II. REVIEW OF LITERATURE

2.1. *Salmonella*

*Salmonella* spp is one of the most important causative agents of food borne diseases throughout the world (Lacey, 1993). The *Salmonella* is a Gram negative bacterium that is responsible for a variety of disease syndromes in humans and domesticated animals. There are more than 2200 serotypes of *Salmonella* and most of them are considered human pathogens (Pelzer, 1989). *Salmonella* is responsible for an estimated 16 million annual cases of typhoid fever, primarily in developing countries and millions of unreported cases of gastroenteritis in both industrialized and developed countries (Groisman *et al*., 1999). Infections with *Salmonella* are typically acquired through the consumption of contaminated water or food (Alford and Palumbo, 1969).

*Salmonella* infection can be manifested in three forms, *viz.*, gastroenteritis - involving an abrupt onset of nausea, fever, vomiting and diarrhea; enteric fever (typhoid fever) – usually caused by *S. Typhi* and septicemia – characterized by fever, chills, anorexia, anemia and focal lesions on visceral organs.

2.2. History

Typhoid fever was the first salmonellosis ever recognized (Le Minor, 1981). The disease was defined with clinical signs and symptoms long before the bacteriological era began. Petit and Serres pointed out the intestinal ulcerations in 1813 for the "enteric Fever" (later came to be called as typhoid). But then, the disease was not clearly distinguished from enteric tuberculosis.

Louis in 1829 described the disease under the present name of typhoid. Budd in 1859 pointed that each case of typhoid was epidemiologically related to the former case and that a specific toxin was disseminated with the patient’s feces. Eberth, in 1880 observed the typhoid bacillus for the first time in spleen sections and mesenteric lymph nodes of a typhoid patient. Koch in 1881 confirmed the finding and in 1884, succeeded in cultivating the organism. Widal in 1896, Grunbaum in 1896 and Widal and Sicard in 1897 showed that the serum of typhoid patients agglutinated typhoid bacillus. It was then the serodiagnosis of typhoid bacillus became possible.

The first antigenic scheme for *Salmonella* (White, 1926) was developed by Kauffman (Kauffman, 1966b; 1978). The Kauffman White Scheme contained 100 serotypes in 1941 (Kauffman, 1941). In 1988, this scheme explained about 2,250 serotypes (Le Minor and
Popoff, 1988). During that time bacteria resembling “paratyphus” was reported from diseased animals. Salmon and Smith in 1885 first successfully isolated the bacteria from pigs with hog cholera and they thought that they had isolated the infectious agent of hog cholera. Also similar bacteria became continuously isolated from food intoxications and animal diseases. This name ‘Salmonella’ was coined in the honor of Dr. Salmon who isolated the then thought to be infectious agent of hog cholera. A bacterial genus was created to include these bacteria and named Salmonella by Ligni’ères (Ligni’ères, 1900).

2.3. Taxonomy and Nomenclature

Salmonella is a member of the family Enterobacteriaceae and has a DNA base composition of 50-53 G+C % (Marmur et al., 1963). Kauffman (1963, 1966a) subdivided the genus into four subgenera, I, II, III and IV. The subgenus III corresponds to bacteria called Arizona. A second taxonomic system was proposed by Edwards and Ewing (1972) according to which the entire Salmonella genus was limited to Kauffman subgenus I. The Kauffman subgenus III corresponding to bacteria Arizona was placed as a separate genus by Edwards and Ewing and the Kauffman subgenera II and IV were considered as atypical strains of either Salmonella or Arizona.

During early days of Salmonella identification the species name was given according to clinical considerations, e.g., S. Typhi. Serological analysis was initiated by White (1926) and the extension of this work was done by Kauffman who defined the species as a group of fermentation and phage types (Kauffman, 1941, 1966a, 1978) and considered each serovar as a species. As the host specificity suggested earlier did not exist, the next chosen methodology was the name derived from the geographical origin of the first isolated strain of the newly discovered serovars e.g., S. London, S. Panama, S. Bareilly, S. Hissar etc. It was after the International Congress in 1966, the geographical names were written as simple names e.g., S. Typhimurium.

Even as Kauffman proposed subgenera, he did not propose a formal nomenclature. The situation became more confusing when serovars of subgenera I, II and IV had species while subgenus III did not have. The latter was designated only by antigenic formulae. Thus, the taxonomy of Salmonella had been a controversial issue until DNA related analysis came in to the picture.

This taxonomic confusion was ultimately put to rest by the rapid advancement of the DNA relatedness studies. These studies showed that all Salmonella serovars form a single DNA hybridization group. The thermal stability of these DNA hybrids delineated seven
subgroups. The level of relatedness among DNA subgroups is consistent with that of subspecies within one Salmonella species, although subgroup V (subspecies Bongori) is somewhat less related and could possibly represent a second species (Cross et al., 1973; Stoleru et al., 1976; Le Minor et al., 1982). Subspecies are designated as Salmonella I, II, III (III a & III b) (corresponding to Kauffman’s subgenera III i.e., monophasic and diphasic Arizona), IV, V and VI. The last three are recently delineated. In short, the nomenclature adopted was as a single species composed of seven subspecies based upon the thermal stability of the DNA hybrids. The species name Salmonella enterica was then adopted and the following names were adopted for subspecies.

subspecies I  \[ S.\text{enterica} \ \text{enterica} \]
subspecies II  \[ S.\text{enterica} \ \text{salamae} \]
subspecies IIIa  \[ S.\text{enterica} \ \text{arizonae} \]
subspecies IIIb  \[ S.\text{enterica} \ \text{diarizonae} \]
subspecies IV  \[ S.\text{enterica} \ \text{houtenae} \]
subspecies V  \[ S.\text{enterica} \ \text{bongori} \]
subspecies VI  \[ S.\text{enterica} \ \text{indica} \]

Subspecies I strains represent more than 99.5% of Salmonella strains isolated from humans and other warm-blooded animals. Subspecies II and III include organisms frequently isolated from cold-blooded animals. Subspecies IV and V are found mainly in the environment and rarely pathogenic to human beings (Le Minor, 1981).

2.4. General characteristics of Salmonella

Genus Salmonella consists of a Gram –ve, oxidase –ve, straight sided, rod shaped bacteria, which are catalase +ve, and have both respiratory and fermentative metabolism of carbohydrates. There are three major groups of Salmonella based on the degree of host adaptation.

1. Human adapted serovars, viz., S. Typhi, S. Paratyphi and S. Sendai usually cause grave disease like Septicemia-typhoid syndrome or enteric fever. These serovars are not usually pathogenic to animals.

2. Serovars like S. Typhimurium are ubiquitous, i.e., they affect both man and a range of animals to cause gastrointestinal infections of varying severity. In addition to classical food poisoning, these serovars are involved in infantile and travellers diarrhea.
3. Serovars highly adapted to animal hosts include *S. Abortovis* (sheep) and *S. Gallinarum* (poultry). They usually produce no or very mild symptoms in man.

Human adapted serovars are generally transmitted by food. Ubiquitous serovars are transmitted either by water or person-to-person contact.

*Salmonella* are facultative anaerobic and non-spore forming bacteria. Motile forms have peritrichous flagella. They produce acid and gas from glucose. *Salmonella* are usually motile but *S. Pullorum*, *S. Gallinarum* and variants of other serovars are nonmotile. Most strains are aerogenic except *S. Typhi*. G+C content of DNA is 50-53%. *Salmonella* may harbor phages or plasmids that code for metabolic characters like H₂S production and lactose or sucrose fermentation, which are used for identification.

### 2.5. Habitat

The principal habitat of *Salmonella* is the intestinal tract of human beings and animals (Le Minor, 1981). *Salmonella* serovars can be found predominantly either in one particular host, ubiquitous or unknown habitat. Those associated with humans cause serious diseases and are often found in blood. The diseases like typhoid and paratyphoid are caused by strictly human adapted serovars. Such serotypes are mostly waterborne though they may be transmitted by person to person contact. *Salmonella* serovars like *S. Gallinarum*, *S. Abortusovis* and *S. Typhisius* have strictly avian, bovine and porcine hosts respectively. These host-adapted serovars cannot grow on minimal medium without growth factors (contrary to ubiquitous serovars).

