	19	18	22	19	27	20	24	29	15	20	20	18	23	21	16	19	16	25
WSK-71	18	21	23	21	20	24	17	23	28	16	19	18	20	27	24	18	22	17	24
WSK-74	20	20	18	21	19	22	19	25	28	16	18	19	20	26	22	17	25	18	23

Table 4.5(e) Antibiogram of *Salmonella* Oslo isolated from seafood

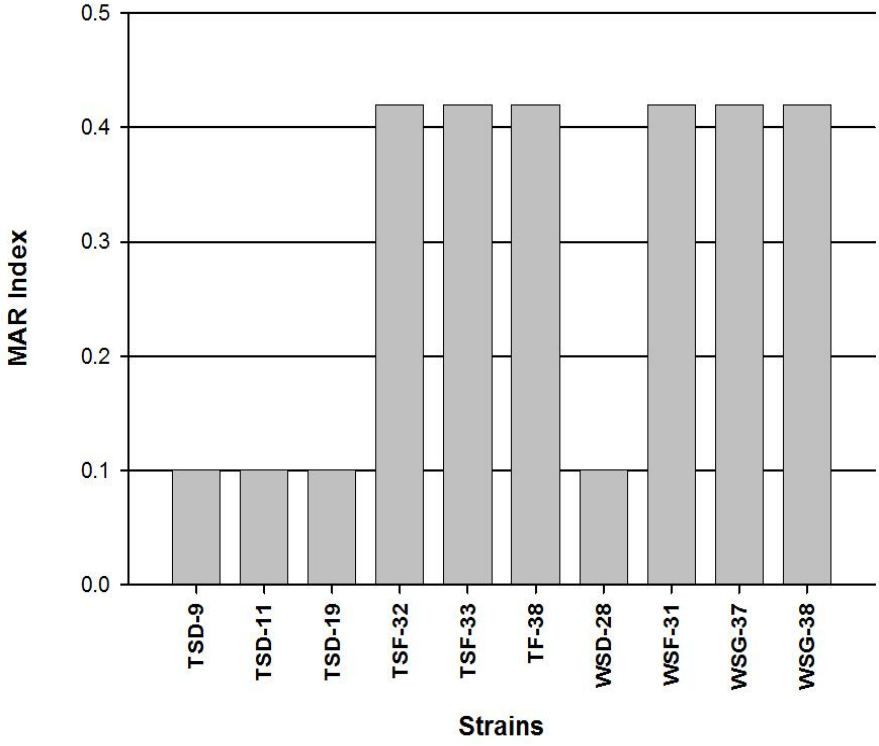
	A	Ak	At	Cb	Cfx	Ci	Cu	C	Cf	Do	G	K	Na	Nx	Nt	S	Su	T	Tr
OSI-50	16	20	24	20	23	27	18	21	28	14	18	20	20	28	24	18	22	16	23
OSI-73	19	21	20	21	18	22	20	25	27	15	20	17	19	30	21	17	22	16	25

A-Ampicillin, Ak-Amikacin, At-Azithromycin, Cb-Carbenicillin, Cfx-Cefixime, Ci-Ceftriaxone, Cu-Cefuroxim, C-Chloramphenicol, Cf-Ciprofloxacin, Do-Doxycycline, Fr-Furazolidone, G-Gentamycin, K-Kanamycin, Na-Nalidixic acid, Nx-Norfloxacin, Nt-Netilmicin, S-Streptomycin, Su- Sulfafurazole, T-Tetracycline, Tr-Trimethoprim

4.3.1. MAR (Multiple antibiotic resistance) Index

Only 10 of the 37 strains showed multiple antibiotic resistance. MAR index of the resistant strains enabled grouping of the *Salmonella* strains into two; with one group having an index of 0.42 and the other with 0.1 (Fig. 4.3).

Fig.4.3 MAR index of the multiple antibiotic resistant *Salmonella* strains

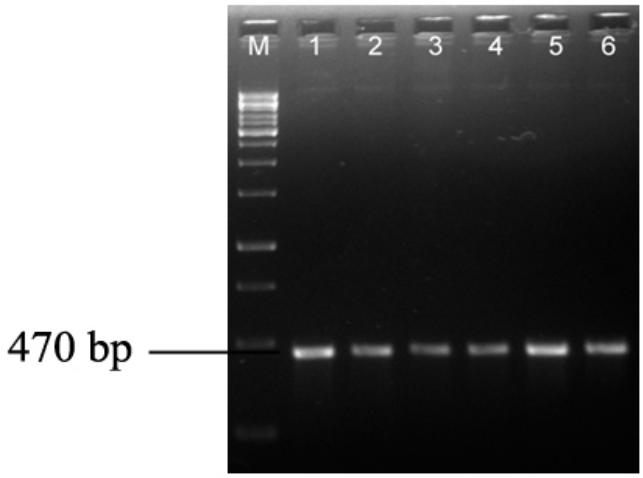


4.4. Detection of mutations in Quinolone Resistance-Determining Region (QRDR) of gyrase A gene

4.4.1. PCR amplification of *gyrA* (partial) gene

Quinolone resistant phenotype was observed in six stains of *Salmonella*: three *Salmonella* Typhimurium strains TSF-32, TSF-33, TSG-38 and three *Salmonella* Weltevreden WSF-31, WSG-37, WSG-39, all resistant to nalidixic acid. This is attributed to point mutations at specific locations in the *gyrA* gene coding for the A subunit of the gyrase enzyme. Partial *gyrA* gene of nalidixic acid resistant bacteria was amplified giving characteristic band of 470bp which includes the QRDR (quinolone resistance-determining region. All the six nalidixic acid resistant *Salmonella* strains produced this band. The results are shown in Fig.4.4.

Fig.4.4. PCR results of amplification of *gyrA* (partial) gene

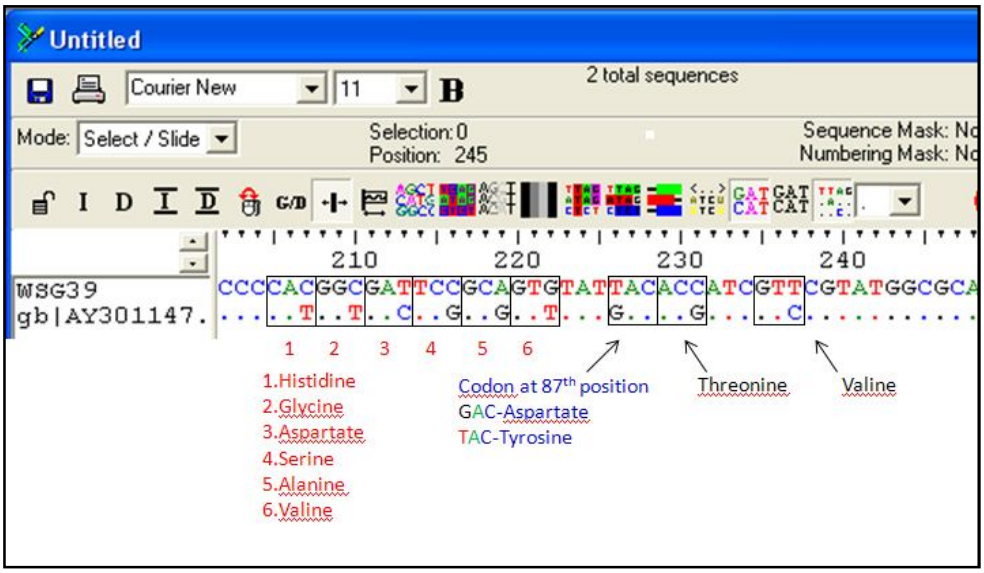


Lane M; 1kb DNA marker, lanes 1-6; TSF-32, TSF-33, TSG-38, WSF-31, WSG-37, and WSG-39 respectively.

4.4.2. Detection of point mutations in the QRDR region

The partial *gyrA* gene amplicons were sequenced and the sequences were compared with the normal *gyrA* gene sequences in the GenBank data base using CLUSTALW software. Alignment of these sequences (Fig 4.5) showed a crucial point mutation at the 87th codon. The codon GAC, encoding aspartate, has been changed to TAC, which codes for Tyrosine. Several other point mutations were also detected. But these mutations do not change the amino acids in the product, showing the degeneracy of genetic code.

Fig.4.5. Multiple sequence alignment showing point mutations in the QRDR region of *gyrA* of WSG-39

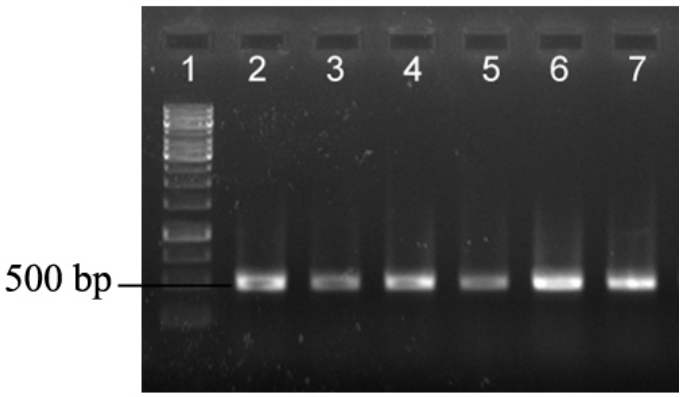


4.5. PCR screening of *Salmonella* Genomic Island 1

The presence of *Salmonella* Genomic Island 1 was screened in all the strains with multiple antibiotic resistance.

PCR screening of the left junction of *Salmonella* genomic island 1 produced an amplicon of 500bp from six *Salmonella* strains. Three *Salmonella* Typhimurium strains (TSF-32, TSF-33, and TSG-38) and three *Salmonella* Weltevreden strains (WSF-31, WSG-37 and WSG-39) were positive for this PCR. The results are shown in Fig.4.6.

Fig.4.6. PCR amplification of left junction of *Salmonella* genomic island 1



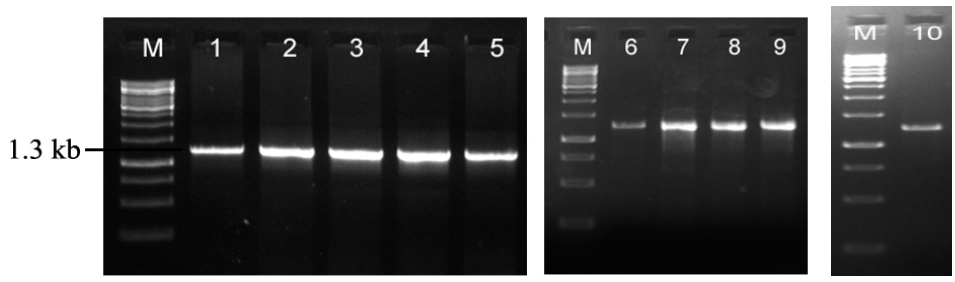
Lane 1; 1kb DNA Marker, lanes 2-7; TSF-32, TSF-33, TSG-38, WSF-31, WSG-37, WSG-39 respectively.

4.6. Class 1 Integrons

4.6.1. PCR screening of class 1 integron and gene cassettes

A portion of class 1 integron, including the antibiotic resistant gene cassette, was amplified by PCR using specific primers. Out of the 64 strains screened, ten (TSD-11, TSD-19, WSD-28, TSF-32, TSF-33, WSF-31, WSG-37, TSG-38 and WSG-39) showed amplifications. Six were *Salmonella* Typhimurium and the remaining was *Salmonella* Weltevreden. All amplicons were of the same size indicating that the same antibiotic resistant gene was present in the integron. The amplification results are shown in Fig. 4.7.

Fig.4.7. PCR results of class 1 integron gene cassette



Lane M; 1 kb DNA marker, lanes 1-10; TSD-9, TSD-11, TSD-19, TSF-32, TSF-33, TSG-38, WSD-28, WSF-31,WSG-37 and WSG-39 respectively.

4.6.2. Sequencing of the amplicon

The amplicons produced by PCR were sequenced for identifying the antibiotic resistant genes present in them and the sequences were compared with those in the GenBank data base using BLAST software (Altschul *et al.*, 1990). Fig.4.8 shows the sequence of the integron amplicon obtained from WSG-37. The sequence was submitted to GenBank (JQ794607).The result showed 100% similarity with *dhfrA1* gene giving resistance to trimethoprim. An open reading frame designated o*rfC* was also present. All the ten strains were found to harbour the same antibiotic resistance gene.

Fig. 4.8. Sequence of partial integron amplicon from WSG-37 submitted to GenBank.

GenBank: JQ794607.1

GenBank Graphics

>gi|390433163|gb|JQ794607.1| Salmonella enterica subsp. enterica serovar Weltevreden strain S37 class 1 integron dihydrofolate reductase (dfrA1) gene, complete cds, and hypothetical protein (orfC) gene, partial cds

ACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCTCTGAGGAAGAATTGTGAAACTATCA
CTAATGGTAGCTATATCGAAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTG
AACAGCTCCTGTTTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAATCAAT
GGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTGACAATGAGAACGTA
TTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAATAACGGATCATGTCATTGTTTCAG
GTGGTGGGGAGATATACAAAAGCCTGATCGATCAAGTAGATACACTACATATATCTACAATAGACATCGA
GCCGGAAGGTGATGTTTACTTTCCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCC
TCTAACATAAATTATAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGGCAGCAACGGATTCGCAAACCT
GTCACGCCTTTTGTACCAAAAGCCGCGCCAGGTTTGCGATCCGCTGTGCCAGGCGTTAAGGCTACATGAA
AATCGTACATTACGAAGCGAATGCACCATGGATAGGAAGAATGAAATGCCCAAACCCAAAGTGTGGGAAG
GAAACTCCTGCCTGGCAATCGAGCGGCATGAGCGACAGTTGCCCGCATTTTTTCTGTGATACTTGCTCGA
ATGTAATCCATAGAGAGCAGGACCATGCATTACTGTATGAAAATGAAATCAATCAAGAGCTCTTGGATCG
AATAGCAGCAACTCTTCCAGATTGCCCTTGCGGGGGTAGGTTTGTTCCTGGTGCAA

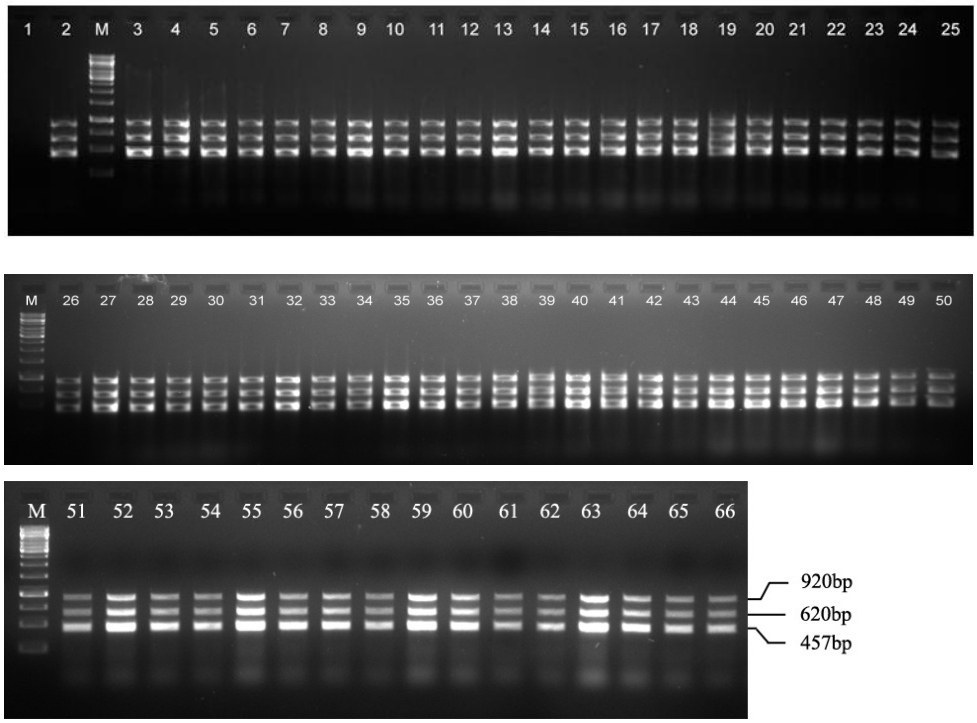
4.7. Class 2 integron

PCR with class 2 integron primers did not yield any amplicons indicating the absence of class 2 integrons.

4.8. Multiplex PCR screening of *ttrC, mgtC* and *inv E/A*

Amplicons of 920bp, 620bp and 457bp were obtained from all cultures which were subjected to PCR screening (Fig.4.9) indicating the presence of *ttr*C, *mgt*C, *invE/A respectively*.

Fig.4.9. Agarose gel of multiplex PCR for *inv E/A, ttrC* and *mgtC*

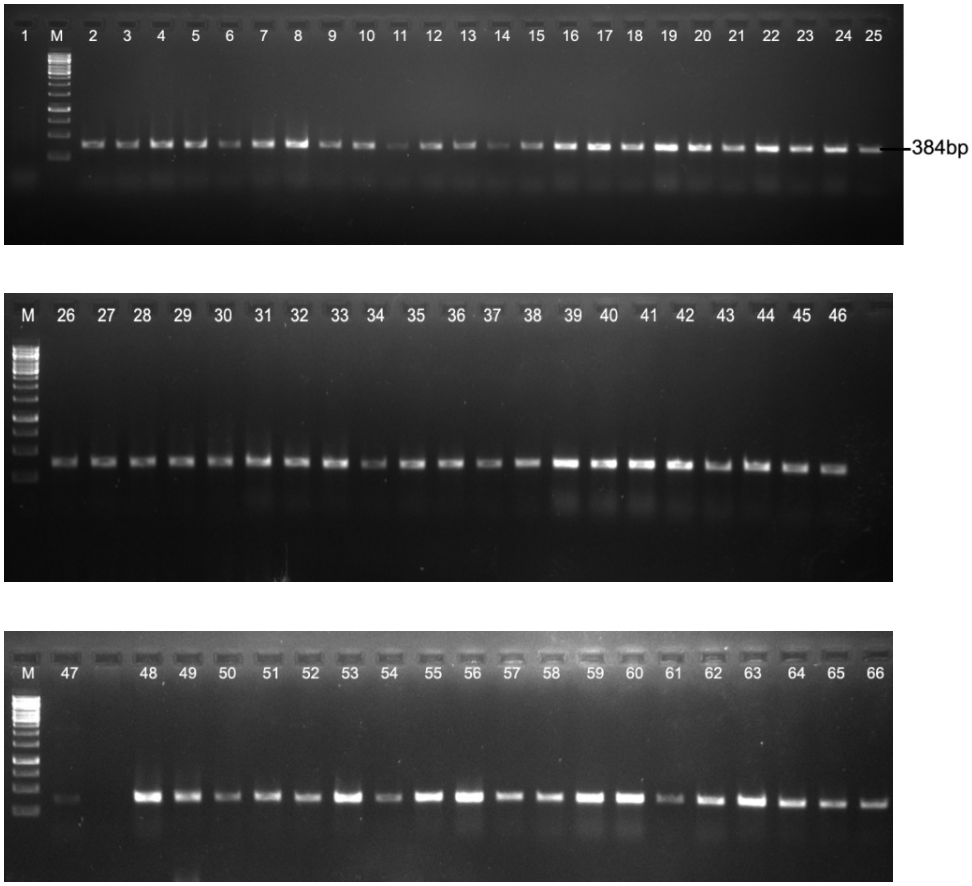


Lane M; 1kb DNA marker, lane 1; Negative control, lane 2; *Salmonella* Typhimurium (ATCC 23564, Positive control), lanes 3-22; *Salmonella* Typhimurium from seafood, lanes 23-37; *Salmonella* Weltevreden, lanes 38 and 39; *Salmonella* Oslo, lanes 40-52; *Salmonella* Typhimurium from poultry and lanes 53-66; *Salmonella* Enteritidis

4.9. PCR screening of *spaM*

A 384bp sized amplicon was obtained from all the sixty four strains indicating the presence of *spam*. The results are given in fig 4.10.

Fig.4.10. Agarose gel picture of PCR screen for *spam*

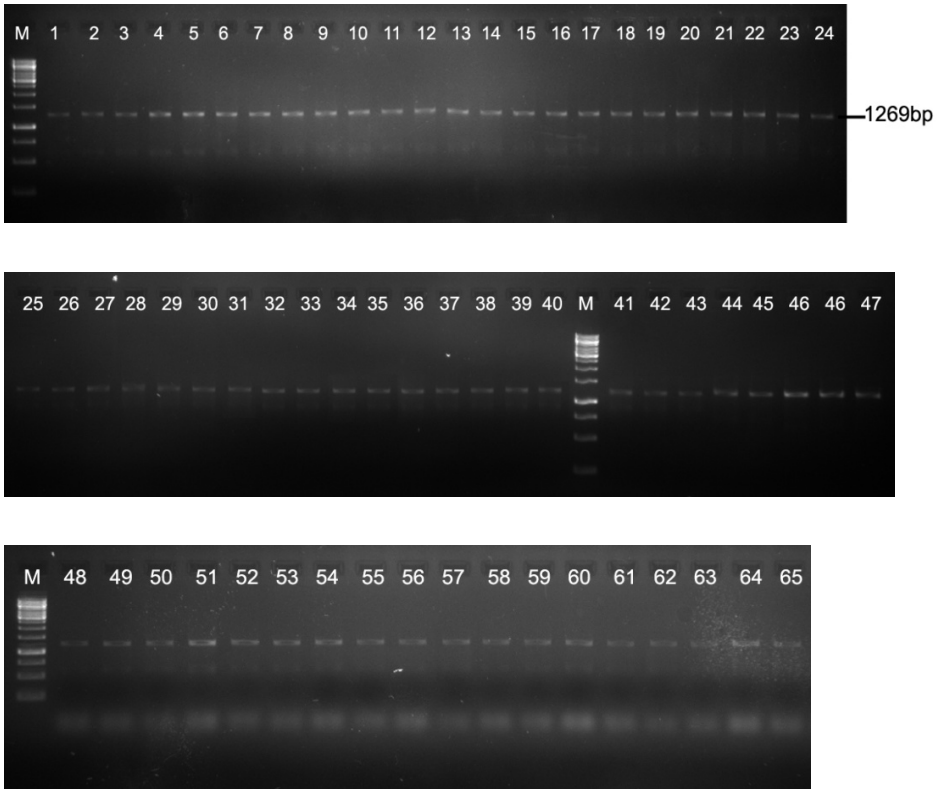


Lane M; 1kb DNA marker, lane 1; Negative control, lane 2; *Salmonella* Typhimurium (ATCC 23564, Positive control), lanes 3-22; *Salmonella* Typhimurium from seafood, lanes 23-37; *Salmonella* Weltevreden, lanes 38 and 39; *Salmonella* Oslo, lanes 40-52; *Salmonella* Typhimurium from poultry and lanes 53-66; *Salmonella* Enteritidis

4.10. PCR screening of *spi4R*

PCR screening with specific primers produced an amplicon of size 1269bp from all the 64 strains (Fig.4.11).

Fig.4.11. Agarose gel picture of PCR reaction for *spi4R*

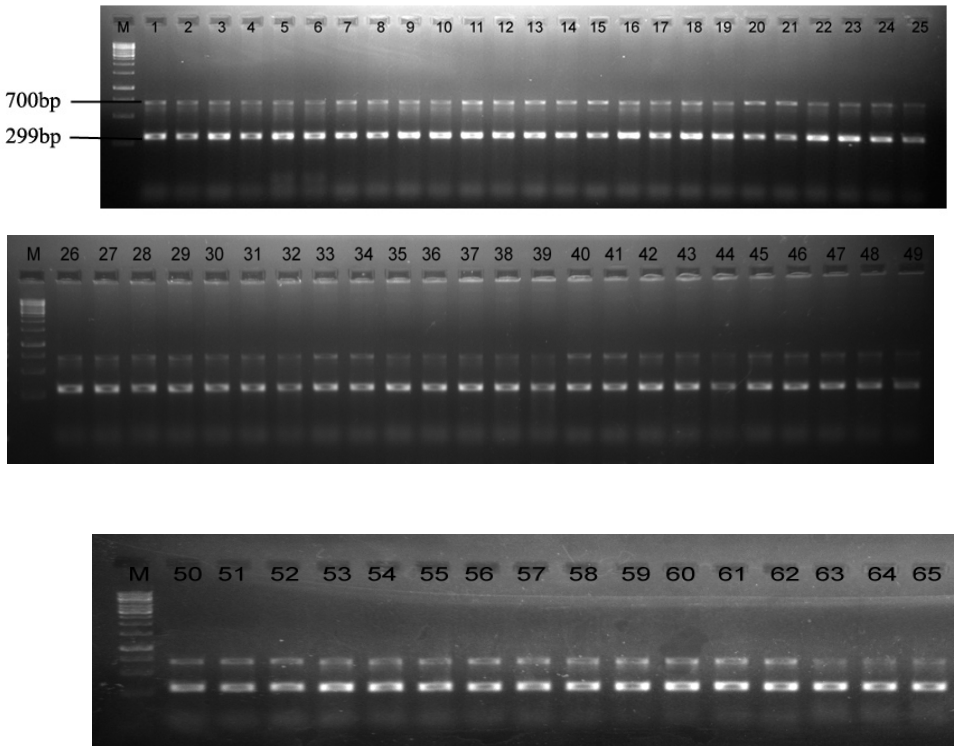


Lane M, 1kb DNA marker; lane 1, *Salmonella* Typhimurium (ATCC 23564, Positive control); lanes 2-21, *Salmonella* Typhimurium from seafood; lanes 22-36 *Salmonella* Weltevreden; lanes 37 and 38 *Salmonella* Oslo; lanes 39-51 *Salmonella* Typhimurium from poultry and lanes 52-65 *Salmonella* Enteritidis

4.11. Multiplex PCR screening of *phoP/Q* and *slyA*

All strains produced two bands of 920bp and 457bp indicating the presence of *pho*P/Q and *sly*A genes respectively (Fig 4.12).

Fig.4.12. Agarose gel of multiplex PCR for *pho*P/Q and *sly*A

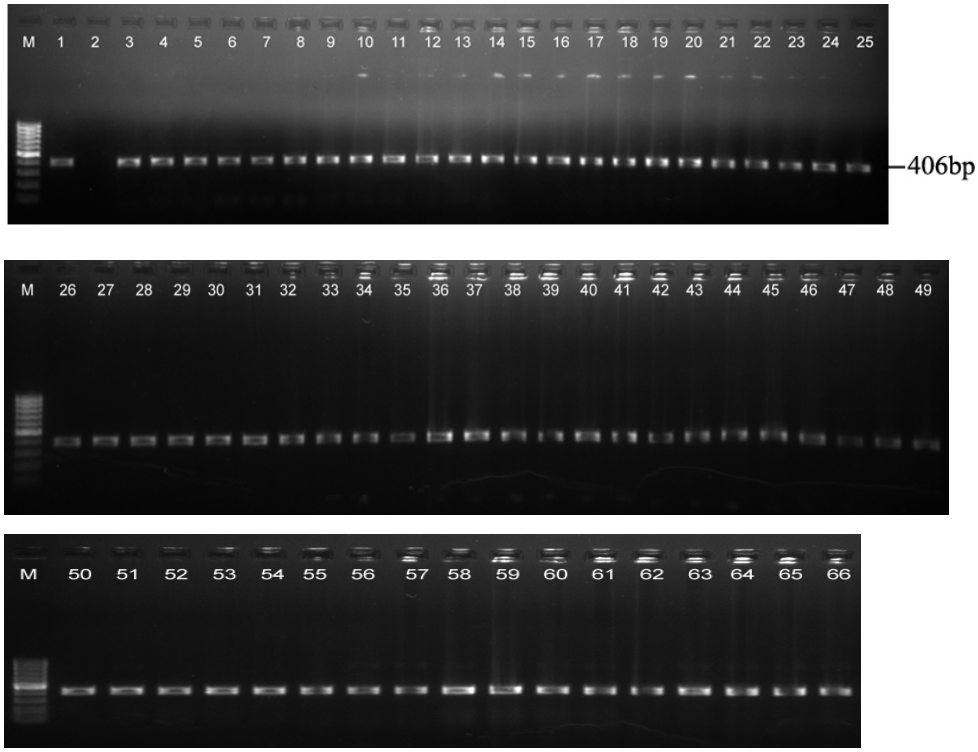


Lane M; 1kb DNA marker, lane 1; *Salmonella* Typhimurium (ATCC 23564, Positive control), lanes 2-21; *Salmonella* Typhimurium from seafood, lanes 22-36; *Salmonella* Weltevreden, lanes 37and 38; *Salmonella* Oslo, lanes 39-51; *Salmonella* Typhimurium from poultry and lanes 52-65; *Salmonella* Enteritidis

4.12. PCR screening of *pipA* gene

PCR screening showed that all *Salmonella* strains were harbouring *pipA* gene. They all produced a characteristic amplicon of size 406bp (Fig 4.13).

Fig.4.13. Agarose gel of PCR reaction for *pipA* gene

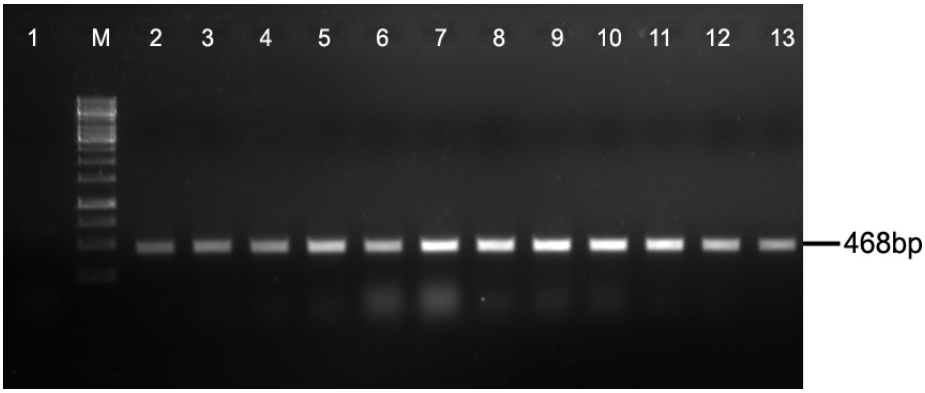


Lane M; 1kb DNA marker, lane 1; *Salmonella* Typhimurium (ATCC 23564, Positive control), lane 2; Negative control, lanes 3-22; *Salmonella* Typhimurium from seafood, lanes 23-37; *Salmonella* Weltevreden, lanes 38 and 39; *Salmonella* Oslo, lanes 40-52; *Salmonella* Typhimurium from poultry and lanes 53-66; *Salmonella* Enteritidis

4.13. PCR screening of *sodC1* gene

Eleven strains showed the presence of *sodC1* gene (Fig.4.14). They include five *Salmonella* Typhimurium, four *Salmonella* Weltevreden and one *Salmonella* Oslo from seafood; one *Salmonella* Typhimurium and one *Salmonella* Enteritidis from poultry.

Fig.4.14. Agarose gel of amplification product of *sodC1* gene

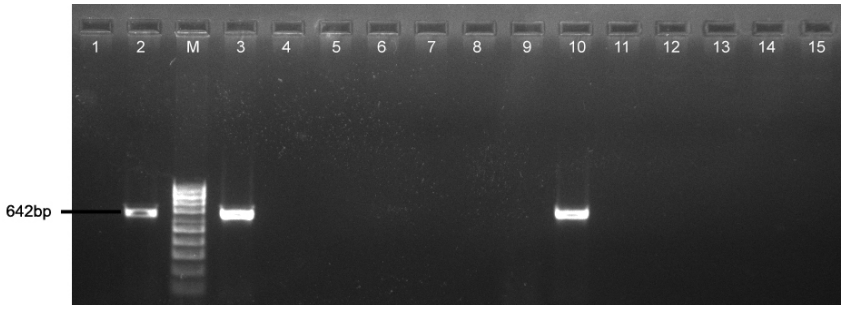


Lane 1, Negative control; lane M, 1kb DNA marker; lane 2-13, TSI-52, TSI-53, TSJ-58, TSJ-59, TSJ-60, WSI-51, WSI-54, WSI-55, WSJ-61, OSI-50, ST-128 and SE-52 respectively.

4.14. PCR screening of *sopE* gene.

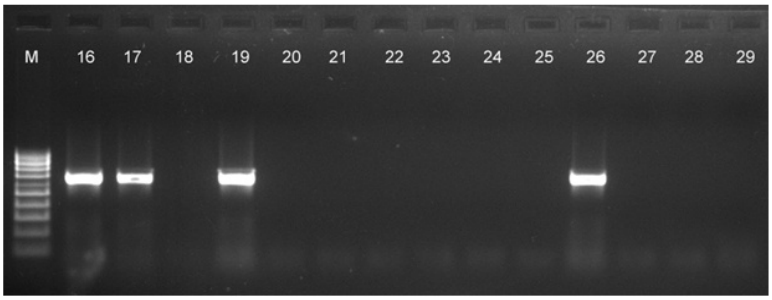
All *Salmonella* Typhimurium, *Salmonella* Weltevreden and *Salmonella* Oslo strains isolated from seafood were negative for *sopE* gene. Two *Salmonella* Typhimurium and four *Salmonella* Enteritidis strains isolated from poultry showed the presence of this gene (Fig. 4.15. and 4.16).

Fig.4.15. Agarose gel of *sopE* gene of *Salmonella* Typhimurium from poultry.



Lane 1; negative control, lane2; *Salmonella* Abony (NCIM 2257), lane M; 100bp DNA marker, lanes 3 and 10; ST-2 and ST-49 respectively.

Fig.4.16. Agarose gel of *sopE* gene of *S*.Enteritidis from poultry.



Lane M; 100bp DNA marker, lanes 16, 17, 19 and 26; SE-24, SE-26, SE-33 and SE-57 respectively.

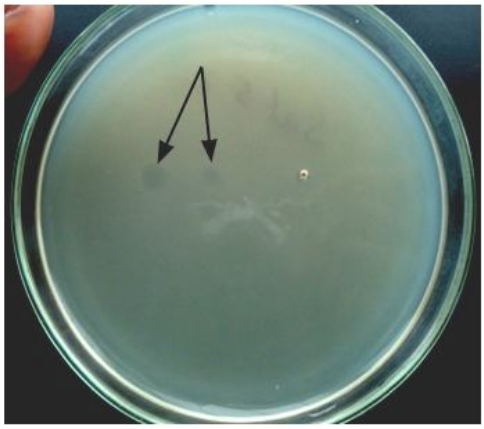
4.15. Phage Induction

Fifteen of the *Salmonella* strains screened showed the presence of lysogenic phages. The cultures containing phages were detected on the basis of characteristic plaques produced on double agar overlay plates (Fig.4.17). The

strains harbouring phage were TQE-14, TSH-16, TSJ-58, TSK-75, OSK-73, ST-32, ST-41, ST-42, ST-44, ST-48, ST-77, ST-128, SE-46, SE-52, and SE-118.

Fig.4.17. Plate showing lysogenic plaque produced by SE-52

Untreated

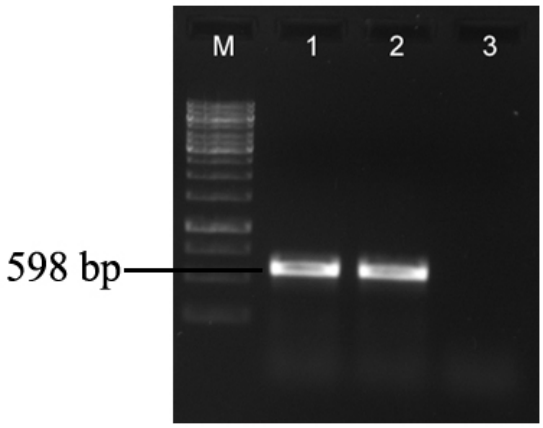


Tetrazolium treated

4.16. PCR screening of Gifsy one gene (*gogB*)

PCR produced a characteristic amplicon of 598bp. Only one *Salmonella* Enteritidis strain (SE-52) was positive for this gene (Fig 4.18), the putative virulence gene carried by Gifsy-1, a *Salmonella* lambdoid prophage.

Fig.4.18. Agarose gel of PCR reaction of *gogB*

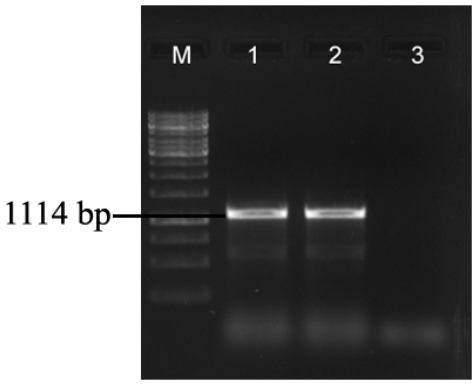


Lane M; 1kb DNA marker, lane 1; Positive control- *Salmonella* Typhimurium, Lane 2; SE-52, lane 3; Negative control

4.17. PCR screening of Gifsy two gene (*gtgE*)

Only one *Salmonella* Enteritidis strain (SE-52) gave characteristic amplification of 1114 bp in the PCR screening (Fig.4.19).

Fig.4.19. Agarose gel of PCR amplification for *gtgE*



Lane M; 1kb DNA marker, lane 1; Positive control- *Salmonella* Typhimurium, Lane 2; SE-52, Lane 3; Negative control

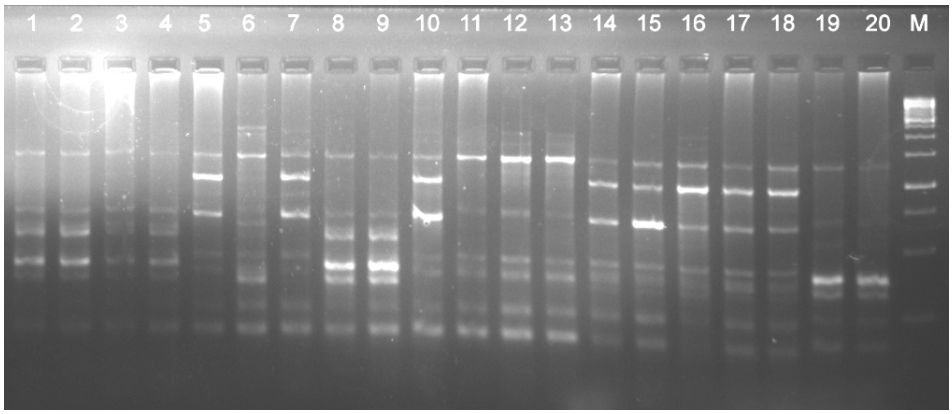
4.18. Molecular Typing

Molecular typing was done using two PCR-based methods. ERIC-PCR and PCR-Ribotyping were used to evaluate the relatedness of the different *Salmonella* strains.

4.18.1. ERIC PCR

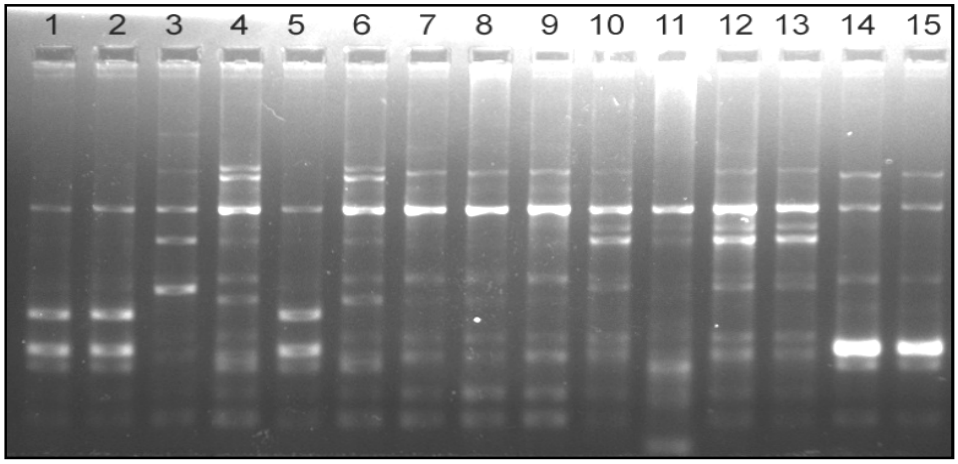
ERIC-PCR produced characteristic banding profiles for each strain. The banding pattern showed variations at the intra-serotype level. Fig 4.20 and Fig 4.21 respectively shows the ERIC profile of *Salmonella* Typhimurium and *Salmonella* Weltevreden obtained by PCR.

Fig.4.20. ERIC-PCR profile of *Salmonella* Typhimurium isolated from seafood.



Lane M;1Kb DNA marker, lanes 1-20; TAB-4,TAC-7,TSD-9,TSD-11,TQE-14,TQE-15,TSH-16,TSD-19,TAC-22,TSH-25,TSF-32,TSF-33,TSG-38,TSI-52,TSI-53,TSJ-58,TSJ-59,TSJ-60,TSK-72 and TSK-75 respectively.

Fig 4.21. ERIC-PCR profile of *Salmonella* Weltevreden strains isolated from seafood



Lanes 1-15; WAB-5, WAB-6, WSH-17, WSD-18, WSD-28, WSH-29, WSF-31, WSG-37, WSG-39, WSI-51, WSI-54, WSI-55, WSJ-61, WSK-71 and WSK-74 respectively.

4.18.1.1. Dendrogram and Discriminative index of ERIC-PCR

ERIC PCR produced six different profiles for *Salmonella* Typhimurium strains (Fig.4.22). Types I, II, III, IV, V and VI includes 6, 2, 3, 5, 1 and 3 isolates respectively. *Salmonella* Weltevreden strains were clustered into 8 types (Fig.4.23). Types I, II, III, IV, V, VI, VII and VIII includes 3,2,1,2,1,3,2, and 1 strain respectively. Discriminative index was calculated based on Simpson's index of diversity and it was found to be 0.832 and 0.916 respectively for *Salmonella* Typhimurium and *Salmonella* Weltevreden.

Fig.4.22. ERIC dendrogram showing dissimilarity among *Salmonella* Typhimurium strains isolated from seafood.

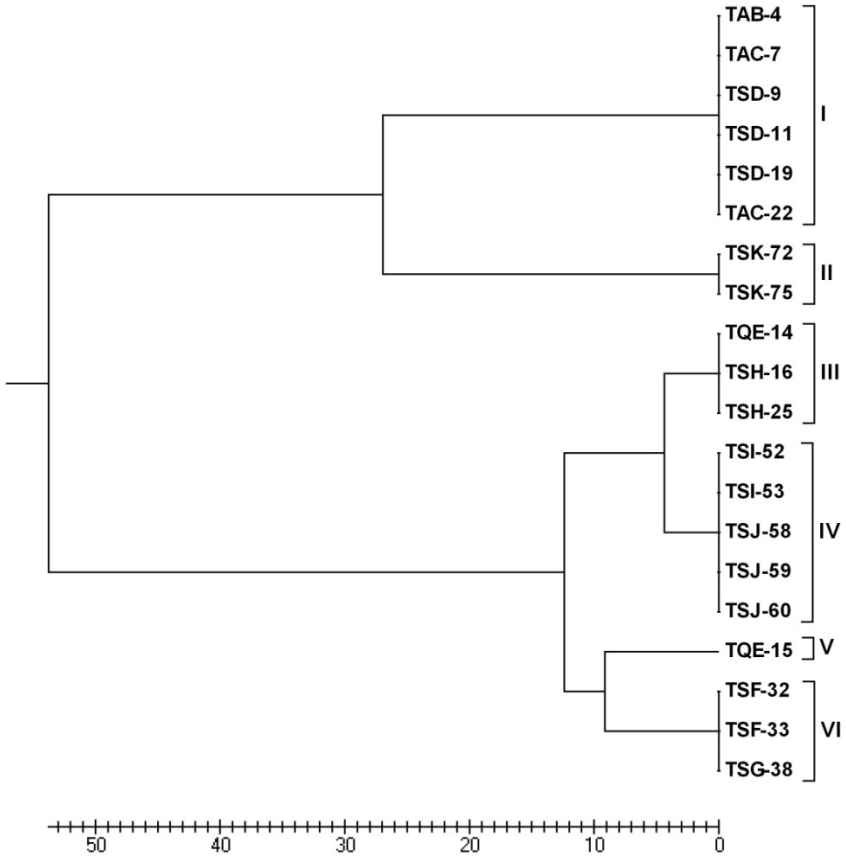
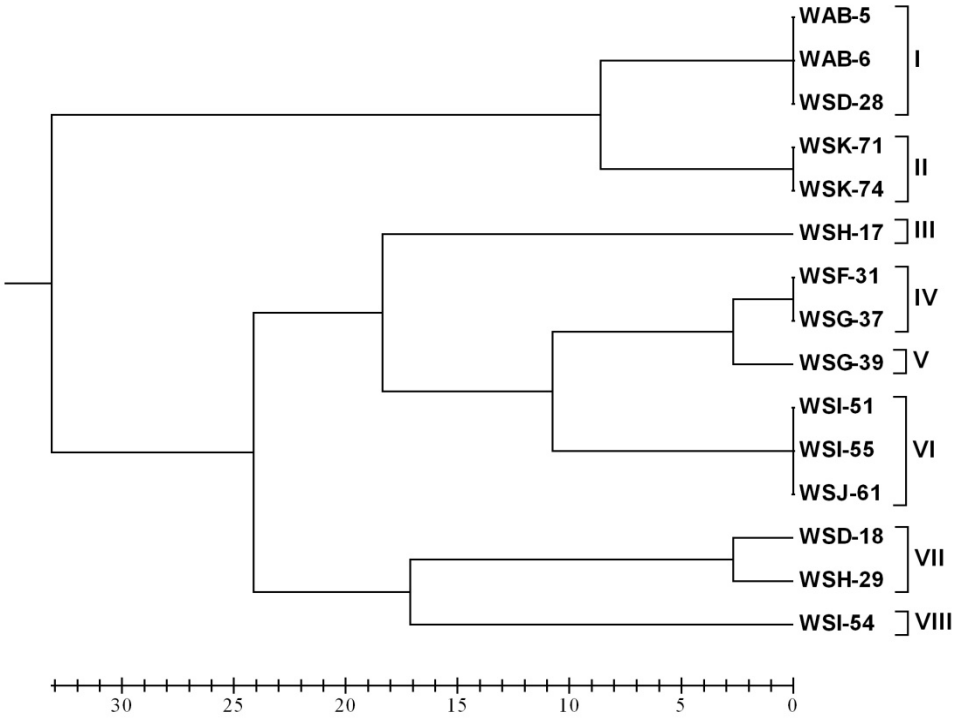


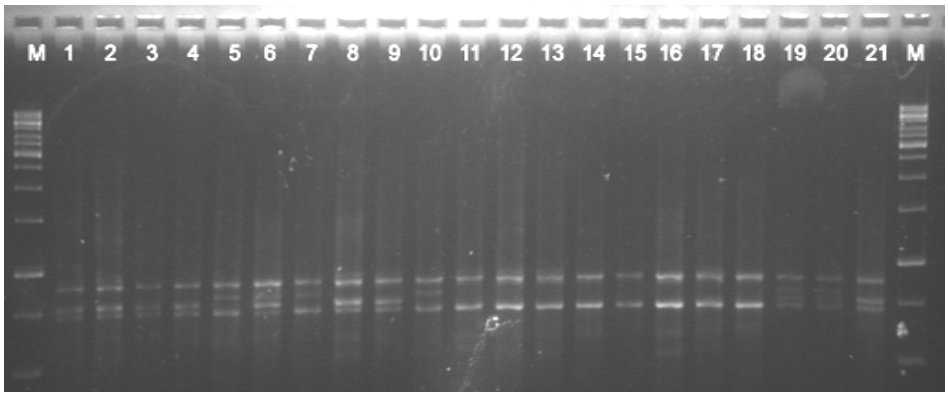
Fig.4.23. ERIC dendrogram showing dissimilarity among *Salmonella* Weltevreden strains isolated from seafood.