Ubiquitous serovars (nonhost adapted), e.g., *S. Typhimurium* cause grave disease symptoms ranging from asymptomatic infections to serious typhoid like syndromes in infants or highly susceptible animals (mice). Mostly, ubiquitous *Salmonella* are responsible for causing food borne infections in human adults.

The pathological roles of many *Salmonella* serovars are still unknown. These serovars mainly belong to the subspecies II to VI which have been isolated rarely in cold-blooded animals.

### 2.6. *Salmonella* survival in water and soil

Water sources serve as reservoirs of *Salmonella* and aid in transmission between the hosts (Foltz, 1969). *Salmonella* are constantly released into the environment from infected humans, farm animals, pets and wildlife (Baudart *et al.*, 2000) and can survive up to 10 to 15
days in a septic system (Parker and Mee, 1982). Seepage from these septic tanks, sewage and storm run off facilitate bacterial entry into the surface waters. Detection of *Salmonella* is always correlated with proximity to sewage discharge area (Baudart *et al*., 2000).

In comparison to other pathogenic bacteria, *Salmonella* has a high survival rate in aquatic environments. *Salmonella* can be detected in the coastal marine environments receiving sewage and its prevalence does not vary seasonally and is independent of water temperature (Alonso *et al*., 1992). The *Salmonella* has a high survival rate following mixture of sewage effluent and brackish water, resulting in a dramatic decrease in salinity helps the survival of *Salmonella* (Mezrioui *et al*., 1995). Thus, *Salmonella* appears to withstand stresses associated with environmental fluctuation and may persist in water for a longer time.

*Salmonella* are widely disseminated in soil and sediment, water currents, underground springs and rain run-offs carrying contaminated material (Chao *et al*., 1987). Perculation of waste water through the soil filters the bacteria that become trapped in the environment where *Salmonella* can survive and multiply for atleast one year (Thomason *et al*., 1977). Soil and sediment particles function as an ecological niche for these pathogens to survive (Brettar and Holfe, 1992). Adhesion of *Salmonella* cells to soil particles correlates with cell surface hydrophobicity, typically manifested by modification of bacterial outer membrane. These adhesive properties of *Salmonella* along with the nutrient rich growth conditions provided by the association with soil may account for the 100% *Salmonella* positivity in the bottom sediment samples rather than surface water samples, obtained downstream of sewage treatment plants (Hendricks, 1971). The ubiquitous nature and high rate of survival of *Salmonella* in water and soil environments may represent a critical adaptation to its cyclic lifecycles involving host and non-host environments.

### 2.7. Foods associated with *Salmonella*

*Salmonella* are usually associated with all kinds of meat. Animal salmonellosis may result in the contamination of meat which ultimately leads to the contamination of the muscles during evisceration, transportation of carcasses, washing, etc (Le Minor, 1981). Surface contamination of *Salmonella* is usually of no significance and proper cooking will eliminate it. But if the meat is ground *Salmonella* may multiply within the meat and superficial cooking may not eliminate the bacteria. For successful infection and symptoms, it is necessary that *Salmonella* be ingested with food, which supports its multiplication. This is because significant amounts of *Salmonella* are needed to initiate an infection.
Prevention of *Salmonella* infection thus involves avoidance of contamination, preventing multiplication of *Salmonella* in food (constant storage at 4°C) and use of pasteurization and sterilization wherever possible (Le Minor, 1981).

### 2.8. *Salmonella* in seafood

The marine fish collected from the open sea is free from *Salmonella* but fish collected from polluted coastal waters contain this organism (Shewan, 1962). Earlier reports have indicated that the contamination of marine products with *Salmonella* from fishing boats (Morris *et al.*, 1970), utensils used for processing (Anderson *et al.*, 1971), food handlers (Lee, 1973), process water (Morris *et al.*, 1970) and surroundings of the processing plant (Shewan, 1962). Such external contaminations play a major role in the presence of *Salmonella* in fish and other foods of aquatic origin. These bacteria being enteropathogens, finds their way from excreta and droppings or the intestinal contents of many animals into sewage, riverine water, estuarine and coastal waters. In the shrimp processing industry, the major sources of *Salmonella* include culture ponds, coastal waters and other materials used for handling and processing. Even rodent and lizard droppings have been implicated as possible sources.

Reports of the widespread presence of *Salmonella* in shrimp culture environment indicate that *Salmonella* may be a part of the natural population of brackishwater cultured shrimp (Leanphibul *et al.*, 1986; Iyer and Varma, 1990; Reilly *et al.*, 1992). Major source of *Salmonella* contamination in the shrimp pond sediment is due to the use of untreated animal manure for pond fertilization (Reilly *et al.*, 1992). Besides, aquatic birds (Kapperud and Rose, 1983; Reilly *et al.*, 1992) and the feeds (clam meat, formulated feed) also contribute to an additional source of *Salmonella* contamination. Reilly and Twiddy (1992) monitored brakish water ponds in one of the major prawn exporting countries in Southeast Asia and found that 16% of the prawns and 22.1% of the mud/water samples contained *Salmonella*. Formation of microbial biofilm by pathogenic *Salmonella* (Dhir and Dodd, 1995; Humphrey *et al.*, 1995; Joseph *et al.*, 2001) on contact surfaces may be undesirable and source of contamination in food processing industries. *Salmonella* strains entering food processing environments either through meat or carriers handling meat may survive in the premises with resultant formation of biofilms on various surfaces such as HDPE (high density polyethylene) used to produce crates to carry food products. Depending upon the processing conditions and the contact time, the biofilm formed upon these surfaces could be a source of contamination for foods passing through the same processing line (Joseph *et al.*, 2001). Such biofilms are
much more persistent and are resistant to the common sanitizers that are used in the food processing industry. The reported worldwide prevalence rates of *Salmonella* have varied widely. Wilson and Moore (1996) noted that 8% of the 433 shellfish samples contained *Salmonella*, while Heinitz *et al.* (2000) have reported 1.2% prevalence of *Salmonella* in domestic shellfish in the US.

### 2.9. *Salmonella* in the Indian seafood

There are reports indicating the detection of *Salmonella* in Indian seafood samples. The bacterium was detected in limited samples of fish and shellfish of retail trade (Nambiar and Iyer, 1990), frozen fish and shrimp (both fresh and frozen), lobster and froglegs, fish meal and poultry feed containing fish meal (Iyer and Srivastava, 1989a). A report from Calcutta mentions that marine prawns and fish from markets were positive for the presence of *Salmonella* (Singh and Kulshreshta, 1993). The samples of water, sediment and shellfish from three estuarine centers along Mangalore coast showed the incidence of *Salmonella* (Srikantiah *et al*., 1985). Nayyar Ahmend *et al.* (1995) could isolate *S*. *Paratyphi* from the sediment of shrimp culture pond. The same pond was negative for *E. coli* and this suggests that *Salmonella* survives longer than *E. coli* is such conditions. The generally accepted notion of *E. coli* and fecal *Streptococci* as indicators of *Salmonella* was questioned by Iyer and Srivastava (1989b) indicating that these bacteria are of little significance as indicators of *Salmonella* in frozen fishery products. Very recently, Sanath Kumar *et al.* (2003) reported that 30% of the finfish, 20% of the clams and 1% of the shrimp samples off mangalore coast are contaminated with *Salmonella*.

### 2.10. Common *Salmonella* serotypes in the Indian seafood

2. 11. Detection of *Salmonella* in seafood.

2. 11. 1. Conventional techniques

Detection of *Salmonella* from seafood involves pre-enrichment, enrichment, selective plating and confirmation by biochemical and serological analysis.

2. 11. 2. Pre-enrichment

Lactose broth and buffered peptone water (BPW) are the most common of the large number of media that are proposed for the pre-enrichment of *Salmonella*. BPW is generally used for most routine works (van Leusden *et al*., 1982; Fricker, 1987). Pre-enrichment is generally recommended for all the situations where sample material is heavily contaminated by other *Enterobacteriaceae* (Varnam and Evans, 1991). The major occurrence of Gram +ve bacteria can be overcome by the addition of low levels of dyes such as brilliant green, crystal violet etc. (North, 1960). An incubation temperature of 37°C is recommended for pre-enrichment of *Salmonella* (Varnam and Evans, 1991).