4.18.2. PCR-Ribotyping

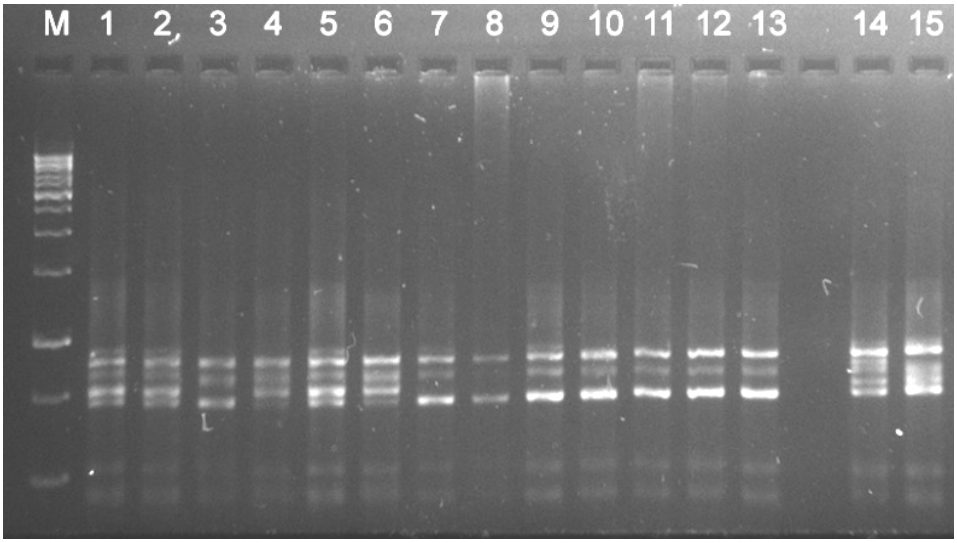
PCR-Ribotyping profile of *Salmonella* Typhimurium and *Salmonella* Weltevreden are shown as Fig 4.24 and Fig 4.25.

Fig 4.24. PCR-ribotype profile of *Salmonella* Typhimurium isolated from seafood



Lane M;1Kb DNA Marker, lanes 1-21; TAB-4,TAC-7,TSD-9,TSD-11, TQE-14,TQE-15,TSH-16,TSD-19,TAC-22,TSH-25,TSF-32,TSF-33,TSG-38, TSI-52,TSI-53,TSJ-58,TSJ-59,TSJ-60,TSK-72 TSK-75 and *Salmonella* Typhimurium (ATCC 23564) respectively.

Fig 4.25. PCR-ribotype profile of *Salmonella* Weltevreden isolated from seafood



Lane M; 1kb DNA marker, lanes 1-15; WAB-5, WAB-6, WSH-17, WSD-18, WSD-28, WSH-29, WSF-31, WSG-37, WSG-39, WSI-51, WSI-54, WSI-55, WSJ-61, WSK-71 and WSK-74 respectively.

4.18.2.1. Dendrogram and discriminative index of PCR-Ribotyping

Twenty *Salmonella* Typhimurium strains clustered into 5 types (I-V) which included 6, 3, 3, 7 and 1 strains respectively (Fig 4.26). Nine types (I-IX) were identified among the 15 *Salmonella* Weltevreden strains (Fig 4.27). They included 3, 1, 1, 2, 4, 1, 1, 1 and 1 strains respectively. The discriminative indices of *Salmonella* Typhimurium and *Salmonella* Weltevreden were 0.914 and 0.905 respectively.

Fig.4.26.PCR-ribotyping dendrogram showing dissimilarity among *Salmonella* Typhimurium strains isolated from seafood.

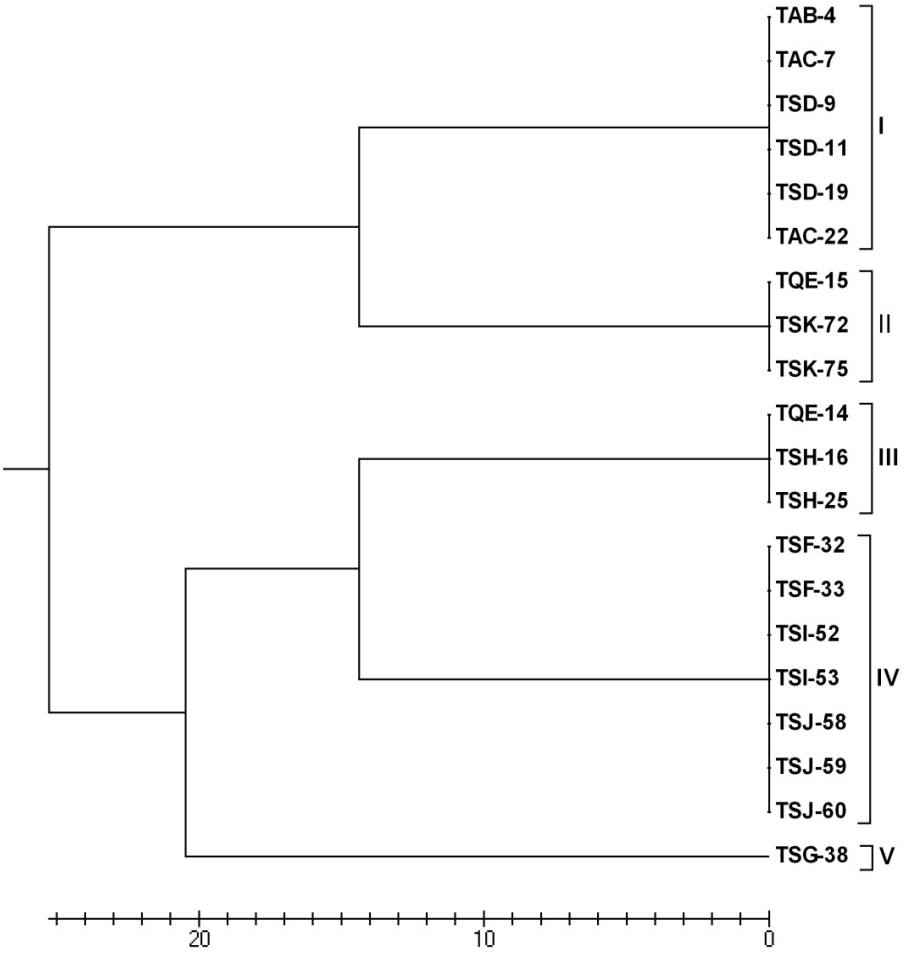
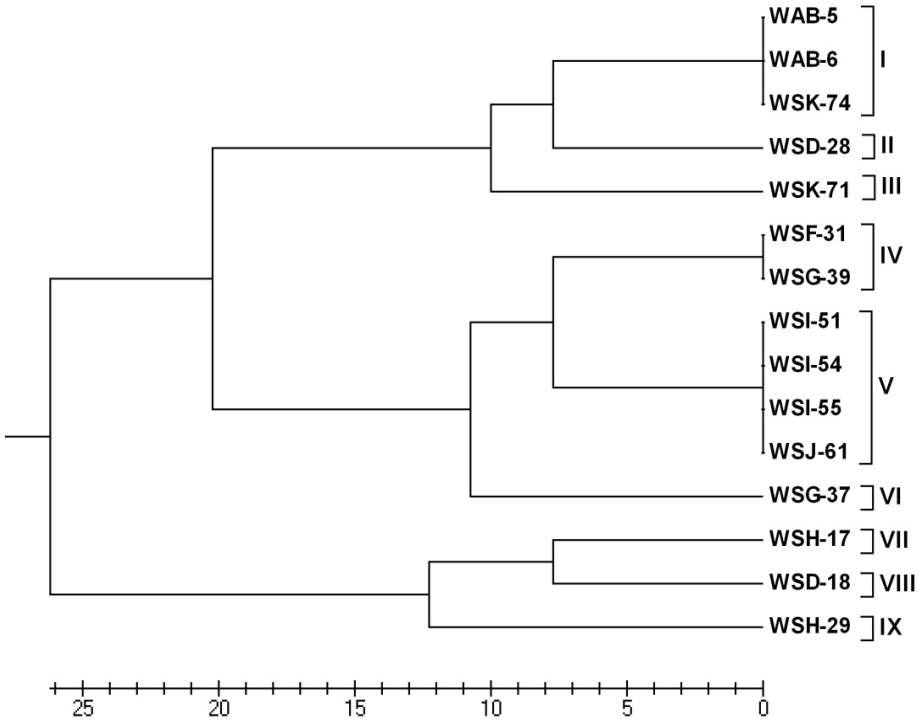


Fig 4.27. PCR-ribotyping dendrogram showing dissimilarity among *Salmonella* Weltevreden strains isolated from seafood.



4.19. Loop mediated isothermal amplification (LAMP)

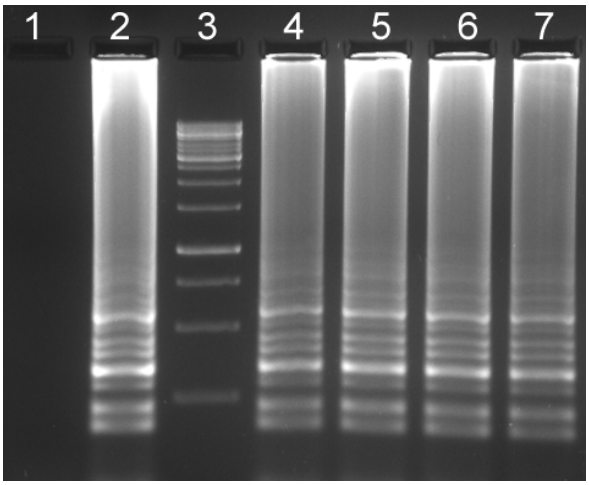
LAMP assay was done for detection of *Salmonella* from experimentally contaminated water and seafood

4.19.1. LAMP assay for detection of different *Salmonella* serotypes in experimentally contaminated water.

LAMP and PCR assay could not detect presence of *Salmonella* immediately after inoculation at zero hour. After 4h of enrichment, positive signals for *Salmonella* were obtained from samples inoculated with 10^5,

$10^4$and 10^3 CFUs. Samples with 200 and 20CFUs gave amplifications only after 6h incubation. LAMP assay after 7h of enrichment produced characteristic ladder like pattern with all 4 standard *Salmonella* serotypes even with initial inoculum of 2 CFUs (Fig 4.28). Water samples inoculated with 2 CFUs of *Salmonella* Typhimurium and 10^4 CFUs of *E.coli* also gave amplifications after 7h of enrichment. The negative control with *E.coli* cells alone did not give any amplification.

Fig.4.28. Agarose gel of LAMP assay with 2 CFU initial inoculum after 7 h enrichment for water samples



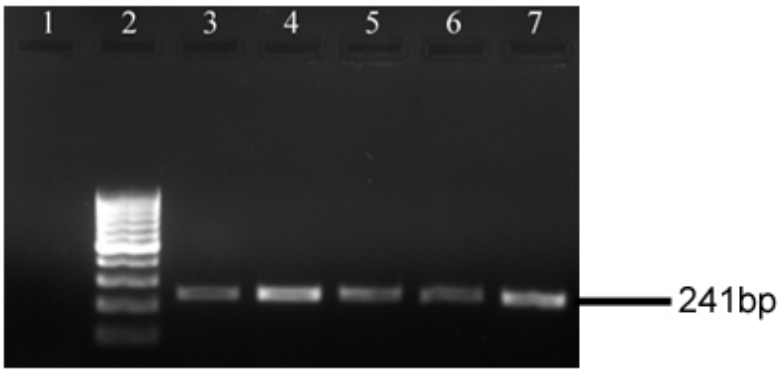
Lane 1, *E.coli* negative control; lane 2, 2 CFUs of *Salmonella* Typhimurium co- inoculated with 10^4 *E coli* CFUs; lane 3, 1 kb DNA marker; lanes 4-7, 2 CFUs initial inoculums of *Salmonella* Abony, *Salmonella* Typhimurium, *Salmonella* Typhi, and *Salmonella* Paratyphi respectively.

Reference. (Varghese *et al.*, 2012)

4.19.2. PCR assay for detection of different *Salmonella* serotypes in experimentally contaminated water.

PCR assay for detection of different *Salmonella* serotypes in experimentally contaminated water, showed positive amplifications after 4h enrichment, for samples inoculated with 10^5 and 10^4 CFUs inoculums. Six hours of enrichment was required to obtain amplification from samples inoculated with 10^3 and 10^2 CFUs. Samples inoculated with 20 and 2 CFUs (Fig 4.29) gave positive signals only after 8h of enrichment. Water samples inoculated only with *E.coli* cells as control did not give any amplification.

Fig.4.29. Results of the PCR assay for water samples with 2 CFU initial inoculum after 8 h of enrichment



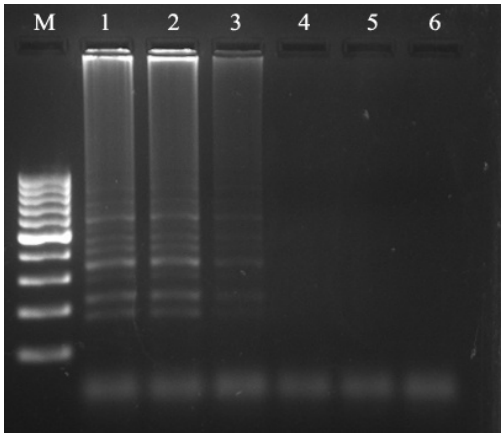
Lane 1; *E.coli* negative control, Lane 2; 100-bp DNA marker, lane 3; 2 CFUs of *Salmonella* Typhimurium co-inoculated with 10^4 *E. coli* CFUs, lanes 4-7; 2 CFUs initial inoculums of *Salmonella* Abony, *Salmonella* Typhimurium, *Salmonella* Typhi, and *Salmonella* Paratyphi respectively.

Reference. (Varghese *et al.*, 2012)

4.19.3. LAMP assay for detection of *Salmonella* in seafood

Five seafood samples were tested for the presence of *Salmonella* using LAMP assay after 5h of enrichment, and could detect *Salmonella* from 2 samples (Fig 4.30). The remaining three samples did not show any positive signal for *Salmonella* even after continued enrichment.

Fig.4.30. Agarose gel electrophoresis of LAMP assay of seafood samples enriched for 5h.

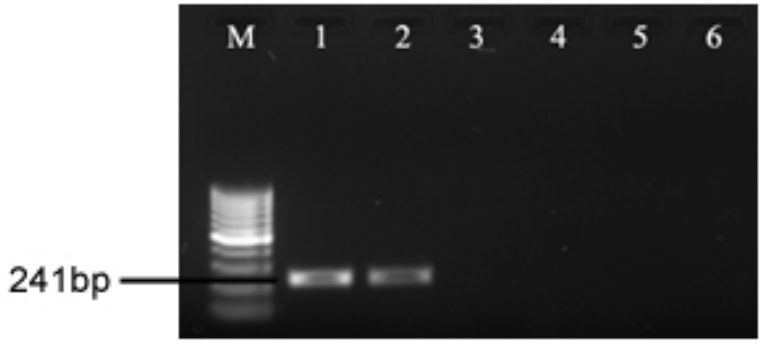


Lane 1; 1kb DNA marker, lane 1; *S*.Typhimurium (ATCC 23564), lanes 2-6; Sardine samples

4.19.4. PCR assay for detection of *Salmonella* in seafood

Out of the 5 five Sardine samples tested by PCR assay, only one gave positive result after 7h of enrichment (Fig.4.31). Further enrichment could not detect *Salmonella* from the remaining samples.

Fig.4.31. Agarose gel electrophoresis of PCR assay after 7h enrichment



Lane M; 1kb DNA marker, lane 1; *S*.Typhimurium (ATCC 23564), lanes 2-6; Sardine samples

DISCUSSION

5.1. Prevalence of *Salmonella* in fish

Salmonella is one among the major bacterial contaminants of seafood (Hatha and Lakshmanaperumalsamy, 1997; Shabarinath *et al.*, 2007). It is a major health concern as these asymptomatic carriers act as reservoir for a wide range of *Salmonella* serotypes. *Salmonella* contaminates the aquatic population by entering the ecosystem from the gastrointestinal tract of various animals which is their natural habitat.

Seafood samples were collected from several stations in Ernakulam and screened for the presence of *Salmonella*. The results show that Sardine shows a high prevalence of *Salmonella*. Twenty nine percent of the Sardine samples were contaminated with *Salmonella*. Next to Sardine, Anchovies also showed a high incidence of *Salmonella.* Two out of the eight samples were contaminated (25%). Only one out of five Squid samples was contaminated with *Salmonella* (20%), specifically *Salmonella* Typhimurium.

This study also indicated the high potential of Sardine and Anchovies to carry multiple serotypes of *Salmonella*. All the positive samples of Sardine were contaminated with multiple serotypes of *Salmonella.* Two samples harboured *Salmonella* Typhimurium, *Salmonella* Weltevreden and *Salmonella* Oslo, while six samples were contaminated with *Salmonella* Typhimurium and *Salmonella* Weltevreden. One of the two Anchovy samples harboured both *Salmonella* Typhimurium and *Salmonella* Weltevreden while the other one was contaminated with *Salmonella* Typhimurium alone. It is noteworthy that all the Mackerel samples were free from *Salmonella*.

Previous studies have shown that *Salmonella* Typhimurium and *Salmonella* Weltevreden are among the most frequently isolated serotypes from seafood and marine environments (Hatha and Lakshmanaperumalsamy, 1997; Baudart *et al.*, 2000; Catalao Dionisio *et al.*, 2000; Shabarinath *et al.*, 2007). This study also indicates a high prevalence of these two serotypes. Out of the 37 strains isolated *Salmonella* Typhimurium and *Salmonella* Weltevreden corresponds to 54.1% and 40.54% respectively. All the contaminated samples except one Anchovy and the only Squid samples harboured both these serotypes.

Salmonella Weltevreden has gained importance as the most important cause of non-typhoidal salmonellosis in South East Asia and Western Pacific (World Health Organisation, 2005). In the US too, *Salmonella* Weltevreden is the most common serotype isolated from seafood (Heinitz *et al.*, 2000; Zhaoa *et al.*, 2003). It is also one among the most common serotypes associated with non-typhoidal human infections in Thailand (Bangtrakulnonth *et al.*, 2004; Padungtod and Kaneene, 2006).

During the 'chaakara' phenomenon, along with other fishes Sardine is available in huge quantities. Due to its high nutritional value and low price it among the most preferred fish for people in Kerala. As fish is asymptomatic carrier of *Salmonella*, the people are unable to detect these hidden pathogens. Undercooked contaminated fish can cause salmonellosis in humans. In addition, human handling of these contaminated fishes can also result in cross contamination. *Salmonella* in food can also be attributed to unhygienic practices, becoming cause for serious infections.

5.2. Antibiotic resistance profiling

Antibiotic susceptibility test of the 37 Salmonellae showed that 10 (27.02%) were resistant to two or more antibiotics, while the remaining were sensitive to all the antibiotics tested. Two different profiles of resistance were observed among the strains. One pattern showed resistance to sulfafurazole and trimethoprim and the other showed resistance to ampicillin, carbenicillin, doxycycline, kanamicin, sulphafurazole, trimethoprim and tetracycline. Similar pattern of antibiotic resistance indicates origin from a common source. *Salmonella* strains isolated from seafood resistant to nalidixic acid, chloramphenicol, tetracycline, co-trimoxazole, gentamicin and beta-lactam antibiotics have been previously reported from India (Ruiz *et al.*, 1999)

All of the multi resistant strains were resistant to sulfafurazole and trimethoprim. Trimethoprim and sulfonamides are synthetic antimicrobials. Since 1968 these two antibiotics are being used in combination (Bushby and Hitchings, 1968). Combination therapy has increased the life span of these antibiotics but the development of co-resistance pose a serious concern for the future treatment of salmonellosis. Their low cost and wide antibacterial spectrum made them popular all over the world. Sulfonamide and trimethoprim resistance is common in *Salmonella* Typhimurium strains isolated from various animals (Wray *et al.*, 1991).

Six of the multidrug resistant strains (TSF-32, TSF-33, TSG-38, WSF-31, WSG-37 AND WSG-39) showed resistance to an array of 8 antibiotics classified under five classes: penicillins, quinolone, tetracyclines, aminoglycoside and folate pathway inhibitor. Three of them were *Salmonella* Typhimurium and the remaining three were *Salmonella* Weltevreden. The antibiotics to which they were resistant include the first-line ones given for salmonellosis. All six MAR strains were isolated from two different Sardine

samples at different times. The result showed that different strains of *Salmonella* serotypes isolated from different source share a common antibiotic resistance profile and such multidrug resistant pathogen harbouring fish are wide spread.

Of particular concern is the resistance to nalidixic acid. Quinolones are the first-line drug of choice for the treatment of invasive salmonollosis in humans. Quinolone resistance in *Salmonella* is mainly chromosome mediated and hence the increase in quinolone-resistant *Salmonella* is attributed to (i) selection of a quinolone-resistant bacterium after exposure to the antibiotic in humans or animals and (ii) the spread of the resistant bacteria to other animals or humans (from animals or human-to-human transfer). Quinolone resistance can often result in decreased susceptibility to fluoroquinolones (Threlfall *et al.*, 1997). Nalidixic acid resistance has been used as an indicator for reduced flouroquinolone resistance (Wain *et al.*, 1997; Threlfall *et al.*, 2000). Transfer of nalidixic acid resistant *Salmonella* to animals or humans and the repeated fluoroquinolone exposure in animals or humans may result in increased resistance due to acquisition of further mutations in *gyrA* or other loci (Heisig, 1993; Everett *et al.*, 1996). This can result in high floroquinolone resistance which is not amenable to fluroquinolone therapy. Hence the nalidixic acid resistant strains isolated in this study pose a potential risk to public health. Nalidixic acid resistance has been previously reported from *Salmonella* isolates from seafood (Khan *et al.*, 2006). The presence of multidrug resistant *Salmonella* in seafood is a potential risk to the efficacy of antibiotics.

The wide spread dissemination of antibiotic resistance among bacteria can be ascribed to some extent, to the presence of integrons. The results of the present study are in conformity with these findings. All the ten multidrug resistant strains in the present study harboured a class 1 integron. Previous

studies (Ruiz *et al.,* 1999; Khan *et al.,* 2006) have reported the presence of class 1 integrons from seafood-borne *Salmonella* strains isolated from India. Resistance to sulfonamides is a characteristic feature of strains harboring class 1 integrons. Most of the class 1 integrons studied so far contains at least a part of a region called the 3' conserved region (Stokes and Hall, 1989; Hall *et al.*, 1994). With a few exceptions (Recchia and Hall, 1995) the 3' conserved region of integrons have a *sul1* gene which gives resistance to sulphonamides (Stokes and Hall, 1989). All the integron bearing strains in the present study were resistant to sulfafurazole.

There was no diversity among the class 1 integron gene cassettes detected in this study. Sequence analysis of the ~1.3kb amplicon obtained by PCR from all these cultures using the InF/InR primer revealed the presence of *dhfrA1* gene cassette, which gives resistance to trimethoprim, and an open reading frame *orfC* gene cassette with unknown function. These gene cassettes were 100% similar with the *dhfrA1and orfC* cassettes reported previously from *Escherichia coli* (Accession No. AB161449), and *Salmonella* spp. (Accession No. AB186122). The strains harbouring integrons comprised two serotypes, both isolated from two different samples at different times. Moreover, they also showed two distinct resistance profiles. Nonetheless, all harboured an identical integron. There can be horizontal transfer of integrons along with the integrated antibiotic resistance gene cassette as these integrons can be part of mobile elements (Heikkila *et al.,* 1993; Correia *et al.,* 2003) or these strains might have been surviving in a region which gave a similar antibiotic stress.

Class 2 integron was absent in all the strains of *Salmonella* tested in this study , especially as it is not found as frequently as class 1 integrons.

In *Salmonella enterica*, *Salmonella* Genomic Island 1(SGI1) is a significant determinant for multiple antibiotic resistance. Notably, all the six

strains which were resistant to more than two antibiotics showed the presence of SGI1. In addition, they all harboured a class one integron. Three of the above were *Salmonella* Typhimurium and the remaining was *Salmonella* Weltevreden. SGI1 has been reported from several *Salmonella* serotypes likes *S.* Paratyphi, *S.* Albany, *S.* Agona and *S.* Meleagridis (Meunier *et al.*, 2002; Doublet *et al.*, 2003; Doublet *et al.*, 2004; Ebner *et al.*, 2004). This is the first report of SGI1 from *S.*Weltevreden. DT104, a dominant lineage of *Salmonella* Typhimurium, is well known for the presence of SGI1. The most common type of resistance pattern showed by SGI1 is ACSSuT (A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfonamides; and T, tetracycline). The multidrug resistant strains in the present study showed a different resistance profile with ampicillin, carbenicillin, doxycycline, kanamycin, nalidixic acid, sulphafurazole, trimethoprim and tetracycline resistance. The resistance profile showed deviation from the common SGI1 resistance profile by the absence of chloramphenicol and streptomycin. These strains show carbenicillin, doxycycline kanamycin, nalidixic acid and trimethoprim resistance in addition but their presence inside the genomic island was not confirmed. SGI1 and its variants with different antibiotic resistance profiles were reported from several serotypes of *Salmonella enterica* by Levings *et al.* (2005). They have reported two new variants, SGI1-I and SGI1-J, both harbouring a *dfrA1*-orfC cassette array. The present study also reports a similar integron cassette array.

The marked deviation of the resistance profile of the SGI1 bearing strains in the present study may be due to homologous recombination between identical segments of the genomic island. Integrons, in single dose or in double dose, are invariably present in *Salmonella* genomic island 1. Integrons are flanked by conserved sequences at the 3' and 5' regions (Stokes and Hall, 1989) which provide the identical regions necessary for homologous

recombination. Thus SGI1 along with the antibiotic resistance gene bearing integrons provide a route for integrating more and more resistance genes and their shuffling among the microbial world. The formation of diverse combinations of antibiotic resistance gene cassettes in integrons supports the crucial role performed by integrons in the adaptive bacterial evolution.

Antibiotics are frequently used in animals along with food and for therapeutic purposes. Misuse and overuse has resulted in an explosive increase in the development of antibiotic resistance in bacteria. Old genetic recombination mechanisms have been modified due to the heavy selective pressure of liberal usage of antibiotics.

Multiple antibiotic resistance (MAR) indexing is widely used to identify high risk contamination. Out of the ten multiple antibiotic resistant strains, six showed a MAR index of 0.42 and the remaining four showed 0.1. In terms of MAR index, the regions from which the above mentioned six strains (with a MAR index of 0.42) were isolated are more anthropogenically contaminated. As the source of *Salmonella* in this study is fish, it is difficult to exactly pinpoint the source of antibiotic contamination. There is another possibility that the antibiotics may be concentrated in the body of the fish, which provided the required antibiotic stress for the bacteria for the integration and reshuffling of antibiotic resistance genes. *Salmonella* serotypes resistant to five antibiotics were reported previously from seafood (Khan *et al.*, 2006). A similar study on strains isolated from poultry reported resistance to ten antibiotics (Singh *et al.*, 2010).

5.3. Nalidixic acid resistance and point mutations in Quinolone Resistance-Determining Region (QRDR)

Quinolones have been used successfully for the treatment of salmonellosis caused by multiple antibiotic resistant strains (Barnass *et al.*, 1990). Topoisomerases II (DNA gyrase) are enzymes responsible, in part, for maintaining the topology of bacterial DNA. These enzymes are the major targets of Quinolones. Quinolone-resistant strains combat the antibiotic by making point mutations in the gyrase gene (Heddle *et al.*, 2000). Quinolones act by preventing the resealing of DNA after it has been cleaved by GyrA. The 3D crystal structure of the N-terminal region of GyrA shows that most of the mutations giving rise to resistance are near the GyrA dimer interface and DNA binding site of DNA gyrase (Cabral *et al.*, 1997).

Human nontyphoidal *Salmonella* infections are frequent in several countries, and the emergence of quinolone-resistant *Salmonella* strains is a serious matter and therefore cause for concern. The present study detected 6 nalidixic resistant strains of *Salmonella*: 3 *Salmonella* Typhimurium and 3 *Salmonella* Weltevreden, all isolated from two Sardine samples. Notably, both samples were contaminated with *Salmonella* Typhimurium and *Salmonella* Weltevreden resistant to nalidixic acid supporting the possibility of similar mutational events or horizontal gene transfer. The aquatic environments may act as an ecological niche for the exchange of antibiotic resistance genes among different *Salmonella* serotypes. *Salmonella* Typhimurium enjoys a wide host range and is one among the major veterinary isolates. Piddock (1996) proposed a hypothesis of selection and spread of quinolone-resistant strains following the introduction of fluoroquinolones in veterinary therapy. Different drugs from an antibiotic class (e.g the fluoroquinolones, enrofloxacin and ciprofloxacin), are used in animals and compared with man bacteria are

unable to distinguish between two such chemically similar molecules and selection of resistance to one drug leads to cross resistance to the other. In addition, the greater understanding of the mechanisms of multiple drug resistance has shown that a single drug can select resistance to several chemically unrelated agents.

PCR assay and sequence analysis of the amplicons showed that all six nalidixic acid resistant strains have a point mutation at the 87th codon of subunit A of the *gyrA* gene. This is a transversion mutation which has converted the codon GAC to TAC, whereby the acidic amino acid aspartate has been replaced by the aromatic amino acid tyrosine. Amino acid sequence comparison from different bacteria ranging *E. coli* to *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and *Enterococcus faecalis* shows that serine-83 and aspartate-87 are hot spots for decreased susceptibility to quinolones (Griggs *et al.*, 1994; Piddock, 1999; Giraud *et al.*, 1999). A mutation involving codon Gly-133 in the *gyrA* gene was also reported for the nalidixic resistant phenotype of *Salmonella* (del Cerro *et al.*, 2003).

In this study several silent mutations were also detected in the *gyrA* amplicon: His-80, Gly-81, Asp-82, Ser-83, Ala-84, Val-85, Thr-88 and Val-90 to mention a few. Similar silent mutations have been reported previously from the *gyrA* of *Salmonella enterica* subspecies *enterica* serotype Panama (del Cerro *et al.*, 2003). This indicates the preferential codon usage in *Salmonella*.

5.4. Screening of Virulence genes

Thirteen *Salmonella* Typhimurium strains and fourteen *Salmonella* Enteritidis strains isolated from poultry were also included in the study to make a comparison of the virulence potential of different serotypes and same serotypes isolated from different source.

Genes *invA, invE, ttrC* and *mgtC* were screened by triplex PCR. A single set of primer was used for the detection of genes. It amplified a region from the junction of these genes. All the strains were found to carry the four genes irrespective of the serotype and the source from which they were isolated. The duplex PCR performed with primers for *phoP/Q* and *slyA* confirmed the presence of *phoP, phoQ* and *slyA*. Uniplex PCR with primers *pipA, spi4R* and *spaM* were positive for all the strains.

Salmonella has a very complex life cycle and requires the expression of a large number of virulence/virulence associated genes. As *Salmonella* cannot evade the attack of macrophages, they have adapted to survive in macrophages by the expression of some genes. MgtC is required for intra-macrophage survival and growth in low Mg^{2+} environment (Blanc-Potard and Groisman, 1997; Blanc-Potard *et al.*, 1999). Gene *mgtC* is regulated by the PhoP/PhoQ two-component system, a part of the phosphorylation regulon which regulates the expression of genes involved in virulence and macrophage survival of *Salmonella* species (Miller *et al.*, 1989; Soncini *et al.*, 1996). SlyA is a hemolysin (Libby *et al.*, 1994), the mutation of its gene, *slyA*, shows attenuated virulence reduced survival in macrophages and M-cell cytotoxicity. The results clearly show that all the strains in the study have the required virulence potential for the successful survival in the macrophages.

Gene product of *ttrC* is a component of the tetrathionate reductase structural protein responsible for tetrathionate respiration. This gene is genetically stable in the genus *Salmonella* (Hensel *et al.*, 1999a). PipA has putative role in systemic salmonellosis (Wood *et al.*, 1998). Gene products of *invA, invE* and *spaM* (*invI*) are required for invasion of the host cells.

The results demonstrates the house keeping nature of *invA*, *invE*, *ttrC*, *mgtC*, *phoP*, *phoQ*, *slyA*, *pipA*, *spi4R*, and *spaM* genes in strains included in the study.

Horizontal transfer of virulence genes through phages have been a major driving force in the evolution of *Salmonella* pathogenicity. Lysogenic phages with their virulence genes have contributed greatly to the bacterial genome composition. Phage induction studies using mitomycin C have shown that fifteen strains comprising different serotypes of *Salmonella* harboured lysogenic phages. They include four *Salmonella* Typhimurium and one *Salmonella* Oslo strain isolated from seafood and seven *Salmonella* Typhimurium and three *Salmonella* Enteritidis strains isolated from poultry. All the *Salmonella* Weltevreden strains in this study were devoid of phages.

Gene *sopE* was absent from all the *Salmonella* Typhimurium, *Salmonella* Weltevreden and *Salmonella* Oslo strains isolated from seafood in the present study. However, it was present in two *Salmonella* Typhimurium and four *Salmonella* Enteritidis isolated from poultry. SopE is an SPI-1 dependent translocated protein, which is a bacterial toxin modulating host cell RhoGTPase function. Even though the Type III secretion system coded by the SPI-1 is well conserved among the Gram negative bacteria, the various effector proteins secreted by them vary greatly among closely related strains (Galan and Bliska, 1996). Gene *sopE* enjoys a varied distribution among the different serotypes of *Salmonella*. This gene was originally cloned from *Salmonella* Dublin (Wood *et al.*, 1996). Later, it was found to be located on a P2-like cryptic bacteriophage (SopEΦ) in the *Salmonella* Typhimurium genome at centisome 61 (Hardt *et al.*, 1998a). However, they are not present in all strains of *Salmonella* Typhimurium. The presence of this gene suggests the probable presence of the phage in the genome or the occurrence of an event of

horizontal gene transfer through phages. The present study could not induce any phages from the strains which harboured *sopE* gene and thus supports the presence of cryptic phages in their genome. The presence of this gene also indicates previous horizontal gene transfer mediated by phages.

The majority of *Salmonella* Typhimurium isolates that were found to carry *sopE* have belonged to epidemic strains that have persisted in humans and food animals for a long period of time (Mirold *et al.*, 1999). Gene *sopE* in *Salmonella* plays an important role in modifying the genome for equipping these pathogens to make epidemics. Since 6 out of the 27 *Salmonella* strains isolated from poultry harboured *sopE,* poultry can be considered a potential source of epidemics. So it is assumed that those strains carrying *sopE* might have been persistent in poultry for a long. In poultry, transovarian route is the major way of transmission of *Salmonella*. *Salmonella* Typhimurium and *Salmonella* Weltevreden are among the important serotypes that are transmitted through the transovarian route. The eggs of the infected flocks, contaminated by transovarian infection, act as the major reservoir of *Salmonella*. Hence, these pathogens can persist in poultry for generations.

Phage-type conversion occurs when lysogenic phages are integrated into *Salmonella* genome (Mmolawa *et al.*, 2002; Tucker and Heuzenroeder, 2004). The presence of prophages in the strains of the present study indicates the ameliorated virulence potential in them. Prophages have been reported to harbour a wide variety of virulence genes (Figueroa-Bossi and Bossi, 1999; Bakshi *et al.*, 2000; Stanley *et al.*, 2000; Ho *et al.*, 2002) necessary for the establishment, survival and increased pathogenicity of *Salmonella* in their hosts. Interestingly, previous studies from our lab showed that two *Salmonella* specific lytic phage designated ΦSP-1and ΦSP-3 isolated using two of the *Salmonella* strains isolated from poultry which are used in the present study

has the potential to be used as an effective biocontrol agent (Augustine *et al.*, 2013a; Augustine *et al.*, 2013b). The ability of *Salmonella* serotypes to adapt to a wide range of animal hosts is attributed mainly to the variable distribution of effector protein genes carried by the phages (Prager *et al.*, 2000; Figueroa-Bossi *et al.*, 2001).

The presence of two lambdoid phage mediated gene, *gogB* and *gtgE,* mediated by Gifsy-1 and Gifsy-2 respectively, in one of the *Salmonella* Enteritidis strain (SE-52) isolated from poultry was shown by PCR assay. The presence of the two Gifsy phages in the same isolate was reported previously (Figueroa-Bossi *et al.*, 1997). Their presence has been reported in all epidemic isolates of *Salmonella* Typhimurium (Figueroa-Bossi *et al.*, 2001). This strain was shown to harbour prophage by phage induction studies also, but the plaques formed by it were large. Gifsy phages are known to produce very small plaques which are very difficult to detect by standard plate assay (Figueroa-Bossi and Bossi, 1999). This suggests the presence of some other inducible phage in SE-52. This strain was positive for *sodC1* also. The presence of *gtgE* and *sodC1* genes indicates the possible presence of Gifsy-2 or a related prophage. Previous studies have shown that genes g*tgE* and *sodC1* are among the major virulence determinants carried by Gifsy-2 (Ho *et al.*, 2002). Bacciu *et al*. (2004) reported the presence of Gifsy-2AO, a prophage equivalent to Gifsy-2 in *Salmonella enterica* subspecies *enterica* serotype Abortusovis.

Phages have been detected from four *Salmonella* Typhimurium and one *Salmonella* Oslo strains isolated from seafood, but none of them harboured the Gifsy-1 or Gifsy-2 phages. Induction studies have shown that TSJ-58 harboured a lysogenic phage. Gene *sodC1* was also detected in this strain by PCR. In this strain *gtgE* and *gogB* were absent, eliminating the possibility of

the presence of Gifsy-1 and Gifsy-2. However, Gifsy-related or other phages may be present in them.

PCR assay using specific primers showed that strains TSI-52, TSI-53, TSJ-58, TSJ-59, TSJ-60, WSI-51, WSI-54, WSI-55, WSJ-61, OSI-50, SE-52 and ST-128 harboured the *sodC1* gene. These strains were not positive for *gtgE,* indicating the absence of Gifsy-2 phage. Induction studies showed the absence of other inducible phages as well in all these strains except TSJ-58. The presence of *sodC1* in the prophage is proved by restriction studies (Figueroa-Bossi *et al.,* 1997) and is supported by the fact that tail-like genes are present near to it (De Groote *et al.,* 1997; Farrant *et al.,* 1997). The presence of the *sodC1* gene without the full genome of Gifsy-2 indicates the occurrence of an event of horizontal gene transfer that might have taken place previously. Representation of the *sodC1* gene is there in the three different serotypes of *Salmonella* isolated in the study. The presence of the full genome of the Gifsy phages is not essential for the increased virulence potential of *Salmonella.* Only the *sodC1* gene is enough for change in the virulence potential of the pathogen. Gene *sodC1* is usually associated with highly virulent strains causing extra intestinal infections (Fang *et al.,* 1999; Sansone *et al.,* 2002; Uzzau *et al.,* 2002). Inactivation of *sodC* gene can result in the substantial attenuation of virulence of *Salmonella* (De Groote *et al.,* 1997; Farrant *et al.,* 1997). *Salmonella* deficient in copper and zinc cofactored super oxide dismutase shows reduced survival in macrophages. This enzyme catalyses the conversion of superoxide radicals to hydrogen peroxide, a potentially useful detoxification reaction in the bacterial defence against macrophage oxidative burst.

All the virulence/virulence associated genes except the phage mediated genes are present in all the strains under the present study irrespective of the

serotype and source. Gene *sopE* enjoys a restricted association with the poultry related strains. But *sodC1* was detected more from seafood related isolates.

The presence of *Salmonella* in fish is cause for alarm. Moreover, the condition is aggravated by the presence of the phages or the phage encoded virulence genes like *sodC1* and *sopE*.

5.5. Typing

Two molecular typing methods have been used for clustering the different strains of each serotype based on genetic variations. ERIC-PCR produced 6 and 8 different clusters for *Salmonella* Typhimurium and *Salmonella* Weltevreden respectively. PCR-Ribotyping produced 5 and 9 different profiles respectively for *Salmonella* Typhimurium and *Salmonella* Weltevreden. The result shows that comparatively more diversity among the *Salmonella* Weltevreden strains could be established by PCR-Ribotyping. Conversely, ERIC-PCR established more diversity among the *Salmonella* Typhimurium strains. PCR-Ribotyping has been considered inferior to the other commonly used typing methods, when it is used for serotype differentiation. The discriminative index is less when compared with other methods (Lim *et al.,* 2005). This study shows that it can be very effectively used for strain differentiation with in serotypes.

The number of *Salmonella* Weltevreden strains included in the study was less compared to that of *Salmonella* Typhimurium, but the number of clusters produced was more. A comparatively less number of *Salmonella* Weltevreden strains producing more clusters indicated high intra-serotype diversity among them. Previous studies on genotyping of 12 *Salmonella* Weltevreden produced 6 clusters suggesting the prevalence of genetically diverse *Salmonella* Weltevreden in seafood (Shabarinath *et al.,* 2007). ERIC-

PCR included a maximum of 6 (20%) *Salmonella* Typhimurium strains in a cluster, whereas it was 7 for PCR-Ribotyping (35%). For *Salmonella* Weltevreden it was 3 (20%) with ERIC-PCR and 4 (26.7%) with PCR-Ribotyping. Even though PCR-Ribotyping could establish genetic similarity among one group of the strains the remaining were clustered into more groups with one or few members in each cluster.

The discriminative index for both the typing methods was more or less the same with the exception of ERIC-PCR for *Salmonella* Typhimurium. PCR-ribotyping clustered the *Salmonella* Typhimurium and *Salmonella* Weltevreden strains with a discrimination index of 0.914 and 0.905 respectively, whereas it was 0.832 and 0.916 respectively with ERIC-PCR. The index of ERIC-PCR is comparatively less for *Salmonella* Typhimurium indicating less diversity. The high discriminative index supports the high intra-serotype genetic diversity occurring in *Salmonella*.

Stains in cluster I and III are same for both ERIC-PCR and PCR-Ribotyping. ERIC-PCR cluster II has two strains and PCR-Ribotyping has three strains. The two strains present in cluster II of ERIC-PCR were present in PCR-Ribotyping cluster as well. PCR-Ribotyping could cluster all the strains present in cluster IV and VI, except for one strain from cluster (VI), into a single cluster (cluster IV), signifying the superiority of PCR-Ribotyping over ERIC-PCR in grouping strains of the same serotypes.

Strains isolated from the same source were clustered together with a few exceptions. The two *Salmonella* Typhimurium strains isolated from Squid (TQE-14 and TQE-15) were included in separate clusters in both ERIC-PCR and PCR-Ribotyping.

WSH-29 and WSH-17 were clustered separately by both methods. WSH-17 represented a separate cluster which included only this strain in both ERIC-PCR and PCR-Ribotyping. Strains WAB-5 and WAB-6 were included in the same cluster in both typing methods. WSK-71 and WSK-74 which were isolated from the same source were included in the same cluster in ERIC-PCR but they were included in different clusters in PCR-Ribotyping.

Salmonella Typhimurium strains TSF-32, TSF-33 and TSG-38 showed similar antibiotic resistance pattern and integron gene cassette. They were clustered together by ERIC-PCR but were clustered in two groups by PCR-Ribotyping. TSG-38 was clustered alone and TSF-32 and TSF-33 were clustered along with five other strains. Although *Salmonella* Weltevreden strains WSF-31, WSG-37, WSG-39 showed similar resistance pattern, they were not included in the same cluster. Strains WSF-31 and WSG-37 were clustered together and WSG-39 was clustered separately by ERIC-PCR. But PCR-Ribotyping clustered them into two groups with WSF-31 and WSG-39 in one cluster and WSG-37 in the other.