2. 11. 3. Selective Enrichment

Most widely used inhibitors for the selective enrichment of *Salmonella* are bile salts, tetrathionate, selenite and dyes including brilliant green, malachite green etc. The most common media used are tetrathionate broth (Kauffman, 1935), selenite cystine broth (North and Bartram, 1953), brilliant green–MacConkey broth (King and Metzger, 1968) and Rappaport-Vassiliadis broth (Harvey and Price, 1982).

Selenite cystine broth (SCB) and tetrathionate broth (TTB) are not sensitive to the amount of sample added (Varnam and Evans, 1991). If RV medium is to be used, it is important that the correct ratio of the sample to the broth is used. For SCB and TTB, an inoculation of 1:10 is most common while for RV, a much smaller inoculation of 1:100 is enough (Varnam and Evans, 1991). SCB and TTB are incubated at 37°C, while RV is incubated at 43°C.

2. 11. 4. Selective plating

Selective plating media for *Salmonella* contain selective agents like bile salts, sodium deoxycholate, brilliant green etc. Differential agents such as sugars and amino acids with appropriate indicators will permit the differentiation of the organism from non-*Salmonella*. 
This is based on the observation that most *Salmonella* cannot ferment lactose or in other cases other carbohydrates like sucrose and salicin. Most commonly used media are hektoen-enteric agar with bile salts as inhibitory agent (King and Metzger, 1968), bismuth sulphite agar with bismuth sulphite, sodium sulphite and brilliant green as inhibitory agents (Wilson and Blair, 1927), xylose-lysine-deoxycholate agar with desoxycholate as inhibitory agent (Taylor, 1965) and brilliant green agar with brilliant green as inhibitory agent (van Leusden *et al.*, 1982). Incubation of the selective media plates is done at 37°C for 24 hours. The typical colonies that appear in the plates are subjected to the following biochemical (Table 1) and serological tests.

2.11.5. **Biochemical and serological analysis**

The typical colonies that appear in the plates are subjected to the following biochemical and serological tests as shown in Table 1. The colony characteristics of *Salmonella* on different selective plates are provided in Table 2.

2.11.6. **Rapid detection of *Salmonella* using Polymerase Chain Reaction (PCR)**

2.11.6.1. **General considerations**

Cultural and immunological methods of detection generally rely at least to some extent on selective growth of bacterial cells to achieve sufficient numbers of bacterial cells (Pillai and Ricke, 1995). Such methods that are based on cell growth are seriously limited by cellular metabolism and reproduction, often not considering viable but non-culturable cells. Molecular methods are based on genetic material and thus may overcome these disadvantages. Earlier, such methods linked hybridization assays to a color indicator which became the basis of several commercial kits (Pillai and Ricke, 1995). Hybridization assays can be used to detect *Salmonella* from feed samples but require at least $10^3$ to $10^4$ copies of the target sequence to observe the positive result. As the incidence of *Salmonella* in feed and environmental samples is observed to be low, this would obviously require a selective enrichment (Hill and Keasler, 1991; Wolcott, 1991; Pillai and Ricke, 1995). Such complex samples, environmental samples in particular necessitate such detection techniques are sensitive to low numbers and highly specific in the presence of a highly complex background.
Table 1: Biochemical and serological tests of *Salmonella* (FDA, 1992)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Test/substrate</th>
<th>Positive</th>
<th>Negative</th>
<th>Salmonella spp Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose (TSI)</td>
<td>Yellow butt</td>
<td>Red butt</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Lysine decarboxylase</td>
<td>Purple butt</td>
<td>Yellow butt</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>H₂S (TSI &amp; LIA)</td>
<td>Blackening</td>
<td>No blackening</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Urease</td>
<td>Purple red colour</td>
<td>No colour change</td>
<td>_</td>
</tr>
<tr>
<td>5</td>
<td>Lysine decarboxylase broth</td>
<td>Purple colour</td>
<td>Yellow colour</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Phenol red dulcitol broth</td>
<td>Yellow colour /gas</td>
<td>No gas; no colour change</td>
<td>_B</td>
</tr>
<tr>
<td>7</td>
<td>KCN broth</td>
<td>Growth</td>
<td>No growth</td>
<td>_</td>
</tr>
<tr>
<td>8</td>
<td>Malonate broth</td>
<td>Blue colour</td>
<td>No colour change</td>
<td>_C</td>
</tr>
<tr>
<td>9</td>
<td>Indole test</td>
<td>Violet colour at the surface</td>
<td>Yellow colour at surface</td>
<td>_</td>
</tr>
<tr>
<td>10</td>
<td>Polyvalent flagellar test</td>
<td>Agglutination</td>
<td>No agglutination</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Polyvalent somatic test</td>
<td>Agglutination</td>
<td>No agglutination</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Phenol red lactose broth</td>
<td>Yellow colour /gas</td>
<td>No gas; no colour change</td>
<td>_C</td>
</tr>
<tr>
<td>13</td>
<td>Phenol red sucrose broth</td>
<td>Yellow colour /gas</td>
<td>No gas; no colour change</td>
<td>_</td>
</tr>
<tr>
<td>14</td>
<td>Voges-proskauer test</td>
<td>Pink to red colour</td>
<td>No colour change</td>
<td>_</td>
</tr>
<tr>
<td>15</td>
<td>Methyl red test</td>
<td>Diffuse red colour</td>
<td>Diffuse yellow colour</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>Simmons citrate test</td>
<td>Growth; blue colour</td>
<td>No growth; no blue colour</td>
<td>V</td>
</tr>
</tbody>
</table>
Table 2: Colony characteristics of *Salmonella* on different selective agars (FDA, 1992)

<table>
<thead>
<tr>
<th>Selective agar</th>
<th>Colony characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hektoen enteric agar</td>
<td>Blue green to green colonies with or without black center. Rarely as yellow colonies with or without black center.</td>
</tr>
<tr>
<td>Bismuth sulphite agar</td>
<td>Brown, black or gray with or without black centers. Some strains also produce green colonies with little or no surrounding medium.</td>
</tr>
<tr>
<td>Xylose lysine desoxycholate agar</td>
<td>Pink colonies with or without black centers. Many have large, glossy black centers and may appear as almost completely black. A few appear as yellow colonies.</td>
</tr>
</tbody>
</table>

2.11.6.2. Polymerase chain reaction (PCR)

PCR has revolutionized many facets of molecular medicine and microbiology over the past few years (Hayden *et al*., 1991; Persing, 1991). The primary advantages of PCR tests are increased sensitivity and the less time required to process samples in the laboratory when compared to standard culture methods. The development of rapid and sensitive PCR methods for the detection of infectious microbial agents from clinical and food samples have been of great importance to microbiologists (Hayden *et al*., 1991; Persing, 1991). The specificity of PCR assay has been extensively reviewed (Bej and Mahbubani, 1994; Pepper and Pillai, 1994; Swaminathan and Feng, 1994; Ricke and Pillai, 1999). Detection of pathogenic bacteria by PCR in food and environmental samples has been reviewed by many authors (Wolcott, 1991; Candrain, 1995; Pillai and Ricke, 1995; Ricke *et al*., 1998) and in fecal samples by Bej and Mahbubani (1994). Though detection protocols have not consistently succeeded in achieving reliable detection of pathogenic bacteria in food or feed samples, this method possess great potential for rapid detection. Recent advances include commercialization of PCR kits, multiplex PCR detection of multiple pathogens and evaluation of population dynamics in complex environments.

2.11.6.3. *Salmonella* spp. detection by PCR

Many commercial kits are available to detect *Salmonella* from food samples. They include BAX™ for screening *Salmonella* (Qualicon, Wilmington, DE), Probelia™ (Sanofi Diag. Pasteur, Marnes La Coquette) and Taqman™ (PE Applied Biosystems, formerly Perkin-Elmer, Foster City, CA). BAX™ received Performance Tested Method certification
by AOAC Research Institute in 1998 with a sensitivity of ≥ 98% and a specificity of ≥ 97% in inoculated milk, beef, pork and pure culture samples (Mrozinski and Betts, 1998; Hoorfar et al., 1999). But the use of BAX™ has yielded mixed results. Bailey (1998) detected *Salmonella* spp. in chicken rinses and ground turkey, and Bennet et al. (1998) achieved 96% agreement between BAX™ and cultural methods in a variety of food matrices. Laubach et al. (1998) noted that cultural methodology detected a higher incidence of *Salmonella* spp. in deboned swine head meat than BAX. Wan et al. (2000) investigated the Probelia™ PCR system in milk powder and cheese and found that, in 54 samples, PCR results mirrored cultural results. Probelia™ has additionally been tested in minced beef, egg, fish, milk and water. The results had 98% correlation with cultural methods (Coquard et al., 1999; Fach et al., 1999). The Taqman™ PCR assay has been investigated with pure culture of *Salmonella*, raw chicken, chicken carcass rinses, ground beef, pork and shrimp (Chen et al., 1997; Kimura et al., 1999; Nogva and Lillehaug, 1999). Kimura et al. (1999) noted agreement between conventional and Taq-PCR results in 99/100 samples of meat.