Salmonella Typhimurium strains are considered monophyletic (Boyd *et al.*, 1993) but PCR-Ribotyping has created different spacer region profiles in the present study. Similar results were obtained in previous studies also (Nastasi and Mammina, 1995; Lagatolla *et al.*, 1996)

The results also show that strains isolated from same samples are clustered together with some exceptions. Some clusters show only one strain showing much genetic variation from other strains of the same serotype. There is considerable polymorphism in the genomic DNA among the different strains of same serotypes of *Salmonella*. Shabarinath *et al*. (2007) reported similar variations among *Salmonella* employing RAPD and ERIC-PCR methods.

A strain included in one cluster by one typing method is included in another cluster by the other method. The resulting genotyping profiles are of considerable epidemiological importance as they can be used for strain differentiation when the traditional typing methods could not discriminate among the different strains of the same serotypes provided the appropriate typing method or a combination of different typing method is selected.

5.6. .Loop Mediated Isothermal Amplification

Water is one of the main routes of the transmission of *Salmonella*. Detection of *Salmonella* by the conventional protocol based on biochemical characters is laborious and requires about 5 days (Andrews *et al.*, 1998). There is a long interval between the sampling and final decision. In many cases, it would be beneficial if this time interval could be reduced. Many researchers have standardized protocols for rapid detection of *Salmonella* using PCR assay (Ferretti *et al.*, 2001; Riyaz-Ul-Hassan *et al.*, 2004). Its sensitivity and detection limit was also superior to the culture method (Riyaz-Ul-Hassan *et al.*, 2004; Kumar *et al.*, 2008), but requires expensive equipments.

Loop mediated isothermal amplification (Notomi *et al.*, 2000) has emerged as an alternative for PCR in detection microbial pathogens. It can be performed under isothermal conditions. It is a cost effective method which can be effectively used for the rapid and sensitive detection of *Salmonella* (Hara-Kudo *et al.*, 2005; Ohtsuka *et al.*, 2005; Ueda and Kuwabara, 2009). 10^2 *Salmonella* CFU/ml of broth were detected using LAMP assay previously (Ueda and Kuwabara, 2009). Such a high concentration of *Salmonella* cells, which will rarely be seen in water used for drinking and cooking purposes, can be achieved only after enrichment. Even though the LAMP assay was carried out after enrichment, the present study was designed to detect very low initial

inoculum of *Salmonella* in water, which is of more importance than the final load after enrichment.

Lamp assay after 7 hours of enrichment could detect two CFU initial inoculum of *Salmonella* in a 25ml water sample inoculated into 225ml broth, making a final volume of 250ml. Hence the detection limit is 2 CFU/250ml prior to enrichment. PCR assay conducted with the same sample took 8 hours of incubation for detection. There are previous reports about detection of 2 CFUs of *Salmonella* in food by PCR after 8 h of non selective enrichment (Kumar *et al.*, 2008). The present study also confirmed these conditions.

Lactose broth which is used for pre enrichment of the samples is a non selective medium hence, favours the growth of other enteric organisms also. The results showed that co inoculation of 10^4 CFUs of *E. coli* with 2 CFUs of *Salmonella* had no inhibitory effect on LAMP assay after 7 hours of enrichment.

Detection assay done with naturally contaminated fish samples also proved the superiority of LAMP assay. Out of the 5 Sardine samples tested, two were detected positive for *Salmonella* after 5 h of enrichment. PCR assay could detect *Salmonella* from only one sample and that also after 7 h of enrichment.

Positive result or amplification can be directly observed as turbidity due to accumulation of magnesium pyrophosphate (Mori *et al.*, 2001) or as colour change by addition of calcein and manganese ions (Tang *et al.*, 2011). LAMP assay is more specific than PCR as it targets six specific regions in the template DNA instead of two in the case of the latter. It is considered more sensitive than PCR in detecting various microbial pathogens (Horisaka *et al.*, 2004; Wang *et al.*, 2008; Ueda and Kuwabara, 2009; Techathuvanan *et al.*, 2010; Tang *et al.*, 2011).

The usefulness of a non selective pre enrichment prior to LAMP assay for the rapid detection of low number of *Salmonella* has been revealed by this study. This method caters to the need for a rapid, sensitive and cost effective detection assay for *Salmonella* spp. in water and fish. This assay is a potential tool for routine monitoring of low number of *Salmonella* spp. in water, which can help in the immediate implementation of control measures. *Salmonella* is a major bacterial contaminant in seafood responsible for product recalls. Rapid and sensitive detection of *Salmonella* by LAMP can save much time and money in seafood industries.

This study demonstrates that *Salmonella* are extensively prevalent in seafood, especially Sardine and Anchovies. This is also one of the first studies that points to the higher prevalence of *Salmonella* in Sardine samples. The observation of a high MAR index in some of the isolated strains indicates the high antibiotic stress to which these microbes were exposed. Integrons and SGI1 help them in combating these unfavorable conditions in a coordinated and efficient way. A variable distribution of the phage-mediated genes was also detected in the present study. The study further showed that the strains of the same serotype can be genotyped using typing methods and the data can be effectively used for molecular epidemiological studies. The superiority of LAMP-based method over PCR method on the rapidity and sensitivity of *Salmonella* detection was also proved by the present study.

Chapter 6

SUMMARY AND CONCLUSION

Salmonella is a major bacterial contaminant of seafood. Seafood samples were collected from different locations and screened for the presence of *Salmonella*. All *Salmonella* isolates were identified by biochemical and serological methods. 23.8% of the samples showed the presence of different serotypes of *Salmonella*. Thirty seven strains comprising three serotypes were isolated from the different samples screened. *Salmonella* Typhimurium and *Salmonella* Weltevreden were the predominant serotypes encountered. *Salmonella* Oslo was also detected in less numbers. It was observed that Sardine samples were highly contaminated with *Salmonella* followed by Anchovy and Squid. Thirteen *S.* Typhimurium and fourteen *S.* Enteritidis strains isolated from poultry were also included.

Most of the samples were contaminated with multiple serotypes. The seven Sardine samples were contaminated with *Salmonella* Typhimurium and *Salmonella* Weltevreden. Two samples among them also showed contamination with *Salmonella* Oslo. One of the two positive Anchovy samples was contaminated with *S.* Typhimurium and *S.* Weltevreden.

Antibiotic sensitivity profiling with 19 antibiotics coming under 10 classes produced two patterns of resistance. Three *Salmonella* Typhimurium (TSF-32, TSF-33 and TSG-38) and three *Salmonella* Weltevreden strains (WSF-31, WSG-37 and WSG-39) were resistant to an array of eight antibiotics which include ampicillin, carbenicillin, doxycycline, kanamicin, nalidixic acid, sulfafurazole, tetracycline and trimethoprim. The MAR index of these strains was 0.42. Three *Salmonella* Typhimurium strains (TSD-9, TSD-11 and TSD-19) and one *Salmonella* Weltevreden strain (WSD-28) were resistant to

sulfafurazole and trimethoprim with a MAR index of 0.1. Sulfonamides and trimethoprim are given in combination for the last 45 years. The antibiotics to which they are resistant includes the ones which are the first-line ones given for salmonellosis. These strains were resistant to five classes of antibiotics. The high MAR index indicates the high antibiotic stress prevalent in the environment which is the result of indiscriminate use of these antibiotics in feeds and therapeutic purpose.

PCR assay and sequencing of the partial *gyrA* gene of the nalidixic acid resistant strain showed that there was a point mutation at the 87th position of the *gyrA* gene. This transversion mutation (GAC →TAC), located in the QRDR, has replaced amino acid aspartate with tyrosine giving resistance to nalidixic acid. Several silent mutations were also detected showing the preferential codon usage in this gene.

The six nalidixic acid resistant strains harboured the *Salmonella* genomic island 1 (SGI1). The left junction of SGI1 was detected by PCR assay. *Salmonellae* harbouring SGI1 were reported to show a common pattern of multiple antibiotic resistance (ACSSuT phenotype) with some exceptions. The present study reports the presence of SGI1 in *Salmonella* Weltevreden strains. The resistance pattern demonstrated by these strains is also different from the common pattern.

All the 10 multiple antibiotic resistant strains harboured a class 1 integron. PCR assay and sequencing of the amplicon revealed the presence of *dfrA1* gene, giving resistance to trimethoprim. An open reading frame, *orfC*, with unknown function was also present in the integron.

Induction studies showed that four *Salmonella* Typhimurium (TQE-14, TSH-16, TSJ-58 and TSK-75) and one *Salmonella* Oslo (OSK-73) harboured

lysogenic phages. Seven *Salmonella* Typhimurium strains (ST-32, ST-41, ST-42, ST-44, ST-48, ST-77 and ST-128) and three *Salmonella* Enteritidis strains (SE-46, SE-52, and SE-118) isolated from poultry also harboured phages. One of the *Salmonella* Enteritidis strains (SE-52) showed the presence of genes encoded by two Gifsy phages.

Virulence profiling was determined by PCR-based screening and the distribution of the genes in the 64 *Salmonella* strains under study is shown in table 6.1.

Table 6.1. No. of strains positive for the screened virulence genes

Sl.No.	Genes	No. of strains
1	*invE*	64
2	*invA*	64
3	*ttrC*	64
4	*mgtC*	64
5	*spaM*	64
6	*spi4R*	64
7	*phoP*	64
8	*phoQ*	64
9	*slyA*	64
10	*pipA*	64
11	*sodC1*	12
12	*sopE*	6
13	*gogB*	1
14	*gtgE*	1

Screening to detect the presence of 14 genes (*invA, invE, ttrC, mgtC, phoP, phoQ, slyA, pipA, spi4R, spaM, sodC1, sopE, gogB* and *gtgE*) showed that genes *invA, invE, ttrC, mgtC, phoP, phoQ, slyA, pipA, spi4R* and *spaM* were present in all strains irrespective of the serotype and source. The phage-mediated genes *sodC1, sopE, gogB,* and *gtgE* showed a restricted distribution. Twelve strains showed the presence of *sodC1* gene which includes five *Salmonella* Typhimurium (TSI-52, TSI-53, TSJ-58, TSJ-59 and TSJ-60), four *Salmonella* Weltevreden (WSI-51, WSI-54, WSI-55, WSJ-61) and one *Salmonella* Oslo (OSI-50) from seafood; one *Salmonella* Typhimurium (ST-128) and one *Salmonella* Enteritidis (SE-52) from poultry. *Salmonella* Enteritidis SE-52 also harboured the two Gifsy genes. Gene *sopE* was detected from only the strains isolated from poultry.

Molecular typing by ERIC-PCR and PCR-Ribotyping revealed considerable intra-serotype polymorphism. ERIC PCR produced six different profiles for *Salmonella* Typhimurium and 8 for *Salmonella* Weltevreden. It differentiated the strains of *Salmonella* Typhimurium and *Salmonella* Weltevreden strains with a discriminative index of 0.832 and 0.916 respectively. PCR-Ribotyping produced 5 and 9 clusters respectively for *Salmonella* Typhimurium and *Salmonella* Weltevreden with discriminative indices 0.914 and 0.905. Both methods revealed a comparatively high polymorphism for the *Salmonella* Weltevreden strains. The high discriminative index shows the substantial polymorphism existing among the different strains of the same serotype of *Salmonella* which can be successfully used for molecular epidemiological study. The results showed that majority of the samples isolated from the same source were clustered together.

Rapid and sensitive detection of pathogen is of prime importance in any surveillance program. Molecular methods have several advantages over

the conventional culturing and subsequent biochemical identification methods. The present study investigated the superiority of Loop-mediated isothermal amplification (LAMP) over PCR assay in detecting *Salmonella* from experimentally contaminated water and naturally contaminated seafood. LAMP assay detected 2 CFUs of *Salmonella* Typhimurium, *Salmonella* Abony, *Salmonella* Typhi and *Salmonella* Paratyphi in experimentally contaminated water after 7h of enrichment. PCR assay took 8h to produce the same result. Out of the 5 Sardine samples screened for the presence of *Salmonella*, 2 samples were detected positive by LAMP assay after 5h of enrichment. PCR assay could detect *Salmonella* from only 1 sample after 7h of enrichment. Continued enrichment could not produce any positive signal.

CONCLUSION

The gastrointestinal tract is the primary habitat of *Salmonella* in animals. Aquatic animals are asymptomatic reservoirs of several of *Salmonella* serotypes. The inevitable delay in the detection of *Salmonella* contamination and the low sensitivity of the conventional methods is a serious issue, affecting the seafood industry as well as being a cause for health concerns. Emergence of antibiotic resistant phenotypes of *Salmonella* is due to indiscriminate use of antibiotics. In addition, genetic integrons and genomic islands have a role in acquisition and reshuffling of antibiotic resistance genes. The virulence potential of *Salmonella* is determined, not only by the presence of phages or phage mediated genes in the bacterial genome, but also other virulence factors. No direct correlation has been detected between the virulence gene profile and the serotype or source except for *sopE* which was detected form poultry related strains only. *Salmonella* show much intra-serotype polymorphism and

epidemiological studies rely on genetic resemblance of isolated strains. Proper identification of the strain employing the traditional and molecular techniques is therefore essential for epidemiology.

In this context, this study was undertaken to determine the prevalence of different *Salmonella* serotypes in seafood and to characterize the isolated *Salmonella* strains by antibiotic resistance profiling, virulotyping and molecular fingerprinting. In addition, an attempt was made to elaborate a protocol for rapid detection of *Salmonella* using Loop-mediated isothermal amplification.

The occurrence of different serotypes of *Salmonella* in Sardines, Anchovies and Squids were observed, with Typhimurium and Weltevreden as the prevalent serotypes in this study. The *Salmonella* strains in seafood showed multiple antibiotic resistance. Presence of *Salmonella* Pathogenic Island 1 and class 1 integron supported the antibiotic resistance phenotype of these strains. Virulence gene profiling showed that all the genes except the phage-mediated ones were present in all strains irrespective of the serotype and the source. Phage-mediated genes showed a variable assortment indicating the occurrence of horizontal gene transfer or the presence of lysogenic phages. Genotyping using ERIC-PCR and PCR-Ribotyping showed that significant polymorphism existed among the strains of the same serotype, which could be utilized for molecular epidemiological studies. The study also demonstrated the superiority of LAMP assay over PCR assay in detecting *Salmonella* from seafood.

The results stress the need for a continuous surveillance of *Salmonella* in seafood. The high MAR index exhibited by some of these strains expose the indiscriminate use of antibiotics, which may result in horizontal transfer of antibiotic resistance, leading to decreased efficacy of the antibiotics to combat *Salmonella* infections. However, more molecular typing methods need to be

investigated for epidemiological studies, as also utilization of rapid detection methods for detecting *Salmonella* from seafood and other sources, all working towards a major goal to reduce human health risks.

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APPENDIX

Lactose broth

Peptone	:	5g
Beef extract	:	3g
Lactose	:	5g
Distilled Water	:	1000mL

Dissolve 13g of dehydrated medium in 1000mL distilled water. Mix well and dispense 225 mL portions into 500 mL Erlenmeyer flasks and autoclave at 15 lbs pressure for 15 minutes. Final pH- 6.9 ± 0.2.

Tetrathionate Broth base

Polypeptone	:	5g
Bile Salts	:	1g
Calcium Carbonate	:	10g
Sodium Thiosulfate	:	30g
Distilled Water	:	1000mL

Suspend ingredients in 1 liter distilled water, mix, and heat to boiling. Cool to less than 45°C. Final pH- 8.4 ± 0.2.

Add 2 mL of iodine solution (6.0 g of iodine crystals and 5.0 g of potassium iodide in 20.0 mL of water). Do not autoclave the medium after the addition of iodine solution and use immediately.

Rappaport-Vassiliadis broth

Tryptose	:	4.59g
Papaic digest of soyabean meal	:	4.50g

Sodium chloride	:	7.20g
Mono-Potassium phosphate	:	1.44g
Magnesium chloride	:	36g
Malachite green	:	0.036g

49.2g of medium (Himedia, Mumbai, India) 1000mL distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired into tubes and sterilze by autoclaving at 10 lbs pressure (115^{o}C) for 15 minutes. Final pH 5.2±0.2

Xylose Lysine Desoxycholate Agar

Yeast extract	:	3g
L-Lysine	:	5g
Lactose	:	7.5g
Sucrose	:	7.5g
Xylose	:	3.5g
Sodium chloride	:	5g
Sodium desoxycholate	:	2.5g
Sodium thiosulphate	:	6.8g
Ferric ammonium citrate	:	0.8g
Phenol red	:	0.08g
Agar	:	15g

Suspend 56.68g of medium (Himedia, Mumbai, India) in 1000 mL distilled water. Heat with frequent agitation until the medium is boiled. Transfer immediately to a water bath at 50°C. After cooling, pour into sterile Petri plates. Final pH -7.4±0.2

Hektoen Enteric Agar

Proteose peptone	:	12g
Yeast extract	:	3g
Lactose	:	12g
Sucrose	:	12g
Salicin	:	2g
Bile salt mixture	:	9g
Sodium chloride	:	5g
Sodium thiosulphate	:	5g
Ferric ammonium citrate	:	1.5g
Acid fuchsin	:	0.1g
Bromo thymol blue	:	0.065g
Agar	:	15g

Suspend the ingredients in 1 liter distilled water and mix. Heat the medium to boiling with frequent agitation. Transfer immediately to a water bath, at 50°C. After cooling, pour into sterile Petri plates. Final pH -7.5±0.2

Bismuth Sulfite Agar

Peptic digest of animal tissue	:	10 g
Beef extract	:	5g
Dextrose	:	5g
Disodium phosphate	:	4g
Ferrous sulphate	:	0.3g
Bismuth sulphite indicator	:	8g
Brilliant green	:	0.025g
Agar	:	20g

Suspend 52.33g of medium (Himedia, Mumbai, India) in 1000 mL distilled water. Heat the medium to boiling to dissolve the constituents completely. Pour into sterile Petri plates and it should be stored in dark for one day. Final pH - 7.7±0.2

Triple Sugar Iron Agar

Peptic digest of animal tissue	:	2g
Beef extract	:	3g
Yeast extract	:	3g
Lactose	:	10g
Sucrose	:	10g
Glucose	:	1g
Ferric citrate	:	0.3g
Sodium chloride	:	5g
Sodium thiosulphate, pentahydrate	:	0.3g
Phenol red	:	0.024g
Agar	:	12g

Suspend 64.51g of medium (Himedia, Mumbai, India) in 1000 mL distilled water. Heat the medium to boiling to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Allow the medium to set in sloped form with a butt about 1 inch long. Final pH -25°C 7.4±0.2

Lysine Iron Agar

Peptic digest of animal tissue	:	5g
Yeast extract	:	3g
Dextrose	:	1g
L-Lysine	:	10g

Ferric ammonium citrate	:	0.50g
Sodium thiosulphate	:	0.04g
Bromocresol purple	:	0.02g
Agar	:	15g

Suspend 34.56 g of medium (Himedia, Mumbai, India) in 1000 mL distilled water. Heat the medium to boiling to dissolve completely. Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool the tubes in slanted position to form slants with deep butts. Final pH- 6.7±0.2

Tryptone broth

Tryptone	:	1.5g
Sodium Chloride	:	0.5g
Distilled Water	:	100mL

Suspend ingredients in 100mL distilled water, mix and heat to boiling. Sterilize by autoclaving at 15 lbs pressure (121°C) for 10 minutes and plate.

Carbohydrate Fermentation test

Peptone	:	10 g
NaC1	:	5 g
Beef extract (optional)	:	1 g
Phenol red	:	0.018 g
Distilled water	:	1 litre
Carbohydrate*		

*Dissolve either 5 g dulcitol, 10g lactose, or 10g sucrose in this basal broth. Dispense 2.5 mL aliquots into culture tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Final pH -7.4 ± 0.2.

Christensens' Urea agar

Peptone	:	1.0g
Sodium Chloride	:	5 g
Monopotassium phosphate	:	2g
Glucose 0.1%	:	1g
Phenol Red	:	0.012g
Agar	:	15g
Distilled Water	:	900mL
Urea	:	20g
Distilled Water	:	100mL

Dissolve all the ingredients except urea in 900mL water (basal medium). Autoclave for 15 min at 121°C. Cool to 50-55°C.

Filter-sterilized urea; added aseptically to cooled basal medium and mix well. Dispense in to sterile tubes and convert to slants with 2 cm butt and 3 cm slant. Final pH- 6.8 +/- 0.2 at 25°C

Luria Bertani Broth

Casein enzymic hydrolysate	:	10g
Yeast extract	:	5g
Sodium chloride	:	10g

Suspend 25 g of medium (Himedia, Mumbai, India) in 1000 mL distilled water. Heat the medium to dissolve completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH- 7.5±0.2

Tryptone Soya Broth

Pancreatic Digest of Casein	:	17g
Papaic digest of soyabean meal	:	3g
Sodium chloride	:	5g
Dipotassium hydrogen phosphate	:	2.5g
Dextrose (Glucose)	:	2.5g

Suspended 64.51 g of medium (Himedia, Mumbai, India) 1000 mL distilled water. Heat the medium to boiling to dissolve the ingredients completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Final pH -7.3 ± 0.2.

Nutrient Medium

Peptone	:	5g
Sodium chloride	:	5g
Beef extract	:	1g
Yeast extract	:	2g

Dissolve 1.3g of medium in 100ml of distilled water. Mix well, autoclave at 15 lbs pressure for 15 minutes and cool to 50-55°C. When used as solid agar medium, 2.0% agar (w/v) is to be added to the medium for agar plate preparation. Final pH-7.4±0.2

Agarose gel (1%)

Agarose	:	1.0 g
1X TAE (see TBE buffer)	:	100 mL

Melt agarose in microwave oven and allow to cool to 50^{O}C before pouring the gel.

Bromophenol blue loading dye

Bromophenol blue	:	0.05 g
Glycerol	:	5.0 mL
EDTA	:	0.186 g
1X TAE	:	volume to 10 mL

Aliquot the dye in fresh microfuge tubes and store at 4^{O}C.

Disodium ethylenediamine tetraactete (EDTA) - 0.5 M

EDTA	:	186.1 g
Distilled water	:	1000 mL
NaOH	:	~20 g

Dissolve EDTA in 800 mL distilled water and stir vigorously on a magnetic stirrer. Adjust pH to 8.0 using NaOH pellets and make the final volume to 1000 mL. Autoclave before use.

Ethidium Bromide (10 mg/mL)

Ethidium Bromide	:	0.1 g
Distilled water	:	10 mL

Stir the solution using a magnetic stirrer for several hours to ensure that the dye has dissolved completely. Wrap the container in aluminium foil and store at 4^{o}C. For staining agarose gels, a working solution of 0.5 µg/mL can be made.

Hexadecyltrimethylammonium bromide (CTAB) – 10 % in 0.7 M NaCl

CTAB	:	10 g
NaCl	:	4.1 g
Distilled water	:	80 mL

Dissolve 4.1 g NaCl in 80 mL distilled water and slowly add 10 g CTAB, while heating and stirring. If necessary, heat to 65^{O}C to dissolve. Adjust final volume to 100mL.