### 2.11.6.4. Specificity of primers

The basic requirement of a PCR assay is the specificity of the primers to the pathogen of interest. Different primers have been used for pathogen detection, since the optimal primers for such detection is still a matter of debate. A fragment of *hns* gene, encoding for a DNA binding protein have been used for the specific detection of *Salmonella* (Marsh and Hillyard, 1990; Jones et al., 1993; Bej et al., 1996). A fimbriae protein (SEF21) based primer *fimA* also has been widely investigated (Cohen et al., 1996; Clouthier et al., 1998) to investigate among 376 strains of *Salmonella* and 24 non *Salmonella* strains. Sequences of enterotoxin gene *stn* has been used by Makino et al. (1999) and Trkov et al. (1999) and concluded that these sequences can also be used as primers. To distinguish between viable and nonviable *Salmonella* by the production of mRNA, both reverse transcriptase (RT) PCR and nucleic acid sequence-based amplification (NASBA) have been used (Szabo and Mackey, 1999; Simkins et al., 2000).

Comparison of primers under identical conditions, Endley et al. (2001) noted that *fimA* may be a superior primer to *hns* due to the non-specific amplification of *Clostridium* DNA by *hns* primers. Earlier Gooding and Choudary (1999) compared five sets of primer pairs for *Salmonella* spp. The primers included a 429 bp chromosomal fragment of DNA (Aabo et al., 1993), an uncharacterized sequence explained by Jitrakpakee et al. (1995), the primer *oriC* targeting the origin of replication of *Salmonella* spp. (Fluit et al., 1993; Mahon et al., 1994), *invA* and *invE* (virulence genes) (Stone et al., 1994; Fratamico and Strobaugh, 1998) and *ompC* coding an outer membrane protein (Kwang et al., 1996). Gooding and
Choudary (1999) recommended that repeat sequence of Jitrakpakee et al. (1995) as the optimal sequence but also stated that this may cross react with *Shigella sonnei*. A list of the PCR primers used in detecting *Salmonella* is provided in Table 3.

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hns</em></td>
<td>LHNS, RHNS</td>
<td>Jones <em>et al.</em>, 1993; Bej <em>et al.</em>, 1994</td>
</tr>
<tr>
<td><em>fimA</em></td>
<td>Fim1A, Fim2a</td>
<td>Cohen <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>mdh</em></td>
<td>MDH 31, MDH 2</td>
<td>Lin and Tsen, 1999</td>
</tr>
<tr>
<td><em>oriC</em></td>
<td>Primers 1 and 2</td>
<td>Fluit <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><em>stn</em></td>
<td>ST11, ST15</td>
<td>Trkov <em>et al.</em>, 1999</td>
</tr>
</tbody>
</table>

2.11.6.5. Multiplex PCR

Multiplex PCR uses several primers at the same time to screen multiple pathogens in a single sample. Many reports has evaluated combinations of mixed *Salmonella* spp., *S. Enteritidis, S. Typhimurium* and multi drug resistant *S. Typhimurium* in environmental samples (Mahon *et al.*, 1994; Fratamico and Strobaugh, 1998; Way *et al.*, 1993; Carlson *et al.*, 1999; Soumet *et al.*, 1999; Khan *et al.*, 2000). Fratamico and Strobaugh (1998) used multiple primers to detect both the *invA* gene of *Salmonella* and *eaeA* gene of *E. coli* O157:H7 simultaneously in the samples of fruit and meat samples.

2.11.6.6. Different approaches for DNA extraction

To achieve most rapid response time for PCR assays, it would be desirable to directly retrieve *Salmonella* DNA from the environmental sample. Strategies for DNA extraction and purification have varied according to the author and substrate but have the potential to decrease inhibitory factors. Pillai *et al.* (1991) used a sucrose-CaCl$_2$ gradient to separate bacterial from soil samples and subsequently lyse the cells by incubating at 98°C for 10 minutes. Bej *et al.* (1994) used Teflon Fluorpore filters (Millipore, Bedford, MA) to concentrate microorganisms from large volumes of water and noted that both nitrocellulose and cellulose acetate filters inhibited the PCR reaction. Cohen *et al.* (1994a, 1994b) detailed a DNA extraction procedure involving lysozyme and proteinase K digestion, an extraction with a phenol-chloroform-isoamyl alcohol mixture and a commercial filter (Glass MAX, Gibco...
BRL, Gaithersburg, MD). Burtscher et al. (1999) used a DNA extraction procedure for organic wastes involving lysis buffer containing N-Lauro sarcosine and EDTA, proteinase K, NaI and isopropanol to precipitate DNA and two minicoloumns (Promega, Madison, WI) for template purification.

2.11.6.7. Inhibitory factors in PCR

One of the major problems associated with the application of molecular techniques in complex environmental samples is the isolation of DNA without the inclusion of proteins, lipid, fiber and other potentially inhibitory factors (Ricke et al., 1998; Ricke and Pillai, 1999). Kwon et al. (1999) indicated that either DNA extraction or a dilution of sample matrix increased amplification efficiency in poultry litter samples. Bovine serum albumin and T4 gene 32 protein provide protection from inhibition from excess Fe$^{+3}$, fecal extracts and either humic, tannic or fulvic acids, but they are not universally effective against all inhibitory factors, especially bile salts, EDTA, NaCl, SDS or Triton X-100 (Kreader, 1996). Löfström et al. (2004) pointed out that the presence of inhibitory compounds in animal feed matrices in addition to low levels and uneven distribution of Salmonella contamination, severely handicaps the processing of feed samples for PCR analysis.

2. 12. Molecular Characterization of different Salmonella serotypes

Rapid typing methods to differentiate Salmonella are needed for monitoring outbreaks and epidemiological investigations. Several typing methods, both phenotypic and genotypic have been developed. Four criteria need to be satisfied for a typing method to be efficient: rapidity, reproducibility, ease of use and the ability to differentiate strains with similar phenotypes. Serotyping with differentiation within O and H antigens is the most widely used phenotyping method for Salmonella (Le Minor and Rhode, 1974). The different genotyping methods used to differentiate different Salmonella spp. include PFGE (Pulse field gel electrophoresis), PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism), RAPD (Random amplified polymorphic DNA), ERIC-PCR (Enterogenic repetitive intergenic consensus sequence) and SSCP (Single strand conformational polymorphism) (Helmuth and Schroeter, 1994; Kerouanton et al., 1996; Millemann et al., 1996; Lehner et al., 1999; Tsen et al., 2000). Molecular genotyping of Salmonella isolates as a valuable tool in epidemiology and study of the genomic diversity between the isolates obtained from human and environmental isolates has been made (Martinetti and Altweg, 1990; Williams et al., 1990; Tenover et al., 1995) and would yield useful information in tracking the spread. Detailed accurate data obtained from the molecular
epidemiological studies of *Salmonella* spp. are crucial for effective surveillance and developing techniques for preventing the contamination by pathogenic strains. The role of molecular subtyping to elucidate the genomic diversity between the different *Salmonella* isolates is well documented (Reeves *et al*., 1989; Williams *et al*., 1990; Welsh and McClelland, 1991) and this would yield valuable information on epidemiology and in tracking the source of contamination. Recently many DNA based techniques have been developed to compare and differentiate pathogenic bacteria including *Salmonella*.

2.12.1. Restriction fragment length polymorphism (PCR-RFLP)

In PCR-RFLP, a known sequence is amplified, cut with a restriction enzyme and the restriction fragment profiles compared between different strains. This typing method was suggested by Grimont and Grimont (1986). The use of RFLP in discriminating different serotypes of *Salmonella* has been reported (Cocolin *et al*., 1998). Different genes have been utilized for the amplification and analysis by PCR-RFLP. Most favored genes include rRNA operon (Shah and Romick, 1997; Lim *et al*., 2005), flagellin genes (Dauga *et al*., 1998; Hong *et al*., 2003). This methodology of PCR-RFLP can be used for limited epidemiological typing.

2.12.2. RAPD (Random amplified polymorphic DNA)

RAPD is a modification of the polymerase chain reaction (PCR) where arbitrary oligonucleotide primers promote DNA synthesis at low stringency conditions in order to determine genomic diversity. It can generate simple and reproducible fingerprints of genomic DNA in a PCR reaction by using single primer chosen irrespective of the genome sequence to be fingerprinted (Williams *et al*., 1990; Welsh and McClelland, 1991). This provides a discriminatory, reproducible and easy method to differentiate different *Salmonella* serovars. RAPD has been widely used for differentiating *Salmonella* serotypes (Soto *et al*., 1999; Lim *et al*., 2005).

2.12.3. ERIC PCR (Enterobacterial repetitive intergenic consensus sequence PCR)

The ERIC sequence is a short intergenic repetitive nucleic acid sequence originally found in *Salmonella* and *E. coli*. Using outward facing primers complementary to each end of the repeat in a PCR has been described as ERIC PCR (Versalovic *et al*., 1991). This tool has been proved to be very efficient in the epidemiological investigations of *Enterobacteriaceae*.
members (Georghiou et al., 1995; Lipman et al., 1995). This characterization technique has been used to differentiate different serotypes of Salmonella (Millemann et al., 1996; Lim et al., 2005).

2. 12. 4. SSCP (single strand conformation polymorphism)

Single strand substitutions can alter the mobility of DNA when subjected to electrophoresis on nondenaturing polyacrylamide gels (Orita et al., 1989). These mobility shifts are due to the conformational changes resulting from single stranded substitutions, deletions or insertions. As this was used initially to screen nucleic acid polymorphisms (Orita et al., 1989) it has been termed as single strand confirmatory polymorphism (SSCP). This method offers several advantages over other methods currently used for polymorphism detection. The single base substitution need not affect a restriction enzyme recognition site and this can be detected by SSCP, while a substitution in the restriction site is necessary for RFLP analysis. SSCP was first used to detect point mutations and allelic variations in oncogenes. Here, the amplified PCR product is denatured and subjected to nondenaturing polyacrylamide gel electrophoresis. The mobility of the single stranded DNA product varies not only on its length but also on its secondary structure based on the nucleotide sequence. Very limited studies report the characterization of different serotypes of Salmonella (Nair et al., 2002; Lim et al., 2005).

2. 13. The Salmonella pathogenesis

The first step in the disease process is the transmission to a susceptible host. In the case of Salmonella, this is through the consumption of contaminated food. Volunteer studies point out that $10^5$ to $10^{10}$ bacteria are required to initiate an infection (Glaser and Newman, 1982) but the exact amount varies with the strain and the physiological condition of the host. The large inoculum competes with the normal flora of the host intestinal tract (Tauxe and Pavia, 1998). The food with which the bacterium finds its way also matters. The infectious dose is generally less with food that traverses the stomach rapidly (liquids) or with those which neutralizes the stomach acidity milk, cheese etc. (Tauxe and Pavia, 1998).

The pathogenesis of Salmonella is host specific. While serotypes like S. Typhi and S. Paratyphi A (typhoidal serotypes) are highly host adapted to man, the serotypes like S. Typhimurium (non typhoidal serotypes) affect a wide range of animals. In man, the infection by the former leads to potentially fatal typhoidal or enteric fever while by the latter, the condition leads to a self limiting gastroenteritis. Whether the differences between the
different disease outcomes are due to genetic differences between the two remain to be identified. The different disease outcomes may also be due to the genetic differences between the host species, which points to the difference in the innate immune response (Zhang et al., 2003) and also the ability of the macrophage to control bacterial replication (Barrow et al., 1994). In short, *Salmonella* translocate the epithelial barrier through M cells that overlie lymphoid follicles (Clark et al., 1994; Jones et al., 1994). They then subvert the normal function of M cells to invade the host cell. *Salmonella* then encounter the resident tissue macrophages that are intimately associated with the M cells and rather than being destroyed by these professional phagocytes after internalization, *Salmonella* survive intracellularly (Fields et al., 1986). The bacteria then multiply in the spleen and liver, and large numbers are released into the blood stream. This stage is characterized by high fever, chills and anorexia. From the liver, they move to the gall bladder. They are then shed along with the bile into the intestine. Several ulcerations occur in the intestine at this point. An infection that proceeds to this stage is likely to become fatal.

2. 13. 1. Role of Pathogenicity islands in *Salmonella* virulence

In the 1980’s the concept of the pathogenicity island was put forward by Hacker (Knapp et al., 1986; Hacker et al., 1990). Earlier such DNA segments coding for virulence were termed as ‘virulence gene blocks’ (High et al., 1988; Hacker, 1990). The fact that a single deletion event leads to the loss of two linked virulence gene expression resulted in the epithet “pathogenicity DNA islands” or simply “pathogenicity islands” (Hacker et al., 1990; Blum et al., 1994). The general genomic features of a pathogenicity island (PAI) are that they are large genomic sequences containing virulence genes, that are present only in the pathogenic species and that they have a different base composition than the rest of the genome (Hacker et al., 1997).

The *Salmonella* are identified as having ten pathogenicity islands (Hensel, 2004). They were all identified when comparing large genomic sequences of *Salmonella* with that of *E. coli* K12 (Groisman et al., 1999).

2. 13. 2. *Salmonella* pathogenicity island I (SPI-I)

SPI-I (Fig. 1) is believed to have been acquired by horizontal transfer from another pathogenic bacterial species during its evolution (Groisman and Ochman, 1993). They form an insertion of 40kb and have a base composition significantly lower than the average base composition of *Salmonella* genome (52%). They encode a T3SS (type III secretion system
that mediates the contact dependent translocation of a complex set of effector proteins into eukaryotic cells (Galan, 2001). One subset of SPI-I effector proteins mediates the invasion of non phagocytic cells involving the modification of actin cytoskeleton. The second subset is associated with enteropathogenesis with the role of proteins in the inflammation of intestinal epithelium and diarrheal symptoms observed in animal models (Walis and Galyov, 2000).

SPI-I contain a group of genes called \textit{inv} genes (Miras \textit{et al.}, 1995) which code for membrane ruffling associated invasion in \textit{S. Typhimurium}. These along with other genes \textit{viz.} \textit{spa, prg, org} etc. code for a type III secretion system, their regulatory proteins, secreted effector proteins and chaperons (Galan and Curtiss, 1989; Galan \textit{et al.}, 1992; Ginocchio \textit{et al.}, 1992; Altmeyer \textit{et al.}, 1993; Behlau and Miller, 1993; Eichelberg \textit{et al.}, 1994; Kaniga \textit{et al.}, 1994; Collazo \textit{et al.}, 1995; ; Hueck \textit{et al.}, 1995; Johnson \textit{et al.}, 1996; Jones and Falkow, 1996; Fu and Galan, 1998). It also includes a gene called \textit{sptP}, which code for a tyrosine phosphatase protein which mimics signal transduction system in eukaryotic cells, and play a role in altering the response of eukaryotic cells to outside stimuli. Other main proteins include SipA (Collazo and Galan, 1997) and SopE (Wood \textit{et al.}, 1996). SopE is an injected protein, which activates G protein. This is directed to control the actin polymerization upon which ruffling phenomenon depends. It is also observed that ruffling is a very much localized phenomenon and this localization is believed to be due to the role of SipA. This protein binds directly to actin and inhibits depolymerisation, increasing polymerized actin in the vicinity resulting in localization of ruffling process (Fig. 2). In short, the genes present in SPI-I are important in the initial phase of infection, rather than on the systemic phase (Abigail and Dixie, 2002).

![Fig. 1. Schematic diagram of the SPI-I. Genes indicated in red show regulatory protein. Genes indicated in black show apparatus proteins. Genes in green encode chaperone. Genes in light purple show secreted proteins (Adapted from Darwin and Miller, 1999).](image-url)
SPI-I is present in all serotypes of *Salmonella enterica* analysed so far. The Genes associated with DNA mobility was absent in some environmental isolates. This led to the hypothesis that SPI-I is a rather recent acquisition gained at the separation of *Salmonella* and *Escherichia* from a common ancestor.