Proteinase K (20 mg/ml)

Proteinase K	:	10 mg
Sterile distilled water	:	0.5 mL

Stored at -20^{O}C in 50 µL aliquots.

Sodium dodecyl sulphate (SDS) – 10%

SDS	:	10 g
Distilled water	:	100 mL

Tris-Acetate EDTA (50 X)

Tris base	:	242 g
0.5 M EDTA (pH 8.0)	:	100 mL
Glacial acetic acid	:	57.1 mL
Distilled water	:	to 1000 mL

The stock solution was diluted to 1X for gel runs.

Tris EDTA (TE) buffer

Tris-HCl 10 mM	:	2 mL 1M Tris-HCl (pH 8.0)
EDTA (pH 8.0)	:	1 mM 0.4 mL 0.5 M EDTA

Distilled water to 200 mL

Physiological saline (0.85%)

NaCl	:	0.85g

Dissolve in 100mL distilled water

McFarland standard (.5)

Sulfuric Acid 0.18 M : 99.5 mL

Barium Chloride 0.048 M : 0.5 mL

Mix the components, vortex thoroughly and determine the absorbance at 625nm. It should be 0.08 to 0.10.

LIST OF PUBLICATIONS

1. Peer Reviewed

Augustine, J., Louis, L., **Varghese, S. M.**, Bhat, S. G., and Kishore, A. (2013): Isolation and partial characterization of ΦSP-1, a *Salmonella* specific lytic phage from intestinal contents of broiler chicken- candidate for biocontrol agent. *Journal of Basic microbiology* **53**, 111-120.

Augustine, J., **Varghese, S. M**., and Bhat, S. G. (2013): ΦSP-3, a *Salmonella*-specific lytic phage capable of infecting its host under nutrient-deprived states. *Annals of Microbiology* **63**, 381-386.

Raghul, S., **Varghese, S. M**., and Bhat, S. G. (2013): Isolation and characterization of polyhydroxyalkanoates accumulating *Vibrio* sp. strain BTTC26 from marine sediments and its production kinetics. *Journal of Scientific & Industrial Research* **72**, 228-235.

Varghese, S. M., Augustine, J., and Bhat, S. G. (2012): Rapid and Sensitive detection of low number of *Salmonella* in water by Loop-Mediated Isothermal Amplification. *Advanced Biotech* **11**, 28-30.

2. Full papers in proceedings

Jeena Augustine , **Siju M. Varghese**, Sartia G Bhat and Hatha A A M (2011) "Phenotyping, Genotyping and virulence gene profiling of two *Salmonella* strains isolated from Chicken Gut". In Proceedings of National symposium on "Emerging trends in Biotechnology" organised by the Department of Biotechnology, CUSAT, Cochin, September 1-2, 2011. pp 128-135, **ISBN- 978-93-80095-30-1**

Raghul Subin S**, Siju M. Varghese** and Sarita G Bhat (2011).Genotypic approach in characterisation of polyhydroxyalkanoates (PHAs) accumulation in *Vibrio* sp. isolated from marine sediments. In Proceedings of the National Symposium Emerging Trends in biotechnology, 2011, organised by the Department of Biotechnology, CUSAT, Cochin, September 1-2, 2011. pp 25-36, **ISBN-978-93-80095-30-1**

3. Genbank submissions

GenBank Accession No. JQ794607. *Salmonella enterica* subsp. *enterica* serovar Weltevreden strain S37 class 1 integron dihydrofolate reductase (dfrA1)

gene, complete cds, and hypothetical protein (orfC) gene, partial cds. **Siju,M.V**., Jeena,A. and Sarita,G.B.

GenBank Accession No. HM635765. *Salmonella enterica* subsp. *enterica* serovar Typhi strain STY116S ribosomal RNA gene, partial sequence. **Siju,M.V**., Jeena,A., Raghul,S.S., Toby,A. and Sarita,G.B.

Genbank Accession Number - HQ268499 - *Salmonella enterica* subsp. *Enterica.* strain S 37 - 16S ribosomal RNA gene, partial sequence – Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ268500 - *Salmonella enterica* subsp. *Enterica.* strain S 49 - 16S ribosomal RNA gene, partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260701 – Virulence gene , tetrathionate reductase complex, subunit C (*ttrC)* from S 49 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260702 – Virulence gene , surface presentation of antigens protein (*spam)* from S 49 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260703 – Virulence gene , Aggregative Fimbriae (*agfA*) from S 49 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260704 – Virulence gene , invasion protein (*InvE*) from S 37, partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260705 – Virulence gene , invasion protein (*InvE*) from S 49 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260706 – Virulence gene , Regulatory system (*pho P/Q*) from S 37 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260707 – Virulence gene , Regulatory system (*pho P/Q*) from S 49 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260708 – Virulence gene , Transcriptional regulator(*slyA)* from S 37 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - HQ260709 – Virulence gene, Transcriptional regulator (*slyA)* from S 49 , partial sequence, Jeena Augustine , **Siju M Varghese**, Raghul Subin S, A A M Hatha , Sarita G Bhat

Genbank Accession Number - GU904006 - Vibrio sp. BTTC27 16S ribosomal RNA gene, partial sequence, Raghul,S.S., Sarita,B.G., Smitha,S., Jeena,A., **Siju,V.M**., Vijaya,A.and Helvin,V.

Genbank Accession Number - GU904004 - Vibrio sp. BTTN18 16S ribosomal RNA gene, partial sequence, Raghul,S.S., Sarita,B.G., Smitha,S., Jeena,A., **Siju,V.M**., Vijaya,A. and Helvin,V.

GenBank Accession No. JQ868589. Uncultured bacterium clone MG1, Helvin Vincent, Sarita G. Bhat, **Siju M. Varghese** and Harisree P. Nair.

GenBank Accession No. JQ868594. Uncultured bacterium clone MG18, Helvin Vincent, Sarita G. Bhat, **Siju M. Varghese** and Harisree P. Nair.

GenBank Accession No. JQ868590. Uncultured bacterium clone MG3, Helvin Vincent, Sarita G. Bhat, **Siju M. Varghese** and Harisree P. Nair.

GenBank Accession No. JQ868591. Uncultured bacterium clone MG5, Helvin Vincent, Sarita G. Bhat, **Siju M. Varghese** and Harisree P. Nair.

GenBank Accession No. JQ868592. Uncultured bacterium clone MG7, Helvin Vincent, Sarita G. Bhat, **Siju M. Varghese** and Harisree P. Nair.

GenBank Accession No. JQ868593. Uncultured bacterium clone MG17, Helvin Vincent, Sarita G. Bhat, **Siju M. Varghese** and Harisree P. Nair.

Rapid and Sensitive Detection of Low Number of *Salmonella* in Water by Loop-Mediated Isothermal Amplification

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Abstract

A Loop-Mediated Isothermal Amplification (LAMP) method for rapid, specific, and sensitive detection of four *Salmonella* serotypes from artificially contaminated water has been developed. 10^5 to 2 colony forming units (CFU) of these *Salmonella* serotypes were given pre-enrichment in lactose broth for varying intervals of time. Primers specific for *invA* gene were used for LAMP assay. *E coli* was co- inoculated with *Salmonella* to determine the influence of non-target DNA on sensitivity of LAMP assay. The results were compared with that of PCR. Even though the specificity of both the methods was 100%, LAMP assay could detect 2 CFUs after 7 hrs, whereas it was 8 hrs for PCR. The presence of non-target DNA showed no inhibitory effect. As the LAMP method is more cost effective, easy, rapid and sensitive than PCR, we suggest this assay, in combination with a non selective pre-enrichment, as a routine diagnostic test for *Salmonella* spp. in water.

Keywords: Loop Mediated Isothermal Amplification; PCR; *Salmonella*; Enrichment; *invA*

Introduction

Salmonella is one of the major food-borne pathogens, transmitted mainly through contaminated water. They inhabit the gastrointestinal tract of the host and find their way into the environment along with the fecal matter and finally reach water bodies. Contaminated water cross contaminates the aquatic organisms making them potential carriers. Several methods like the conventional culture method, polymerase chain reaction (PCR), Loop Mediated Isothermal Amplification (LAMP) etc have been developed and validated for the detection of these microbes. Culture method is tedious and time consuming, requiring at least 6 days (Andrews *et al*., 1998). Polymerase chain reaction method has widely been used for the detection of *Salmonella* in food (Ferretti *et al*., 2001; Riyaz-Ul-Hassan *et al*., 2004). It is more sensitive than the culture method as the detection limit is very low (Riyaz-Ul-Hassan *et al*., 2004; Kumar *et al*., 2008) but requires expensive instruments. Recently, a novel loop-mediated isothermal amplification (LAMP) method for DNA amplification was developed (Notomi *et al*., 2000). Due to high sensitivity and amplification efficiency, LAMP method is currently used as an alternative to PCR for amplification and detection of specific genes. This cost-effective method can be performed in a water- bath under isothermal conditions and its sensitivity and specificity for *Salmonella* detection has been proved previously (Hara-Kudo *et al*., 2005; Ohtsuka *et al*., 2005; Ueda , Kuwabara, 2009). LAMP is considered more sensitive than PCR in detecting microbial pathogens (Horisaka *et al*., 2004; Wang *et al*., 2008; Ueda and Kuwabara, 2009; Techathuvanan *et al*., 2010; Tang *et al*., 2011). Considering the role of water as one of the primary sources of infection as well as a medium for cross contamination, the present study was aimed to develop a rapid, sensitive and cost effective protocol using LAMP assay for detection of *Salmonella* in water. Attempts have been made to ascertain the minimum number of cells that can be detected by giving enrichment. The results were compared with that of PCR. In addition, the inhibitory effects, if any, due to presence of non-*Salmonella* cells on the efficiency of LAMP were also checked.

Materials and Methods

Four *Salmonella* serotypes (Table 1) were cultured overnight in nutrient broth and 10-fold serial dilutions were prepared using physiological saline. 25 ml sterile water was spiked with known number of CFUs ranging from 2 X 10^5 to 2 and inoculated to 225 ml lactose broth. The CFU was estimated by plating appropriate dilutions on XLD plates (Difco). The inoculated lactose broth was incubated with shaking at 37°C. 2 ml culture was retrieved from each flask at regular intervals. All the experiments were done in triplicates. The specificity of LAMP assay was determined using three non-*Salmonella* cultures (Table 1). LAMP and PCR assays were carried out using DNA isolated from these cultures at regular intervals of enrichment in lactose broth. 10^4 CFUs of *E coli* were co inoculated separately with 2 CFUs of 4 *Salmonella* serotypes in lactose broth and incubated at 37°C. LAMP and PCR assay were performed with DNA isolated from these cultures. Template DNA for the assays was prepared by the boiling method as follows. 2 ml culture suspension was centrifuged at 10000 x g for 10 min. The harvested pellet was washed twice with sterile water and resuspended in 100 µl Tris-EDTA buffer (pH 8). The tubes were kept in a boiling water bath for 10 min. and chilled immediately on ice. It was then centrifuged at 10000 x g for 15 min. The supernatant containing DNA was directly used for LAMP and PCR assay. Four primers (Sigma) were used for the LAMP assay(Wang *et al*., 2008) . The primers FIP5′- cccagatccccgcattgttgattttttccgccccatattatcgctat - 3′, BIP 5′-gaccatcaccaatggtcagcattttattggcggtatttcggtgggg – 3′, F3 5′-gttcaacagctgcgtcatga – 3′ and B3 5′-cgctattgccggcatcatta – 3′, target 6 distinct regions of the *Salmonella* specific invasion protein gene (invA). The reaction was carried out in 25 µL reaction mixture containing 1.4 µM each of FIP and BIP primers, 0.2 µM each of F3 and B3 primers, 1.6 mM of dNTPs, 1M betaine (Sigma), 1X thermopol buffer [20mM Tris-

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No.	Serotype	Source
1	*Salmonella enterica* subsp.*enterica* ser. Abony	NCIM 2257
2	*Salmonella enterica* subsp.*enterica* ser. Typhimurium	ATCC 23564
3	*Salmonella enterica* subsp. *enterica* ser. Typhi	MTCC 734
4	*Salmonella enterica* subsp. *enterica* ser. Paratyphi	MTCC 735
5	*Escherichia coli*	ATCC 9961
6	*Proteus vulgaris*	ATCC 13315
7	*Klebsiella pnemoniae*	NCIM 2957

Table 1. Cultures used in this study [Note: NCIM – National collection of industrial microorganisms, Pune, Maharastra India; MTCC –Microbial type culture collection and gene bank, Chandigarh, India].

HCl, 10mM $(NH_4)_2SO_4$, 10mM KCl, 2 mM $MgSO_4$ and 0.1% Triton X-100 (pH 8.8)] and 4.5 µL of template DNA. The tubes with the reaction mixture were kept at 95°C in a water bath for 5 min to denature the template DNA and then plunged into ice and 8 units of *Bst* DNA polymerase large fragment (New England Biolabs) were added. The reaction mixture was then heated at 65°C for 1hr and the reaction was terminated at 80°C for 10 min. A negative control was also kept with all the ingredients except the template DNA. 5 µL of the LAMP products were observed under UV light after electrophoresis in a 2% agarose gel. Ethidium bromide was used to stain the products. The image of gel was captured using gel documentation system (Syngene,UK). PCR was done with the same template DNA as that for LAMP. Primers F3 and B3 of Lamp assay were used as primers for the reaction (Wang *et al*., 2008). The reaction was performed in 20 µL volumes containing 0.5mM each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1X *Taq* buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1 unit Taq DNA polymerase and 5 µL template DNA. Conditions for PCR assay were as described previously (Wang *et al*., 2008): initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, primer annealing at 55°C for 30 s and extension at 72°C for 30 s. The reaction was terminated by giving an additional 7 min extension at 72°C after the 30th cycle. 5 µL of the amplified product was resolved on 1.5% agarose gel and visualized under UV light after staining with ethidium bromide. Gel pictures were captured using gel documentation system (Syngene,UK)

Results

LAMP and PCR assay could not detect *Salmonella* spp. at the time of inoculation, but at regular intervals they gave positive signals depending upon the number of CFUs present in each sample. The minimum incubation time for detection decreased with increasing concentration of initial inoculums. Amplification could be obtained by LAMP assay after 4 hrs of enrichment from samples with initial inoculums of 10^5, $10^4$and 10^3 CFUs, while those with 200 and 20 CFUs were obtained after 6hrs incubation (data not shown). LAMP assay after 7 hrs of enrichment produced the characteristic ladder like pattern with all 4 *Salmonella* serotypes with initial inoculums of 2 CFUs (Fig.1). Amplification by PCR was obtained with 10^5 and 10^4 CFUs inoculum after 4 hrs enrichment and with 10^3 and 10^2 CFUs after 6 hrs enrichment (data not shown). Amplicons were observed for 20 (data not shown) and 2 CFUs only after 8 hrs of enrichment (Fig. 2). Samples inoculated with 2 CFUs of *Salmonella* along with 10^4 CFUs of *E. coli* gave positive signals with

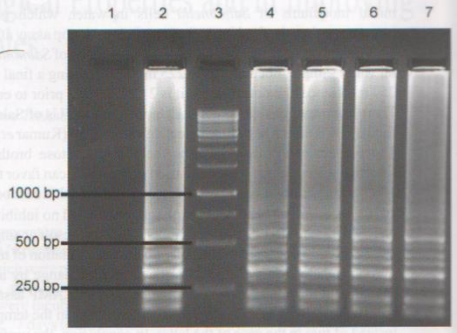

Figure 1. Agarose gel electrophoresis of LAMP assay after 7 hrs enrichment of samples. [Note: Lane 1, *E.coli* negative control; lane 2, 2 CFUs of *Salmonella* Typhimurium co inoculated with 10^4 *E coli* CFUs; lane 3, 1 kb DNA marker; lanes 4-7, 2 CFUs initial inoculums of *Salmonella* Abony, *Salmonella* Typhimurium, *Salmonella* Typhi, and *Salmonella* Paratyphi respectively].

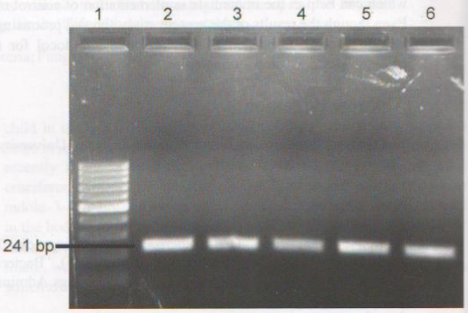

Figure 2. PCR assay after 8 hrs enrichment of samples [*Note*: Lane 1, 100-bp DNA marker; lane 2, 2 CFUs of *Salmonella* Typhimurium co-inoculated with 10^4 *E. coli* CFUs; lanes 3-6, 2 CFUs initial inoculums of *Salmonella* Abony, *Salmonella* Typhimurium, *Salmonella* Typhi, and *Salmonella* Paratyphi, respectively].

LAMP and PCR after incubation for 7 hrs and 8 hrs respectively (Fig. 1& 2). Samples with only *E. coli* culture showed no amplification in LAMP assay (Fig. 1 & 2).

Discussion

Salmonellae are pathogenic and one of the main routes of their transmission is contaminated water. A cost effective rapid method for routine monitoring of water for the presence of *Salmonella* is very much needed. Many researchers have standardized protocols for detection of *Salmonella* in naturally and artificially contaminated food materials after enrichment. 10^2 *Salmonella* CFU/ml of broth were detected using LAMP assay previously (Ueda and Kuwabara, 2009). Such a high concentration of *Salmonella* cells, which will rarely be seen in water used for drinking and cooking purposes, can be achieved only after enrichment. Even though the LAMP assay was carried out after enrichment, our experiments had been designed to give an estimate of

initial inoculums of *Salmonella* cells in water, which is of more importance than the final load after enrichment. Lamp assay after 7 hrs of enrichment could detect two CFU initial inoculums of *Salmonella* in a 25 ml water sample inoculated into 225 ml broth making a final volume of 250 ml. Hence, the detection limit is 2 CFU/250ml prior to enrichment. There are previous reports about detection of 2 CFUs of Salmonella in food by PCR after 8 hrs of non selective enrichment (Kumar *et al.*, 2008). The present study also confirms the result. Lactose broth, the non selective pre enrichment medium used in the study, can favor the growth of other enteric organisms also. Our results showed that co inoculation of 10^2 CFUs of *E .coli* with 2 CFUs of *Salmonella* had no inhibitory effect on LAMP assay after 7 hrs of enrichment. Positive result or amplification can be directly observed as turbidity due to accumulation of magnesium pyrophosphate (Mori *et al.*, 2001) or as colour change by addition of calcein and manganese ions (Tang *et al.*, 2011). LAMP assay is more specific than PCR as it targets six specific regions in the template DNA instead of two in the case of the latter. In conclusion, the usefulness of a non selective pre enrichment prior to LAMP assay for the rapid detection of low number of *Salmonella* has been revealed by this study. This method caters to the need for a rapid, sensitive and cost effective detection assay for *Salmonella* spp. in water. This assay is a potential tool for routine monitoring of low number of *Salmonella* spp. in water, which can help in the immediate implementation of control measures. Even though the results of the present study showed promising results, further research is needed to standardize the protocol for naturally contaminated water and food samples.

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ORIGINAL ARTICLE

ΦSP-3, a *Salmonella*-specific lytic phage capable of infecting its host under nutrient-deprived states

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Abstract *Salmonella* is a robust pathogen capable of surviving under various hostile conditions. The ability of a *Salmonella*-specific lytic phage, ΦSP-3, to infect its host under various nutrient-deprived conditions was studied. This phage was isolated from the intestinal contents of chicken. The identity of ΦSP-3 was confirmed by sequence analysis whereby ΦSP-3 showed maximum similarity towards T5-like phages of family *Siphoviridae*. The genome size of ΦSP-3 was estimated to be 88.43 kb by pulsed-field gel electrophoresis (PFGE) analysis. ΦSP-3 was able to infect its host under stationary phase, multiple nutrient-starved states, carbon-starved and nitrogen-starved conditions. ΦSP-3 failed to multiply only under phosphate-starved condition. Host range studies revealed the genus specificity of ΦSP-3. A wide host range within the genus and the capability of infecting bacterial host cells in ideal as well as nutrient-deprived conditions makes ΦSP-3 a desirable candidate as a biocontrol agent.

Keywords *Salmonella* · Phage · Stationary phase · Stress

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Introduction

The prospects of lytic phages as biocontrol agents against pathogenic bacteria are being reconsidered worldwide with the surfacing of antibiotic resistance (Sulakvelidze et al. 2001). *Salmonella*, the causative agent of gastroenteritis, is one amongst many bacterial pathogens against which phages are experimented as therapeutic agents (Kropinski et al. 2006). However, the capability of *Salmonella* to survive under diverse stress environments (Foster and Spector 1995) makes it a difficult target for phages. The physiological state of the host is an important factor for successful phage-host interaction (Capra et al. 2006; Chibani-Chennoufi et al. 2004). The standard protocol followed for the isolation of phages involves using a host in exponential growth phase (Adams 1959), and hence the isolated phages are capable of propagating only in fast-growing host cells. Bacteria can be maintained in the log-phase only when there is no nutrient limitation, which can be achieved only under laboratory conditions (Robb and Hill 2000). In natural environments, bacteria exist as "long-term stationary-phase cultures" where a set of stress response genes and metabolic pathways are essential for survival (Finkel 2006). These stress conditions are experienced in the laboratory set-up when the culture reaches the stationary phase (Chibani-Chennoufi et al. 2004). Thus, it can be inferred that phages infecting stationary phase bacteria can infect bacteria in natural conditions. Phages intended for use as biocontrol agents have an added advantage when they have the ability to infect the host under stationary as well as nutrient-

deprived conditions. There are only a few reports on this aspect. The first case of phage infection in stationary phase was reported in α3, a phage infecting *Achromobacter* (Woods 1976). Another was regarding a *Pseudomonas* phage that successfully infected host cells that were starved for 5 years (Schrader et al. 1997). Infection of MS2 virus on glucose-, sulfur- and nitrogen-starved cells of *Escherichia coli* resulted in production but no progeny release (Propst-Ricciuti 1976).

In the present study, a *Salmonella*-specific lytic phage, designated ΦSP-3, was characterized and the ability of the phage to infect its host under different nutrient-deprived conditions was studied.

Materials and methods

Salmonella culture

Salmonella Enteritidis, designated as S 49, isolated from a hen egg was used as the bacterial host for phage isolation and propagation. It was isolated following the guidelines of the bacteriological analytical manual of the US Food and Drug Administration (Andrews et al. 2007). The identity of the bacterial host was confirmed by serotyping at the National *Salmonella* and *Escherichia* Centre, Kasuali, Himachal Pradesh, India, and by 16S rRNA gene sequence analysis. The partial sequence was deposited in Genbank (Accession no. HQ268500). The host strain was grown at 37 °C in nutrient broth (HiMedia, Mumbai, India) in all the experiments conducted unless otherwise stated.

Salmonella phage ΦSP-3

The lytic phage, ΦSP–3, isolated from the intestinal contents of broiler chicken as described previously (Augustine et al. 2012) was used for the study.

Identification of ΦSP-3

Phage DNA extraction was carried out as previously described (Sambrook et al. 2000). Briefly, 1 ml of the concentrated phage suspension was incubated at 56 °C with proteinase K (50 μg/ml) and SDS (0.5 %) for 1 h. After incubation, the digestion mix was extracted once with an equal volume of phenol, once with 50:50 phenol and chloroform, and finally with an equal volume of chloroform. The DNA was then precipitated with a double volume of ethanol in the presence of sodium acetate and dissolved in Tris EDTA (TE) buffer (pH 7.6).

Three primer pairs were designed (Table.1) from conserved stretches of the major capsid protein (MCP) of the reported *Salmonella* phages. The specificity of the primers was confirmed by the GenBank database Basic Local Alignment Search Tool (BLAST) program. Assays were performed in 20-μl reaction mixtures containing 100 ng of template DNA, 200 mM each dNTP (Chromous Biotech), 0.5 μm each primer (Sigma Aldrich) and 1 U Taq DNA polymerase (Sigma Aldrich). Amplifications were carried out in BioRad MJ Mini Gradient Thermal Cycler using the following program: an initial denaturation at 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min, ending with a final extension step of 72 °C for 10 min. Samples of 5 μl of the amplification products were analyzed by electrophoresis on 1.2 % agarose gels. The gel was stained with Ethidium bromide and visualized in UV light. The amplicon was sequenced and compared with the sequences in Genbank entries, by BLAST programme (Altschul et al. 1990). The sequence was deposited in the Genbank database (Accession no. JQ638926). A phylogenic tree was generated using sequences of T5-like bacteriophages including Enterobacteria phage SPC35 (HQ406778.1), Bacteriophage T5 strain st0 (AY692264.1), Bacteriophage T5 (AY543070.1) , Bacteriophage T5 strain ATCC 11303-B5 (AY587007.1), Enterobacteria phage EPS7 (CP000917.1), *Salmonella* phage PVP SE1 (GU070616.1), *Escherichia coli* bacteriophage rv5 (DQ832317.1), *Salmonella* phage 7–11 (HM997019.1), Enterobacteria phage Qbeta (FJ483844.1) by the neighbor-joining method (Saitou and Nei 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Sequences were aligned using BioEdit version 5.0.6 software (Hall 2001). The evolutionary distances were computed using the Tajima Nei method (Tajima and Nei 1984) and are in the units of the number of base substitutions per site.

Table 1 List of primers designed for PCR and their estimated amplicon size

Primer	Sequence	Amplicon size	Reference
MCPF1	AGACCAGATCCAGCAGTCC	866	This work
MCPR1	AGCGGTAGTCCTCAACCAC		
MCPF2	AAGACCAGATCCAGCAGTCC	861	This work
MCPR2	AGTCCTCAACCACATAGGCTTC		
MCPF3	GAACTGTCCAGCAAGTTCACC	851	This work
MCPR3	GGGTTGTCGATAACTGAACG		

MCPF major caspid protein-forward primer

MCPR major caspid protein- reverse primer

Evolutionary analyses were conducted using MEGA5 (Tamura et al. 2011).

Genome size determination of ΦSP-3 by Pulsed Field Gel Electrophoresis (PFGE)

ΦSP-3 DNA was run in 1 % agarose gel (SeaKem® Gold Agarose, Pulsed field grade) in 0.5× TBE buffer (50 mM Tris–HCl, 50 mM boric acid, 1 mM EDTA) and electrophoresis was carried out in a CHEF DRII PFGE system (Bio-Rad, USA). The gel was run at 4.5 volts/cm using ramped pulse times from 5 to 120 s for 18 h at 15 °C. Bacteriophage lambda DNA, 1,000 kb (New England BioLabs), was used as molecular weight marker. The gel was stained with Ethidium bromide and photographed. The molecular size was calculated using Quantity One® software (Bio-Rad).

Host range studies

The host range of the phage was assessed on the basis of its ability to form plaques on *Salmonella* Enteritidis strains present in the microbial genetics laboratory culture collection. A total of 96 cultures were tested including the standard strains, *Salmonella* Typhimurium (National Collection of Industrial Microorganisms (NCIM) no. 2501), *Salmonella* Abony (NCIM no. 2257), *Escherichia coli* (NCIM no. 2343), *Klebsiella pneumoniae* (NCIM no. 2957) *Pseudomonas aeruginosa* (NCIM no. 2863), *Bacillus cereus* (NCIM no. 2155), *Staphylococcus aureus* (NCIM no. 2127) and *Proteus vulgaris* (NCIM no. 2027). All the strains used in the study were grown at 37 °C in nutrient broth (HiMedia).

Preparation of log- and stationary-phase, starved and nutrient depleted cultures, plaque assay

Host cells were grown as overnight cultures at 37 °C and were used for the stationary phase cell infection experiments. A fresh 6-h culture was used for exponential phase host cell infection experiments. The starved host cells were prepared by resuspending cell pellet obtained after centrifugation of a 6-h-old culture, in an equal volume of physiological saline and incubating it for 24 h at 37 °C (Kadavy et al. 2000). Nutrient-depleted cultures were prepared by growing the bacteria to log phase in nutrient broth, and the cells harvested by centrifugation was resuspensed in appropriate starvation suspension media, like minimal media without carbon, minimal media without phosphate, and minimal media without ammonium chloride (nitrogen source) (NystrÖm et al. 1992). In all the cases except for the stationary phase, the OD_{600} of the cultures was adjusted to 0.5 [1×10^8 colony forming units (CFU)/ml] using the respective medium prior to the addition of the phage. ΦSP-3 was introduced into each of these cultures at a low multiplicity of infection (MOI) of 0.1, incubated at 37 °C for 24 h and plated (double agar overlay method; Adams 1959) to observe the efficacy of phage multiplication under various nutrient-deprived conditions. All experiments were conducted in triplicate.

Statistical evaluations were done by ANOVA followed by Students–Newman–Keul Test using GraphPad InStat (v.2.04a; San Diego, USA) computer program.

Results and discussion

The ability to infect a nutrient-deprived bacterial host is a desirable attribute for bacteriophages in their application as biocontrol agents. *Salmonella* Enteritidis (S 49) was used as model bacterial pathogen for this study. It is considered as the primary causative agent of salmonellosis (Baumler et al., 2000) infecting humans worldwide (Guard-Petter, 2001). ΦSP-3, a *Salmonella*-specific lytic phage has been characterized and its interaction with S 49 under various nutrient-deprived conditions was studied.

Molecular characterization of ΦSP-3

An amplicon of 750 bp (Fig.1a) was obtained in PCR with MCP primers. The sequence of the amplicon when compared with the Genbank database entries showed maximum similarity (95 %) towards Enterobacteria phage SPC35, which is a T5-like strain (GenBank Accession No. HQ406778.1). T5 phages are members of *Siphoviridae* family and are characterized by a long flexible non-contractile tail attached to an isometric icosahedral capsid containing the double-stranded DNA genome (Effantin et al. 2006). San 2, 12 (Ackermann and Gershman 1992), G5 (Slopek and Krzywy 1985), and Φ1 (Bradley and Kay 1960) are some of the reported *Salmonella* phages which are T5-like. The phylogenetic tree of ΦSP-3 is depicted in Fig. 1b. ΦSP-3 was found to be closely related to the phage *Samonella* 7–11, which is an enterobacterial phage belonging to the order of Caudoviridae group, and its genome closely shares proteins similar to the T5 phage which is evident from its full genome sequence analysis (GenBank Accession No. HM997019.1).

PFGE of ΦSP-3 DNA

The genome size of ΦSP-3 was determined to be 88.43 kb by PFGE (Fig. 2). The genome size falls slightly below the reported genome size of both the T5 phage, which is 121 kb (Wang et al. 2005), and the T5-like coliphage SPC35, which is 118 kb in size (Kim and Ryu 2011).

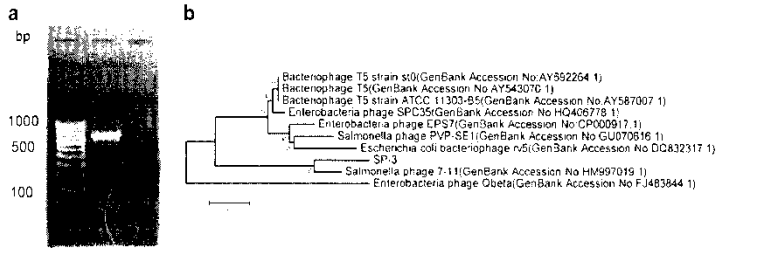

Fig. 1 a *Lane 1* 100-bp ladder, *lane 2* amplicon from ΦSP-3 DNA, *lane 3* negative control. b Phylogentic tree of ΦSP-3 . The optimal tree with the sum of branch length=3.05 is shown. The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates (Felsenstein 1985). *Bar* 2 % sequence divergence

Host range analysis

T5-like phages are by and large considered as coliphages, which are known to infect groups of bacteria like *Salmonella, Klebsiella, Proteus*, and *Vibrio cholerae* (Fauquet et al. 2005). As *Salmonella* phages that infect multiple genera are quite common (Bielke et al. 2007), we investigated the host range of ΦSP-3. Out of 96 cultures tested, ΦSP-3 was able to infect 33 *Salmonella* Enteritidis strains, including the standard strain *Salmonella* Abony and clinical strain *Salmonella* Typhi. However, bacteria belonging to other genera, including the close relative *E.coli*, were found to be resistant to ΦSP-3 infection, indicating its narrow host range in contrast to other reported T5-like phages (Bielke et al. 2007). The broad range within the genus and the inability to infect non-targeted beneficial bacterial populations makes ΦSP-3 a desirable candidate as a biocontrol agent.

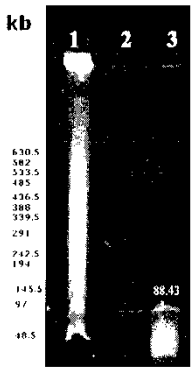

Fig. 2 Pulsed-field gel electrophoresis of ΦSP-3 DNA. *Lane 1* lambda DNA Concatamers, 1,000 kb (New England biolabs); *lane 3* ΦSP- 3 DNA

ΦSP-3 propagation on S 49 under nutrient-depleted state

The physiological state of the host, characterized by levels and activities of host cellular functions, plays a pivotal role in phage infection and propagation (You et al. 2002). The infection and propagation of a phage on a susceptible bacterial host can be modulated with alterations in the growth medium under laboratory conditions (Hedén 1951, Hadas et al. 1997). ΦSP-3 multiplication was maximum when the host was in logarithmic phase [plaque-forming units (PFU): $\log_{10}$ 9.81±0.10] as depicted in Fig. 3. Successful phage infection yielded the maximum number of progeny when phages were added to the bacterial host growing in optimal conditions, a situation not frequent in the natural environment (Lenski et al. 1988). Bacterial cells entering into stationary phase undergo substantial changes in cell morphology, including metabolism and surface characteristics (Kjelleberg et al. 1987) that may

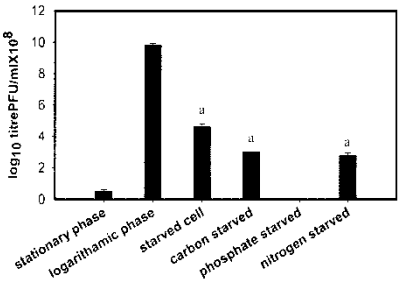

Fig. 3 ΦSP-3 propagation in nutrient-deprived condition. *a* indicates $p<0.001$ when compared to stationary phase

negatively interfere with phage infection (Sillankorva et al. 2004). However, in this study, ΦSP-3 was able to infect the host under stationary phase, although in low numbers (log $_{10}$ 0.51±0.07). Limitations in nutritional factors are known to limit the phage propagation (Miller and Day 2008), though ΦSP-3 was able to multiply even under multiple nutrient-starved states as evidenced by a significant level of increase in phage titer, log $_{10}$ 4.61±0.15 when compared to stationary phase ($p<0.001$). Bacteria grown in carbon-starved condition are reported to defy phage infection (Marcin et al. 2007), but ΦSP-3 was able to infect the host (PFU: $\log_{10}$ 3) ($p<0.001$ when compared to stationary phase) even under such a nutrient-deprived state. However, under phosphate-starved condition, ΦSP-3 failed to multiply. The inability to multiply under phosphate-starved condition can be attributed to the higher nucleic acid to protein ratio in viruses (Bratbak et al. 1993). Nitrogen starvation results in alteration of bacterial cell surface hydrophobicity (Borges et al. 2008) that can temporarily make the bacteria resistant toward phage infection (Sijtsma et al. 1990). This may be the reason for failure of most of the phages to infect the host under such conditions. However, ΦSP-3 was able to successfully propagate in the host S 49 (PFU: 2.81±0.13) ($p<0.001$ when compared to stationary phase) even under nitrogen-limiting conditions. In all the cases, except under stationary phase, phages were able to outnumber the host (data not shown).

In conclusion, the potential of a *Salmonella*-specific lytic phage to infect its host under various nutrient-limited conditions was established. There are only a few reports on phages competent to infect their host under both nutrient-rich and nutrient-deprived conditions (Chibani-Chennoufi et al. 2004). This ΦSP-3 quality, a characteristic required to be an effective biocontrol agent, is of special significance, also considering that the bacterial pathogen *Salmonella* is a tough foe to combat especially in this age of antibiotic resistance (Sulakvelidze et al. 2001).

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Conflict of interests The authors declare that they have no conflict of interests

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Research Paper

Isolation and partial characterization of ΦSP-1, a *Salmonella* specific lytic phage from intestinal content of broiler chicken

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Salmonella enterica subsp. *enterica* serovar Enteritidis is a major causative agent of gastroenteritis with contaminated eggs and chicken meat being the major source of infection. Phages are seriously being considered as a safe and cheaper alternative to antibiotics. The intestinal content of chicken was used as source for isolating phages. Phage designated as ΦSP-1 was selected for the study. Transmission electron microscopy (TEM) of phage ΦSP-1 revealed that it belonged to family *Podoviridae*. The optimal multiplicity of infection (MOI) was 5 phages/cell. Latent and rise period were calculated to be 30 and 55 minutes respectively, while burst size was 44 phages/bacterial cell. The genome size of ΦSP-1 was estimated to be 86 kb from pulsed-field gel electrophoresis analysis (PFGE). The effect of different physical and chemical parameters like temperature, pH, salinity and $CaCl_2$ were analyzed to optimize the conditions for large scale production of phages and to check the viability of ΦSP-1 under different physiochemical conditions. A temperature of 40 °C, pH 8 and 0.25 M NaCl were found to be optimum for phage adsorption and it was able to survive up to a temperature of 50 °C for 3 min. Capability to survive under hostile environmental conditions, absence of virulence genes in genome and genus specificity suggest suitability of ΦSP-1 to be used as a biocontrol agent.

Abbreviations: Transmission electron microscopy (TEM), multiplicity of infection (MOI), pulsed-field gel electrophoresis analysis (PFGE), Plaque forming unit (PFU), Tris EDTA (TE), National Collection of Industrial Microorganisms (NCIM)

Keywords: *Salmonella* Enteritidis / Lytic phage / Biocontrol agent / Antibiotic resistance

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Introduction

Salmonella is Gram negative bacterium known worldwide as the causative agent of gastroenteritis, bacteremia and enteric fever [1, 2]. Majority of human gastroenteritis have been attributed to the consumption of *Salmonella* contaminated eggs and chicken meat [1]. The wide use of antibiotics as therapeutic and growth promoting agents in animal husbandry [3] has enhanced the emergence of antibiotic resistant bacterial strains among farm animals, which in due course reach humans [4]. Phages (bacterial viruses) are now being reconsidered as a potential and safe alternative to antibiotics. Lytic phages were shown to reduce *Salmonella* colonization in broiler chickens [5]. In the study three phages exhibiting broad host range were given in antacid suspension to birds which were experimentally infected and two phages were able to significantly reduce caecal colonization of pathogen. Similar results were reported where phages isolated from poultry and human sewage sources were able to reduce *Salmonella* contamination in chickens both *in vitro* and *in vivo* conditions [6]. Application of bacteriophages at high MOI on chicken carcass surface skin proved to eliminate bacterial contamination efficiently [7]. The potential of

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phages in controlling *Salmonella* in experimentally contaminated sprout seeds was studied [8]. The efficacy of orally administered bacteriophages in reducing the concentration of *Salmonella* Enteritidis in caecal contents of broilers has also been proved [9]. Experiments with the well characterized lytic bacteriophage Felix 01, proved their capability in reducing multidrug resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium burden in mice [10]. The potential of phages as alternatives to antibiotics [11] and for phage therapy [12–14] has been reviewed. The objective of the present study is to isolate and characterize *Salmonella* specific lytic phage from the intestinal content of broiler chicken and to consider its potential application as a biocontrol agent.

Materials and methods

Salmonella host culture

The host, isolated from eggs obtained from the retail market, was used for phage isolation and propagation throughout this study. *Salmonella* was isolated following the guidelines of Bacteriological analytical manual, American food and drug administration. Briefly, samples were incubated in lactose broth at 37 °C for 24 h for pre enrichment followed by selective enrichment in Rappaport–Vassiliadis and tetrathionate broth at 42 °C for 24 h. A loopful of enriched sample was then streaked on to xylose lysine desoxycholate and Hekteon enteric agar. Typical colonies on selective plates were screened based on key biochemical reactions for *Salmonella*. The culture was confirmed to be *Salmonella* Enteritidis by serotyping at National *Salmonella* and *Escherichia* Centre, Kasuali, Himachal Pradesh, India. For further conformation a portion of the 16S rRNA gene (1.5 kb) was amplified from the genomic DNA [15–18]. The amplicon was sequenced and compared with the sequences in Genbank entries, by BLAST programme [19]. The partial sequence was deposited in the Genbank database (Accession number: HQ 268500).

Bacteriophage isolation

The intestinal content of broiler chicken was homogenized in sterile physiological saline, centrifuged at 4000 × g for 10 min at 4 °C, filtered through 0.22 μ membrane filter (Millipore, USA) to make them bacteria-free and the filtrate was screened for the presence of phage (direct method). For the enrichment method, equal volumes of the crude lysate and host (in log phase) were mixed in double strength nutrient broth, incubated for 12 h, after which it was made bacteria-free by centrifugation and filtration. The presence of bacteriophages in the filtrate was confirmed by double agar overlay method [20]. Purification, large scale production and concentration of phage lysate were performed as previously described [21]. For purification, single plaque was picked with a sterile tooth pick and was introduced into 3 ml of log phase culture of host. It was then incubated at 37 °C in an environmental shaker (Orbitek, Scigenics, India) at 120 rpm for 12 h. This was then centrifuged at 10000 × g followed by filtration through 0.22 μ membrane (Millipore, USA). The lysate obtained was then used for double agar overlay. This procedure was then repeated 6 times until uniform sized plaques were obtained on the plate. For large scale production of phages, plates with uniform sized plaques covering the entire plate were overlaid with 10 ml of SM buffer and were incubated overnight with gentle rocking. After incubation the phage suspension from the plate was recovered and pooled. Chloroform was added to this pooled mixture to a final concentration of 5% (v/v). This suspension was mixed well using a vortex mixer and incubated at room temperature for 15 min. The cell debris was removed by centrifugation at 5000 × g for 10 min. The supernatant was transferred to a sterile polypropylene tube and chloroform was added to a final concentration of 0.3% (v/v) and stored at 4 °C until use. Plaque forming unit (PFU) of 1×10^9/ml was produced following this method.

Morphological analysis by Transmission Electron Microscope

A drop of high titer phage sample was spotted onto a carbon-coated TEM grid, stained with 1% uranyl acetate, visualized and photographed using a TEM (Model JOEL JEM-1011) operated at 80 kV. The electron micrograph was taken at a magnification of 180000×.

Optimal multiplicity of infection

MOI is the ratio of phage particles to host bacteria. It is calculated by dividing the number of phage added (volume in ml × PFU/ml) by the number of bacteria added (volume in ml × colony forming units/ml). Optimal MOI was determined [22] and MOI giving maximum yield was considered as optimal MOI.

One-step growth curve

One step growth curve experiment was performed and the graph was plotted with log of PFU against time. The latent period, the rise period and the burst size of the phage were calculated from one step growth curve [23].

ıfluence of physical and chemical parameters n phage viability/infectivity

emperature, NaCl, pH and sugars: The influence of ɔmperature on phage propagation was studied at temeratures ranging from 50 °C to 100 °C [22]. In order ɔ study the influence of NaCl on phage viability, laCl solutions of varying molar concentrations such as .1 M, 0.25 M, 0.5 M, 0.75 M, 1 M, 2 M, and 3 M were repared in deionised water and incubated for 30 min t 37 °C [23]. Influence of pH on phage viability was valuated by incubating the phages in suitable buffers f different pH, ranging from 2–11 [23]. Effect of varius sugars like arabinose, dextrose, galactose, fructose, ıaltose, mannitol, mannose, lactose, rhamnose, ribose nd xylose on phage infectivity was studied [23] with ıinor modifications. Sugars were added to a final conɛntration of 500 mM to each phage sample. All samles after incubation were assayed using double agar verlay plate method to determine the number of surviving PFU. The results were compared with control tre in case of effect of sugars and then expressed as a ercentage of phage inactivation.

ıfluence of physical and chemical parameters n phage adsorption

emperature, NaCl, pH and calcium ions: The adsorpon of phages on the host *Salmonella* Enteritidis was etermined at temperatures of 0 °C, 10 °C, 20 °C, 30 °C, 7 °C, 40 °C, 45 °C and 50 °C [23]. Influence of different ɔncentrations of NaCl (0.1 M, 0.25 M, 0.5 M, 0.75 M ıd 1 M) on adsorption was also investigated [23]. Adɔrption rate of ΦSP-1 was determined at the pH values ınging from 2–11 [23]. The influence of calcium ions ı mM, 1 mM, 10 mM, 20 mM and 30 mM concentraons) on phage propagation was determined [22]. In all ıe experiments the supernatant obtained after cenifugation was assayed using double agar overlay ıethod for unabsorbed free phages and the counts ere compared with control titre. The results were ɛpressed in percentages of adsorption.

latistical analysis

ll the experiments were conducted in triplicates and .otted with ±SD.

acteriophage genome analysis

ıage DNA isolation: Phage DNA extraction was done ı previously described [21]. Briefly, 1 ml of the concenated phage suspension was incubated at 56 °C with ·oteinase K (50 μg/ml) and SDS (0.5%) for one hour. fter incubation, the digestion mix was extracted once ith equal volume of phenol, once with 50:50 phenol and chloroform, and finally with equal volume of chloroform. The DNA was then precipitated with double volume of ethanol in presence of sodium acetate and dissolved in Tris EDTA (TE) buffer (pH 7.6).