2.13.3. *Salmonella* pathogenicity island II (SPI-II)

After entry into the cell, when *Salmonella* are bound inside a phagosome, SPI-II encodes a type III secretion system (Fig. 3) (Hensel et al., 1995; Ochman et al., 1996; Hensel et al., 1997), which injects proteins into eukaryotic cells. This type III secretion system is unique in the sense that this is not used by bacteria bound upon the surface of the host cells to inject protein into the host cells but, rather, used by bacteria inside a phagosome to prevent phagosome-lysosome fusion (Slayers and Whitt, 2002). That is, this island provides a type III secretion system with intracellular function. SPI-2 (Fig. 3) is 40 kb in size and is insertes adjacent to valV tRNA gene (Hensel et al., 1997). A portion of 25kb encodes the type III secretion system activated by intracellular bacteria. A second portion of 15 kb encodes another type III secretion system dispensable in systemic virulence and encodes tetrathionate redctase (Ttr) involved in anaerobic respiration (Hensel et al., 1999). Proteins that are produced via this system translocate across the phagosome membrane to the host cytosol inhibiting the phagosome-lysosome fusion. It is generally this fusion that culminates in the digestion of the membrane bound *Salmonella*. Once the Phagosome-Lysosome fusion is prevented, *Salmonella* remains viable and proliferates as a membrane bound vacuole inside the host cell.
2. 13. 4. *Salmonella* pathogenicity island III (SPI-III)

Analysis of tRNA *selC* locus identified another *Salmonella* specific locus. SPI-3 is a 17 kb locus with a G+C content of 47.3%. The most distinguishing feature inside a phagosome is the Mg$^{2+}$ deficiency, which is bacteriostatic in nature. SPI-III encode for a high affinity Mg$^{2+}$ transporter, important for the survival of *Salmonella* inside a phagosome (Groisman *et al*., 1999). *Salmonella* survives inside the phagosome using SPI-III encoded proteins, which are basically Mg$^{2+}$ transporters. SPI-3 is conserved between *S*. Typhi and *S*. Typhimurium. Genes in the central regions of SPI-3 are flanked by remanents of IS elements indicating an insertion within this SPI.

2. 13. 5. *Salmonella* pathogenicity island IV (SPI-IV)

SPI-4 is an insertion of 27 kb and is located adjacent to tRNA like *ssb* gene. This SPI has not been analysed in detail with several putative virulence factors present. They appear to be conserved between various serovars of *S*. enterica.
2.13.6. *Salmonella* pathogenicity island V (SPI-V)

SPI-5 is a small locus of 7.6 kb inserted adjacent to tRNA serT locus. SPI-5 encodes the effector proteins for both T3SS encoded by SPI-I and SPI-2. SopB is translocated by SPI-I encoded T3SS and expression regulated by HilA, a central transcriptional regulator of SPI-I. PipB is the translocated effector of SPI-2 under the control of SsrAB two component systems.

2.13.7. *Salmonella* chromosomal islands (SPI-6)

A locus of 59 kb in the genome sequence of *Salmonella* Typhi (Parkhill *et al*., 2001a) has been termed as SPI-6 and subsequently SCI for *S*. Typhimurium (Folkesson *et al*., 2002). They are inserted adjacent to aspV tRNA gene and contain the saf gene cluster for fimbriae. pagN encoding an invasin and several genes of unknown function. Deletion of SPI-6 has no effect on system pathogenesis but reduced invasion of cultured cells were observed (Foleksson *et al*., 2002).

2.13.8. *Salmonella* pathogenicity island 7 (SPI-7) or Major pathogenicity island (MPI)

This locus is specific to *S*. Typhi, *S*. Dublin and *S*. Paratyphi C. This is also referred to as MPI of *S*. Typhi (Zhang *et al*., 1997). This is of 133 kb size and inserted adjacent to tRNA pheU (Hansen-Wester and Hensel, 2002). The important virulence factor encoded by SPI-7 is the Vi antigen, a capsular exopolysaccharide. A further putative virulence factor is a type IVB pilus encoded by the pil gene cluster.

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

A locus of 6.8 kb identified in the gene sequence of *S*. Typhi. This locus is located adjacent to pheV tRNA gene (Parkhill *et al*., 2001a). Putative virulence factor include bacteriocin gene but no functional data has been reported so far. The gene encoding an integrase indicates mobility of this element.
2.13.10. *Salmonella* Pathogenicity island 9 (SPI-9)

This locus of 16 kb is located near lysogenic bacteriophage in the chromosome of *S. Typhi* (Parkhill *et al.*, 2001a). The putative virulence factor encoded by SPI-9 includes type I secretion system and a large RTX like protein.

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

This locus a large insertion of 32.8 kb located near tRNA *leuX*. There is a cryptic bacteriophage present within SPI-10 (Parkhill *et al.*, 2001a). Known virulence factors encoded by SPI-10 are *sef* fimbriae. The distribution of *sef* fimbriae is restricted to *S. Typhi* and *S. Enteritidis* and is considered one of the factors that determine host specificity (Townsend *et al.*, 2001). The role of bacteriophage in SPI-10 has not been deduced so far.

Two lysogenic phages, Gifsy-I & Gifsy-II are known to contribute to *Salmonella* virulence. *Sod* gene of Gifsy-II encodes a superoxide dismutase, which help the bacteria to survive the macrophage oxidative burst (Slayers and Whitt, 2002)

2.13.12. Virulence plasmid - pSLT

Several serotypes of *Salmonella* contain a virulence plasmid pSLT ranging in size from 50-90 kb (Gulig, 1990). This Plasmid carries *spv* genes, important for the survival of *Salmonella* in macrophages (Gulig *et al.*, 1992) and in the steps after initial translocation of the bacteria across the mucosa. One of the genes *spvB* encodes an ADP-ribosylating toxin that modifies actin directly and totally disrupts the cytoskeleton of the cell (Slayers and Whitt, 2002). This constitutes a new pathway of cytoskeleton disruption by this bacterium.

2.13.13. Interaction of *Salmonella* with macrophages

2.13.13.1. *Salmonella* induced macrophage death

Multiple serotypes of *Salmonella* have evolved mechanisms to induce macrophage death (Chen *et al.*, 1996a; Guilloteau *et al.*, 1996; Monack *et al.*, 1996). The mechanism of *Salmonella* induced macrophage death was first reported in 1996 (Chen *et al.*, 1996a; Guilloteau *et al.*, 1996; Monack *et al.*, 1996). Since then this has been subject to confusion and controversy. While some groups reported rapid macrophage death (Chen *et al.*, 1996a; Monack *et al.*, 1996) upon infection with *Salmonella*, others reported the need of prolonged infection (24h) for cell death to occur (Guilloteau *et al.*, 1996; Lindgren *et al.*, 1996). Further, some groups observed that macrophages killed by *Salmonella* exhibited the typical signs of
apoptosis such as chromatin fragmentation (Chen et al., 1996a; Monack et al., 1996), caspase 3-activation (Jesenberger et al., 2000) and the presence of nucleosomes in the cytoplasm (Chen et al., 1996a). Some others reported that necrotic features, such as lack of caspase activation and loss of membrane integrity (Chen et al., 1996a; Brennan and Cookson, 2000; Watson et al., 2000) are more consistent. The use of different bacterial mutants as well as mouse strains deficient in relevant pathways did bring some clarification to the death process.

2.13.13.2. *Salmonella* pathogenicity island I (SPI-1) dependent macrophage cell death

*Salmonella* encodes two types of type III secretory systems which through the delivery of specific effector proteins into host cells, operate at different steps of the infection cycle (Galan, 2001). *Salmonella* pathogenicity island I (SPI-1) encodes a Type III secretory system which is involved in the intestinal phase of infection (Galan, 2001). The other one is SPI-2 is essential for a systemic infection (Shea et al., 1999; Hensel, 2000; Waterman and Holden, 2003). These two systems are differentially regulated: expression of SPI-1 is environmentally regulated (high osmolarity, low oxygen tension) present in the intestinal tract, while the expression of SPI-2 is regulated by conditions present inside the hosts cells (low Mg$^{2+}$ concentrations, acidic pH) (Galan, 2001). Both the TTSS have the capacity to induce macrophage cell death, but by different mechanisms and acting at different times after infection.

2.13.13.3. Caspase-1 dependent pathway of rapid macrophage death

*Salmonella* mediated rapid macrophage cell death (within 45 minutes of infection) occurs under conditions that allow optimal expression of SPI-1 type III secretion systems (Chen et al., 1996a; Monack et al., 1996). Also *Salmonella* induced macrophage cell death does not exhibit a homogenous feature which can be characterized into some form of programmed cell death (Boise and Collins, 2001). Some macrophages undergoing cell death exhibit features consistent with apoptosis, such as chromatin condensation while some other macrophages exhibit features consistent with necrotic type of cell death such as, disruption of plasma membrane and internal organelles (Chen et al., 1996a; Watson et al., 2000). The proportion of one or more morphological type is dependent on not only the level of expression of SPI-1 type III secretion systems but also on the multiplicity of infection (Chen et al., 1996a). At low multiplicity of infection or suboptimal expression of SPI-type III secretion systems, the proportion of apoptotic looking macrophage increases while in contrast, maximum expression of SPI-type III secretion system with high multiplicity of infection favours macrophage death exhibiting necrotic features. Reports also clarify that this form of rapid cell death does not occur when the macrophages are infected with a SPI-1.
deficient *Salmonella* strain (Chen *et al*., 1996a; Monack *et al*., 1996; Lundberg *et al*., 1999; Brennan and Cookson, 2000; Jesenberger *et al*., 2000; Forsberg *et al*., 2003).

The difficulty in classifying SPI-1 dependent macrophage cell death into apoptosis or necrosis indicate that more than one mechanism is involved in the process. Both apoptotic and necrotic processes are ultimate manifestations of mechanisms that are encompassed under the umbrella of programmed cell death. The involvement of caspase-1 in programmed cell death is unusual in that this caspase is not implicated in the classical apoptotic mechanisms. Consequently, caspase-3 (involved in classical apoptotic mechanisms) is not activated in *Salmonella*-infected macrophages. Thus, *Salmonella* induced macrophage apoptosis do not resemble classical apoptosis and therefore has been described as ‘programmed necrosis’.

SipB protein of *Salmonella* has been involved in triggering programmed cell death in a caspase-1 dependent manner (Hersh *et al*., 1999). Transient expression of SipB in cells that are relatively resistant to *Salmonella* induced death led to the formation of multilamellar structures that resemble autophagosomes (Hernandez *et al*., 2003). Under the electron microscope these structures are closely found associated with mitochondria or even appearing to contain degrading mitochondria. On some occasions, mitochondria appear to fuse to Sip-containing multilamellar structures. Taken all the factors together, the previous results indicate that SipB mediated macrophage cell death may be the consequence of its ability to induce autophagy by either damaging mitochondria or by altering the balancing between mitochondrial fusion and fission. Autophagy has been linked to the induction of a less known form of programmed cell death called as ‘type II programmed cell death’ (Schwartz *et al*., 1993; Lemasters, 1999; Xue *et al*., 1999; Cohen *et al*., 2002).

### 2.13.13.4. *Salmonella* SPI-2 dependent macrophage cell death

*Salmonella enterica* strains mutant for the SPI-1 type III secretion systems or those that have been grown under conditions that are not optimal for the expression of this system, can still kill macrophages (Guilloteau *et al*., 1996; Libby *et al*., 1997; Lindgren *et al*., 1997; Santos *et al*., 2001; Browne *et al*., 2002). But here, however, killing requires prolonged incubation (up to 24 hours). Though the mechanism of this prolonged cytotoxicity is unknown, it is known that it is dependent upon the activity of the SPI-2 type III secretion system. Strains carrying mutations in this region are known to be defective in their ability to induce this delayed programmed cell death (van der Velden *et al*., 2000; Monack *et al*., 2001a). SPI-2 type III secretion system is known to be required for intracellular survival and survival (Waterman and Holden, 2003). It appears that this activity may not be directly connected to induction of macrophage cell death because strains carrying mutations in this
region are also capable to kill macrophages (van der Velden et al., 2000). It is therefore assumed that the effector proteins delivered into the cells by the SPI-2 type III secretion system specifically trigger the programmed cell death. A candidate protein for this may be plasmid encoded protein SpvB. A deletion in SpvB inhibited the delayed form of programmed cell death (Browne et al., 2002). Expression of SpvB in mammalian cells induced apoptosis (Kurita et al., 2003).

2.13.13.5. Still unrevealed facts about *Salmonella* induced macrophage cell death

Very little is known about the significance of different types of macrophage cell death triggered by *Salmonella* and their relative contribution to pathogenesis or host defence. It is not still clear whether the macrophage cell death triggered by *Salmonella* is to counteract the host defence systems or whether it represents a host defence system to halt bacterial replication. Also not much is known about whether different types of macrophage death a result of the different evolutionary adaptations of the pathogen. Monack et al. (2000) has reported that caspase-1 deficient mice are more resistant to *Salmonella* infection and that this resistance is a consequence of inability of the *Salmonella* to cross the intestinal epithelial barrier in the absence of a profuse inflammation. More studies are undoubtedly needed to understand the biological and immunological significance of different types of cell death, their relative contribution to the pathogenesis and defense against *Salmonella* infections.

2. 14. Antibiotic resistance in *Salmonella*

Indiscriminate use of antibiotics associated with the bacterial adaptability and microbial genome evolution has led to the emergence and dissemination of antibiotic resistant bacteria. The basic mechanisms that contribute to this resistance phenomenon involve decreased antibiotic concentration, physical modification of the antibiotic and alteration of the target by the bacteria. The most recent mechanism elucidated was the presence of an active efflux pump that pumps out the antibiotic (Lacroix et al., 1996; Poole, 2001; Soto et al., 2003). This has become cause of concern especially in the case of pathogenic bacteria. Such resistant bacteria are not only found in the animal flora but also in the commensal flora of humans as in *Salmonella* and strict human pathogens like *Shigella* (Barza, 2002). The resistant determinants can also transfer between unrelated bacteria such as *Bacteroides* and also between related bacteria such as *Salmonella* and *E. coli* (O’Brien, 2002). Thus, there is a gradual spread of resistant strains with a corresponding resistance transfer between human and animal bacteria (O’Brien, 2002). Moreover, the fact that many of the resistance determinants are located on the mobile genetic elements such as plasmids, transposons, gene
cassettes and genomic islands facilitate the easy spread among the different bacteria living in the same habitat. This is of particular importance as the gastrointestinal tract where members of Enterobacteriaceae thrive facilitating the spread of multidrug resistance among its family members.

Multiresistance in different Salmonella serovars has been largely documented and large numbers of different antimicrobial genes have been identified by molecular approaches (Aarts et al., 2001) like hybridization experiments and PCR. The different resistance determinant genes that are present in various Salmonella serotypes are extensively reviewed in Michael et al. (2006). As the results obtained from different gene probes and PCR primers often tend to be biased, the importance of sequence analysis as a reliable approach to confirm the presence of different resistance genes stands indisputable.

2.14.1 Resistance of Salmonella to various types of antibiotics

2.14.2 Resistance to tetracyclines

More than 35 different tetracycline resistance genes have been characterized. Out of them, till date only five are reported to be present in Salmonella. They are tet(A) (Daly et al., 2005), tet (B) (Frech and Schwarz, 1998), tet (C) (Bernardi and Bernardi, 1984), tet (D) (Allard et al., 1993) and tet (G) (Briggs and Fratamico, 1999). All of this code for a membrane associated efflux protein consisting of 12 transmembrane segments which can export tetracycline, oxytetracycline, chloracceacycline and doxycycline (Michael et al., 2006). The five different tet genes are associated with different elements: tet (G) has been exclusively been detected as a component of Salmonella Genomic island (SGI-1) associated multiresistant gene cluster located upon the chromosome. The two genes tet (A) and tet (B) are associated with small non conjugative transposons Tn1721 and Tn10, respectively. Complete copies of these transposons have been found on large multiresistance plasmids. Plasmids conferring resistance to tet (C) and tet (D) have been identified only in single cases (Bernardi and Bernardi, 1984; Allard et al., 1993).