Pulsed-field gel electrophoresis of phage DNA

Phage DNA was run in 1% agarose gel SeaKem® Gold Agarose, (Pulsed-field grade) in 0.5× TBE buffer (50 mM Tris-HCl, 50 mM boric acid, 1 mM EDTA) and electrophoresis was carried out in a CHEF DRII PFGE system (Bio-Rad, USA). The gel was run at 4.5 volts/cm using ramped pulse times from 5 to 120 sec for 18 h at 15 °C. Bacteriophage lambda DNA, 1000 kb (New England BioLabs) was used as molecular weight marker. The gel was stained with Ethidium bromide and photographed. The molecular weight was calculated using Quantity One® software (BioRad, USA).

Screening for virulence/virulence related genes in host and phage

PCR based screening method was adopted to check the presence of virulence genes in the phage and host genome. The details of the primers used are given in Table 1. A total of 11 virulence genes – *invE/A, slyA, pho P/Q, ttrC, mgtC, spi4R, sopE, gog B, sod, gtg E, agfA* were screened. Multiplex PCR was performed with set 1 containing *slyA, invE/A and afgA*, set 2 containing *ttrC, mgtC and phoP/Q*, set3 containing *spi4R, gogB, sod*, and set 4 containing *sopE* and *gtgE* in Bio Rad MJ Mini™ Gradient Thermal Cycler. Reaction volume was set to 20 μl with 100 ng template DNA, 200 μM each dNTP (Sigma-Aldrich), 1 μM each primer (Sigma-Aldrich), 1U Taq DNA polymerase and amplification buffer (Sigma-Aldrich). The program followed was a hot start cycle of 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, ending with a final extension of 72 °C for 5 min.

Host range studies

The host range of the phage was assessed on the basis of their ability to form plaques on *Salmonella* Enteritidis strains present in the microbial genetics lab culture collection. A total of 96 cultures were tested including the standard strains, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (National Collection of Industrial Microorganisms (NCIM) no. 2501), *Salmonella enterica* subsp. *enterica* serovar Abony (NCIM no. 2257), *Escherichia coli* (NCIM no. 2343), *Klebsiella pneumoniae* (NCIM no. 2957) *Pseudomonas aeruginosa* (NCIM no. 2863), *Bacillus cereus* (NCIM no. 2155), *Staphylococcus aureus* (NCIM no. 2127) and *Proteus vulgaris* (NCIM no. 2027).

Table 1. Primers used to screen for the virulence and virulence related genes in ΦSP-1 *Salmonella* Enteritidis.

Virulence gene	Sequence 5′–3′	Function	Reference
invE/A	Forward – TGCCTACAAGCATGAAATGG Reverse – AAACTGGACCACGGTACAA	Invasion to the host	[45]
slyA	Forward – GCCAAAACTGAAGCTACAGGTG Reverse CGGCAGGTCAGCGTGTCGTGC	Production of cytolysin	[45]
pho P/Q	Forward – ATGCAAAGCCCGACCATGACG Reverse – GTATCGACCACCACGATGGTT	Resistance within macrophages	[45]
ttrC	Forward – GTGGGCGGTACAATATTTCTTTT Reverse – TCACGAATAATAATCAGTAGCGC	Vital for tetrathionate metabolism	[46]
mgtC	Forward – TGACTATCAATGCTCCAGTGAAT Reverse – ATTTACTGGCCGCTATGCTGTTG	Survival within macrophages and growth in low Mg^{2+} environments	[46]
spi4R	Forward – GATATTTATCAGTCTATAACAGC Reverse – ATTCTCATCCAGATTTGATGTTG	Intra-macrophage survival and may also contribute to toxin secretion	[46]
sopE	Forward – CCGTGGAACGATTGACTG Reverse – AGCCATTAGCAGCAAGGT	Type III effecter involved in invasion	[47]
Gog B	Forward GCTCATCATGTTACCTCTAT Reverse – AGGTTGGTATTTCCCATGCA	Lambdoid prophage gene	[48]
sod	Forward – TATTGTCGCTGGTAGCTG Reverse – CAGGTTTATCGGAGTAAT	Protects from products of phagocyte NADPH-oxidase and nitric oxide synthase of host cell	[48]
gtgE	Forward – AGGAGGAGTGTAAAGGT Reverse – GTAGAACTGGTTTATGAC	Lambdoid prophage gene	[48]
agfA	Forward – TCCGGCCCGGACTCAACG Reverse – CAGCGCGGCGTTATACCG	Binding to fibronectin of host	[49]

Results

Bacteriophage isolation

A lytic phage designated as ΦSP–1 was isolated and all results are discussed with respect to phage ΦSP-1. ΦSP-1 consistently produced small clear and round plaques of 0.5 mm to 1 mm size, with well-defined edges.

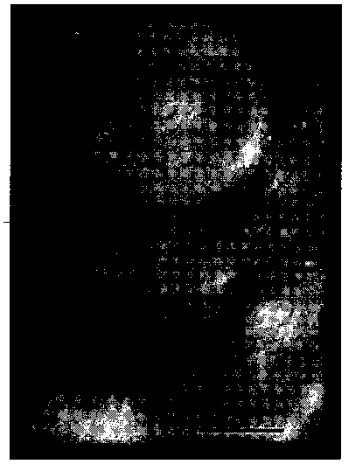

Figure 1. Electron micrograph (TEM) image of phage ΦSP-1 negatively stained with 1% uranyl acetate. Bar: 20 nm.

Morphological analysis

Figure 1 shows the transmission electron micrograph of ΦSP-1 at a magnification of 180000×. The TEM picture revealed identical hexagonal outlines indicating their icosahedral nature, with tail less head.

Optimal multiplicity of infection

MOI for ΦSP-1 was found to be five phage particles per bacterium (Data not shown).

One step growth curve

The one step growth curve using log phase host cells at 37 °C (Fig. 2) was used to study the growth kinetics of ΦSP-1. The calculated latent period was approximately 30 min, the rise period was 55 min and the burst size was 44 phages per bacterial cell. The multiplication period reached a plateau at about 80 min after infection with ΦSP-1.

Influence of physical and chemical parameters on phage viability

Effect of different temperatures and its effect on phage viability are presented in Fig. 3. Phage viability was drastically reduced at higher temperatures. Viable PFU was highest when exposed to 50 °C for 3 min. Although exposure to 80 °C was fatal over an exposure period of 3 min, there were nevertheless a few survivors. ΦSP-1 failed to survive exposure to 90 °C and 100 °C, even for very short time. In all cases, phage count steadily decreased with increase in exposure time to different

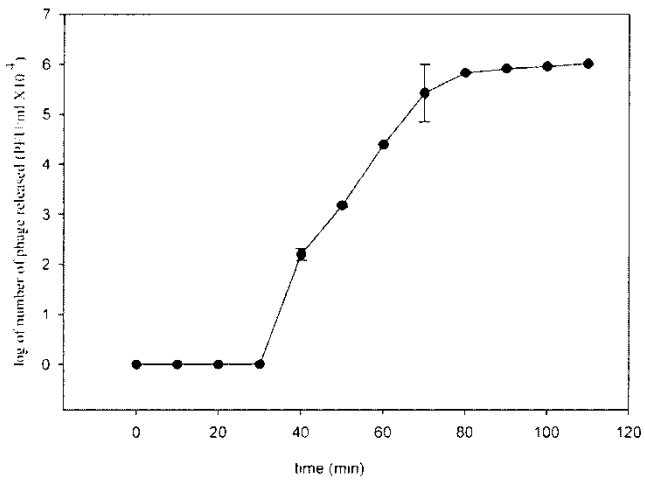

Figure 2. One step growth curve of phage ΦSP-1.

temperatures. The optimum concentration of sodium chloride in the medium for phage survival was found to be 0.1 M (Fig. 4). There was considerable reduction in viability at concentrations higher than 0.1 M NaCl and beyond 3 M NaCl phages did not survived. Fig. 5 shows the effect of pH on phage viability. From the figure it is obvious that pH 8 is optimum for survival of ΦSP-1. Viability was observed even at pH as high as 12, although in small numbers. The influence of eleven different sugars on ΦSP-1 viability is depicted in Fig. 6. Rhamnose, maltose and xylose resulted in a drastic inactivation of ΦSP-1 as much as 95%, 91% and 89% respectively. Phage inactivation by lactose was 85%, 80% by mannose and mannitol and 74% by galactose. Dextrose and ribose caused 65% and 64% inactivation, respectively. Arabinose caused only 35% inactivation, while fructose had the least effect, with only 2% inactivation.

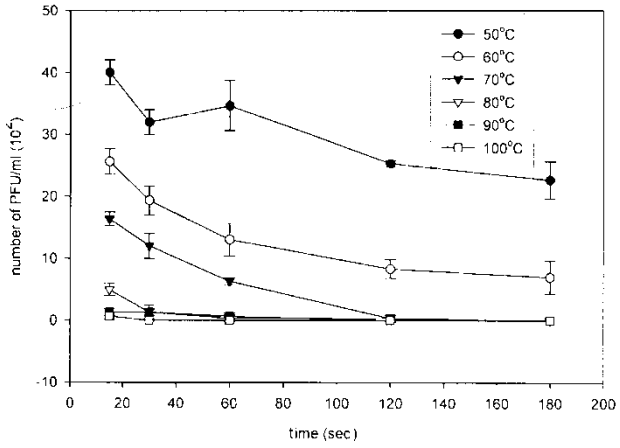

Figure 3. Effect of temperature on viability of ΦSP-1.

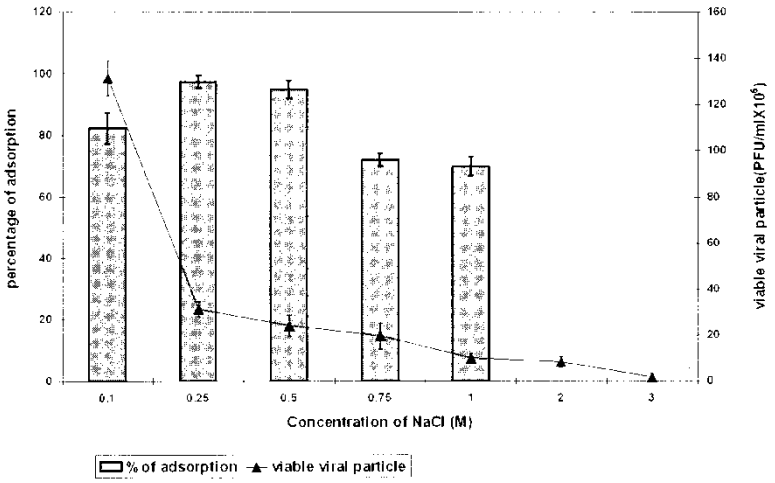

Figure 4. Effect of NaCl on the viability and adsorption of ΦSP-1 to host cells.

Influence of physical and chemical parameters on phage adsorption

At a temperature of 0 °C there was no observable adsorption. But there was a steady increase in adsorption as the temperature was raised to 10 °C. The optimal temperature for maximum adsorption was found to be at 37 °C and 40 °C, 70% adsorption occurred at 45 °C and 45% adsorption at 50 °C (Fig. 7). The influence of sodium chloride on adsorption is presented in Fig. 4. Optimal NaCl concentration for maximal adsorption

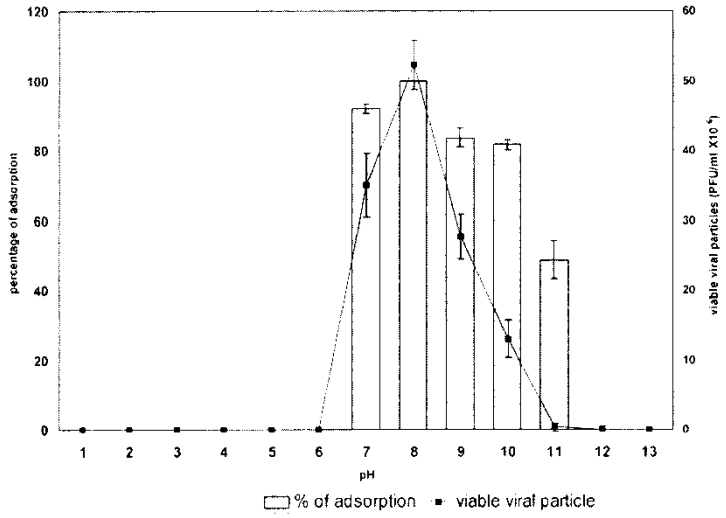

Figure 5. Effect of pH on viability and adsorption of ΦSP-1 to host cells.

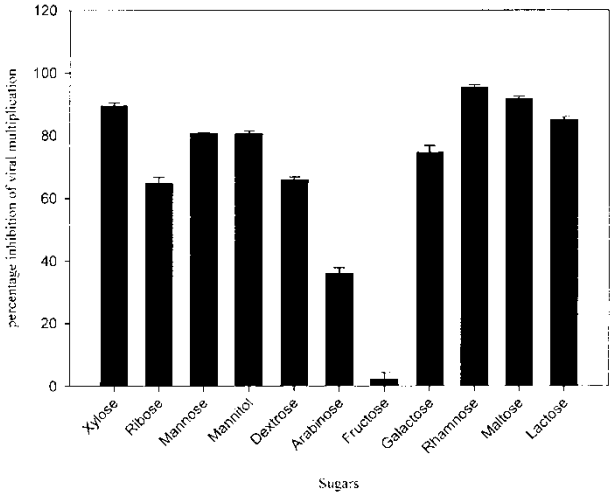

Figure 6. Effect of sugars on the infectivity of ΦSP-1.

was found to be 0.25 M. The influence of pH on the adsorption of ΦSP-1 to host cells is presented in Fig. 5. The optimal pH was found to be 8 for adsorption. In this study phage propagation was found to be optimum at 1 mM as well as at 10 mM $CaCl_2$. At higher concentration of 20 mM and 30 mM PFU dropped significantly (Fig. 8).

PFGE of ΦSP-1 DNA

The genome size of ΦSP-1 was determined to be 86 kb by comparing it with the 1000 kb ladder using Quantity One® software (BioRad, USA) (Fig. 9).

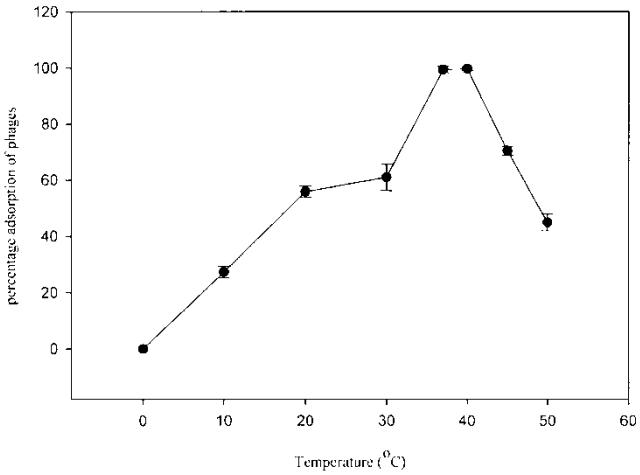

Figure 7. Effect of temperature on adsorption of ΦSP-1 to host cells.

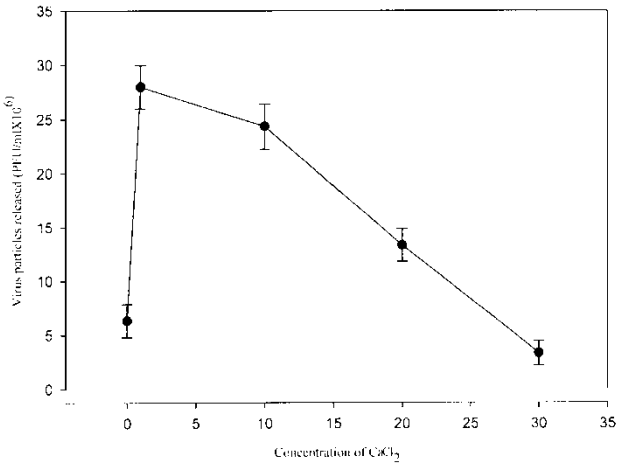

Figure 8. The effect of $CaCl_2$ on propagation of ΦSP-1.

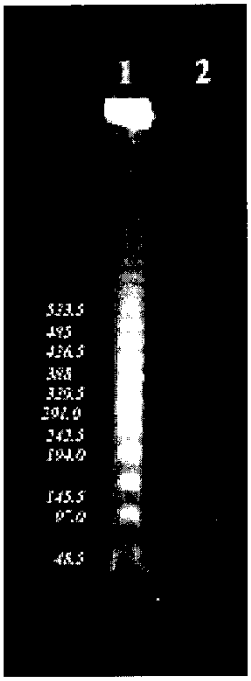

Figure 9. Pulsed-field gel electrophoresis of ΦSP-1 DNA. Lane 1: lambda DNA concatamers, 1000 kb; Lane 2: ΦSP-1 DNA.

Screening for virulence/virulence related genes in host and the phage

A set of eleven virulence genes common among *Salmonella* Enteritidis were selected for screening. The multiplex PCR confirmed the absence of all the eleven virulence genes in the ΦSP-1 genome. PCR with bacterial host indicated the presence of the following virulence genes: *invE/A* (457bp), *afgA* (261bp), *slyA* (700bp), *pho P/Q* (299bp), *ttrC* (920bp), *and mgtC* (655bp) (figure not shown) confirming its pathogenicity.

Host range studies

A total of 31 *Salmonella* Enteritidis strains including the standard strain *Salmonella enterica* subsp. *enterica* serovar Abony were found to be sensitive towards ΦSP-1. But bacteria belonging to other genera including the close relative *E. coli* were found to be resistant towards phage infection.

Discussion

Bacteriophage isolation

Bacteriophages, being natural viral pathogens of bacteria, co-exist with their hosts sharing the same niches [24]. Human infection with *Salmonella* Enteritidis has been attributed mainly to poultry and poultry-derived products [25, 26] and hence chicken caecum, considered to be its primary colonization site [27, 28], was used for phage isolation. A large number of morphologically different plaques were obtained even in a single sam-

pling. The phage ΦSP-1, showing consistent bacterial cell lysis capacity was selected for further study.

Morphological analysis

The TEM picture (Fig. 1) of ΦSP-1 showing icosahedral structure without tail, suggested morphological traits characteristic of family of *Podoviridae* according to International Committee for the Taxonomy of Viruses. These morphotypes have been previously found associated with the genus *Salmonella* [29].

One step growth curve

The growth kinetics of ΦSP-1 shared a similar pattern with the well studied, *Salmonella* specific lytic phage, Felix 01 [30]. However, the results obtained can only be used/evaluated with caution as the phage multiplication kinetics may vary depending on the physiological state of the host cell [31], the environmental conditions and highly controlled lab conditions [32]. The optimal MOI and the data from growth kinetics study of ΦSP-1 were used in subsequent large scale production of ΦSP-1.

Influence of physical and chemical parameters on phage viability and adsorption

Various environmental conditions have a significant effect on viability and most importantly on adsorption, a very crucial step in phage infection [23]. The effects of various physical and chemical parameters such as temperature, salinity, pH, presence of calcium ions and sugars, on these two aspects were studied, as this would aid in optimization of large scale phage propagation in the lab. Ability of ΦSP-1 to survive at temperatures as high as 80 °C is one of the many very desirable traits for consideration, especially in its application as biocontrol agent in surface pasteurization of poultry foods where hot water is applied for a short period of time. Its adsorption at low temperatures finds application as biocontrol agent in cold storage of food products.

Optimal concentration of NaCl in phage preparation is known to confer protection to phages especially against high temperature [33] and is therefore a very significant factor. Taking this into consideration, the viability of phage ΦSP-1 in the presence of varying concentration of NaCl was studied. It was observed that the phages were highly stable at a minimal concentration of sodium chloride (i.e., 0.1 M). The optimum pH range for phage viability and adsorption is between 5 and 8 [20]. ΦSP-1 also exhibited a similar pattern. The affinity of ΦSP-1 for slightly alkaline environment is easily explained, as they were isolated from intestinal content, where the pH normally is 7 and higher in caecum [34].

Capsular polysaccharides of Gram negative bacteria are known to be directly involved in phage host interaction [35]. It can be inferred that sugars like rhamnose, xylose, maltose or their analogues may have a key role as phage receptors on the host surface outer membrane, as their presence in the host phage medium during adsorption stage effectively inhibited the process. Rhamnose was reported to be a determinant of a phage receptor in *Lactobacillus casei*. Bacterial phage inactivation by free sugars like D-glucosamine, D-mannose and L-rhamnose was demonstrated [36]. Francisco and Pasquale also suggested the possibility that phage 2 receptors in lipopolysaccharide contain L-rhamnose, D-glucosamine, and (or) D-glucose, or a structurally related molecule [37].

Adsorption is not only dependent on the presence of specific receptor on the cell surface but also on the presence of certain cations in the media. Phages have a calcium requirement, the concentration of which vary from one phage to another [38]. The optimum calcium chloride concentration was found to be 1 mM for ΦSP-1. Similar results were obtained previously with other bacteriophages [39, 40].

Bacteriophage genome analysis

The nature of phage genome was identified as double stranded DNA on the basis of its sensitivity to digestion by restriction endonucleases (result not shown). The double stranded nature of DNA places them under the order *Caudovirales*. Thus nucleic acid type and the morphology of the phage satisfy the criteria for placing them in the viral family of *Podoviridae*.

Screening for virulence/virulence related genes in Host and phage

Acquisition of virulence factors by bacterial pathogens via mobile genetic elements like bacteriophage is a common affair. In the process phages can convert a non-pathogenic strain to a virulent form or a virulent strain to a more virulent one [12]. So whenever a whole phage preparation is intended for use as biocontrol agent, confirmation of the absence of any associated virulence genes in their genome is important to prevent the possibility of horizontal transfer of virulence genes during the phage host interaction. The multiplex PCR of selected genes confirms that ΦSP-1 does not harbor these virulence genes. Although full genome sequencing is necessary to completely negate the possibility of ΦSP-1 harboring virulence genes, these results take ΦSP-1 a step forward towards its candidature as biocontrol agent against *Salmonella* Enteritidis. The presence of the virulence genes in the host indicates its pathogenic nature.

Host range studies

ΦSP-1 was found to infect a total of 31 *Salmonella* strains belonging to different serotypes, but was unable to infect bacteria belonging to other genera including its close relative *E. coli*. Specificity is the primary requisite of a phage to be used as a biocontrol agent. Even though *Salmonella* bacteriophages are generally host specific and often infect only one bacterial species or only one serotype within a species [41], there are several reports on phages that productively infect a range of bacterial species crossing the genus barrier [42]. *Salmonella* phage that infects multiple genera is also not uncommon [43]. For a biocontrol agent to be effective it should not infect non targeted beneficial bacterial population but at the same time it has to be genus specific infecting different species within a genus. This is especially true in case of *Salmonella* where large numbers of pathogenic strains exist within the genus [44]. ΦSP-1 fulfills these criteria by specifically infecting and causing lysis of only *Salmonella* Enteritidis.

In the present study a very potent biocontrol agent exhibiting excellent properties like high stability, specificity, absence of virulence genes and sturdiness under various physical and chemical exposures was isolated. The candidature of ΦSP-1 as a biocontrol agent is promising. However, more research is required to complete their characterization and to develop a phage cocktail with more phages which will be more effective.

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Competing interests

The authors declare that they have no competing interests.

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