2.14.3 Resistance to phenicols

In Salmonella the enzymatic inactivation of the type A or type B chloramphenicol acetyltransferases (Cat) as well as export of chloramphenicol by the specific efflux proteins is the predominant resistance mechanisms. Consequently there are two types of cat genes; catA and catB. The genes encoding two types of CatA proteins, catA1 and catA2 have been so far
detected in *Salmonella* isolates. *catA1* was detected upon Transposon 9 in different serotypes like *S. Typhimurium* DT104 (Frech *et al.*, 2003), *S. Typhi* (Parkhill *et al.*, 2001), *S. Agona* (Mulvey *et al.*, 2004) and *S. Derby* (Chen *et al.*, 2004). The *catA2* has been detected upon a multiresistant plasmid from *S. Cholerasuis* (Chiu *et al.*, 2005) and *S. Enteritidis* (Chen *et al.*, 2004; Randall *et al.*, 2004). In *catB* three different types (*catB2, catB3* and *catB8*) are known to be present in *Salmonella*. All *catB* resistance genes are located upon gene cassettes identified as class I multiresistance integrons (Tosini *et al.*, 1998; Ahmed *et al.*, 2005; Nogrady *et al.*, 2005).

The chloramphenicol exporter gene *cmlA* is a cassette borne gene which has been found in plasmid located class I integrons in *S. Typhimurium* (Nogrady *et al.*, 2005). Recently, a new *cmlA4* variant has been identified in a plasmid borne class I integron of *S. Agona* (Michael *et al.*, 2005). The gene *floR* encoding proteins that export florphenicol, which is also designated as *flo, flo₄* or *pp-flor* plays an important role as a part of SGI-1 associated multi resistant gene cluster (Briggs *et al.*, 1999).

### 2.14.4 Resistance to aminoglycosides

In *Salmonella*, aminoglycoside resistance is mediated by modifying enzymes which attach certain groups to the aminoglycoside molecule thereby destroying its antibacterial activity. There are three classes of aminoglycoside modifying enzymes: *O*-adenyltransferases, *N*-acetyltransferases and *O*-phosphotransferases. The two alternatively used nomenclatures for the genes encoding the same aminoglycoside modifying enzyme: one designation *aph (3”)-1b* refers to the type of modification (aminoglycoside phosphotransfer at the position 3” and the subtype of the gene 1b) and the other *strA* refers for the streptomycin resistance and the subtype of the gene A. Among the *aad* (aminoglycoside *O*-adenyltransferase) genes, only those which acts at the position 3” (*aadA*) and 2” (*aadB*) are known in *Salmonella*. Streptomycin resistance is mediated by *aadA*. *aadB* mediates gentamycin, kanamycin and tobramycin resistance. Various serotypes of *Salmonella* have been identified with the presence of *aadA1* (Chen *et al.*, 2004; Pezella *et al.*, 2004; Randall *et al.*, 2004), *aadA2* (Guerra *et al.*, 2001; Chen *et al.*, 2004; Pezella *et al.*, 2004; Randall *et al.*, 2004), *aadA7* (Doublet *et al.*, 2004; Levings *et al.*, 2005) and *aadA21* (Fladynova *et al.*, 2003). In addition, a number of unspecified *aadA* have been sequenced from *Salmonella*. Most of these *aadA* have been found on gene cassettes located on class I and class II integrons. Some of these integrons are located inside SGI-I associated multiresistance gene cluster.
2. 14. 5 Resistance to trimethoprim

Trimethoprim resistance is mainly due to the replacement of dihydrofolate reductase by a plasmid, transposon or cassette-borne trimethoprim resistant dihydrofolate reductase. There are so far 30 dfr (dihydrofolate reductase genes) which are subdivided into two on the basis of their structure as dfr1 and dfr2 (Pattishall et al., 1977), nowadays referred to as dfrA and dfrB (Skold, 2001). A total number of 13 dfrA genes were detected in various Salmonella serovars, most of these are located upon class I and class II integrons (Daly and Fanning, 2000; Pai et al., 2003; Daly et al., 2005). The wide variety of dfrA present in Salmonella has been reviewed in Michael et al. (2006).

2. 14. 6. Resistance to sulphonamides

In Salmonella, there are three different sulphonamide resistance genes – sul1, sul2 and sul3. All of these code for sulphonamide resistant dihydropteroate synthetases. sul1 represents the 3’ conserved segment of the class 1 integrons. PCR analysis has revealed the presence of sul1 in many Salmonella serovars (Frech et al., 2003; Chen et al., 2004; Randall et al., 2004; Michael et al., 2005). sul2 has been physically linked to streptomycin resistant genes strA-strB found on plasmids. The recently described sul3 has been detected together with sul1 on a large multiresistance plasmid from S. Choleraesuis (Chiu et al., 2005).

2. 14. 7. Resistance to penicillin and cephalosporins

Resistance to β Lactam antibiotics in Salmonella is mainly mediated by β lactamase enzymes which inactivate the antibiotic. From both Gram positive and Gram negative bacteria about 350 to 400 different β lactamases are identified. Some are inherent to some species while others are widespread among many bacterial species. So far for Salmonella, there is a wide variety of β lactamases encoded by considerable number of genes (bla). Many genes are inherently associated with bacteria of medical importance while some are associated with other bacterial species.

β lactamases in Salmonella constitute a large group of enzymes. At least 10 subgroups of bla exists encoding for TEM-, SHV-, PSE-, OXA-, PER-, CTX-M-,CMY-, ACC-, DHA-, or KPC- type β lactamases have been identified. In Salmonella, many studies report the presence of TEM- (Izumiya et al., 2003; Olesen et al., 2004; Pasquali et al., 2005), SHV- type (Garbarg-Chenon et al., 1990; Hammami et al., 1991; Hasman et al., 2005), PSE-
1 (Briggs and Fratamico, 1999; Boyd et al., 2001), OXA (Tosini et al., 1998) CTX-M (Bauernfeind et al., 1996; Di Conza et al., 2002; Orman et al., 2002; Hasman et al., 2005) etc.

2. 14. 8. **Presence of integrons in Salmonella**

Integrons are associated with multidrug resistance and such multi drug resistant *Salmonella* are a threat to empiric treatment. Integrons are elements that contain genetic determinants of the components of a site-specific recombination system that recognizes and captures mobile gene cassettes (Fluit, 2005). An integron contains the gene for an integrase (int) and an adjacent recombination site. Gene cassettes may not be a part of an operon but once integrated they become part of it.

There are two groups of integrons, resistance integrons and super integrons. All genes known in resistance integrons encode resistance to antibiotics. These integrons can be found on transposons, conjugative plasmids and the bacterial chromosome. Gene cassettes within the super integrons encode a variety of functions. There are three classes of resistance integrons, out of which only two are present in *Salmonella* (Fluit and Schmitz, 2004).

Class 1 integrons consist of a 5’ conserved segment, 3’ conserved segment and gene cassettes in between. The 5’ conserved segment includes a gene for integrase (int) and an adjacent attl site (Fig. 4). At this site the gene cassettes are integrated. The 3’ conserved sequence includes a ΔqacE and a sulI gene (encoding sulphamethoxazole resistance) can be variable in length and even be absent. The gene cassette has a 59 base element called attC that recognizes the integrase. The integrase integrates gene cassettes into the attC. Several gene cassettes, even up to 100, can be present in the integrons. These gene cassettes encode resistance either to antibiotics or disinfectants. Class 2 integrons are embedded within Tn7 family transposons. They have a deletion rendering the integrase ineffective. Only a few class 2 integrons have been described in *Salmonella* (Fluit and Schmitz, 2004).

![Fig. 4. Schematic representation of class I integron (Adapted from Fluit, 2005)](image-url)
Chen *et al.* (2004) identified six integron replicons. The replicons were sized as 0.75, 1.00, 1.20, 1.50, 2.00 and 2.70 kb long. The most important antibiotic resistant genes carried by these integrons were *aadA1* and *aadA2* conferring resistance to streptomycin and *dhfrXII* conferring resistance to trimethoprim. Earlier reports confirm the presence of class 1 integrons in *Salmonella* (Orman *et al.*, 2002; Ploy *et al.*, 2003). Aarestrup *et al.* (2003) could not find the presence of integrons in the environment isolates of *S. Weltevreden.*