Chapter 1

General Introduction

1. GENERAL INTRODUCTION

Food borne infections are a continuing challenge to human health worldwide. It is one of the most important cause morbidity and mortality in both children <5 years old (Bern *et al*., 1992; Kosek *et al*., 2003; O'Ryan *et al*., 2005 and Scallan *et al*., 2013) and the general population (Scallan *et al*., 2005; Alcaine *et al*., 2007; Fleury *et al*., 2008 and Nyenje and Ndip, 2013). It has also been emerged as a growing public health and economic problem in many countries for the past two decades. A wide spectrum of pathogens such as *Salmonella enterica, Escherichia coli* 0157: H7, *Campylobacter* spp, *Shigella* spp, *Listeria* spp and *Yersinia enterocolitica*, are playing an important role in foodborne disease, have emerged as public health problem and have increased in prevalence or become associated with new food vehicles (USFDA, 2010). Of these pathogens, *Salmonella* represents as an important food borne infection causing pathogen worldwide. The bacteria can cause a wide range of diseases depending on the serovar, strain, infective dose, properties of the contaminated food (e.g. fat content) and the host's state of health (Darwin and Miller, 1999 and Schechter and Lee, 2001).

More than 2610 *Salmonella* serotypes have so far been identified, based on somatic (O), flagellar (H) and capsular (Vi) antigenic profile by the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007 and Guibourdenche *et al*., 2009). Most of the *Salmonella* serotypes are pathogenic to humans (Jones *et al*., 2008). It causes infections in humans can range from a self-limited gastroenteritis usually associated with non-typhoidal *Salmonella* (NTS) to typhoidal fever with complications such as a fatal intestinal perforation. Typhoid (enteric) fever is most commonly caused by *Salmonella* Typhi and *Salmonella* Paratyphi A. This is a host restricted serovar, can colonizes only in humans. It was estimated that typhoid fever causes 21.7 million illnesses with 2,17,000 deaths and paratyphoid fever 5.4 million illnesses worldwide

(Crump and Mintz, 2010). The disease is widespread in tropical and subtropical regions in a broad band around the equator, including much of Sub-Saharan Africa, Asia, and the Americas (Layne, 2007). According to the World Health Organization (WHO) reports, annually NTS causes 93.8 million human infections with 1,55,000 deaths worldwide (Majowicz *et al*., 2010). Most of the *Salmonella* serotypes are present in a wide range of hosts. Among these, *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most commonly reported causes of human salmonellosis in developed countries. However, other serovars appear to be more prevalent in specific regions (Olsen *et al*., 2001; Herikstad *et al*., 2002; Bangtrakulnonth *et al*., 2004; Galanis *et al*., 2006 and Ceyssens *et al*., 2013).

Outbreak of the *Salmonella* infection are usually associated with the consumption of faecal contaminated water or animal derived products like poultry, beef, pork, eggs, and milk (Kidd *et al*., 2002; De Medici *et al*., 2003; Angkititrakul *et al*., 2005; Farzan *et al*., 2006; Wierup and Haggblom, 2010; Bayram *et al*., 2011 and Svobodova, 2012). Most of them have a zoonotic origin and have reservoirs in healthy food animals from which they spread to an increasing variety of foods, thriving in conditions of poor sanitation, crowding, and social chaos. Also, pose a risk to travellers visiting in the disease endemic countries (USFDA, 2011).

After oral inoculation, a systemic infection requires bacterial penetration to the intestinal epithelium, a process which occurs primarily through the M cells of Peyer's patches (Jones *et al*., 1994). The bacteria then invade macrophages, neutrophils and enter the reticuloendothelial system, finally it disseminate to other organs including spleen and liver, where further reproduction results in an overwhelming and fatal bacteraemia (Finlay, 1994). Inside of the host cells, to survive and replicate in a variety of physiologically stressful intracellular and extracellular environmental condition like varied pH, oxygen tensions and other complex environments, these environments stimulate the bacterium to express genes that encode proteins which facilitate coexistence. Such virulence factors in a wide number of gram-positive and gram-negative bacteria are controlled by environmentally responsive regulators. *Salmonella*

employs a sophisticated list of virulence factors to overcome antimicrobial and physical barriers at various sites during infection. It is dependent on the products of a large number of genes (up to 200) to cause infection (Finlay and Brumell, 2000). Some of the virulence genes are located on a 90 kb pathogenicity plasmid, of which the *spv* genes are the best characterized (Libby *et al*., 2000; 2002 and Holden, 2002). However, most of the virulence genes are located on the chromosome within *Salmonella* Pathogenicity Islands (SPI) of which SPI-1 and SPI-2 have been the most intensively studied islands (Groisman and Ochman, 1993; 1997; Hacker and Kaper, 1999; Hensel *et al*., 1999; Hensel, 2000 and Galan, 2001). These encode two of the three type III secretion systems (TTSS), the third TTSS being involved in flagellum expression and bacterial motility. Invasiveness of *Salmonella* is attributed to a TTSS, TTSS-1 encoded by SPI1 (Galan and Curtiss, 1989; Lostroh and Lee, 2001 and Zhou and Galan, 2001) required for invasion of mammalian cell. Injection of effector proteins by TTSS-1 directly into host cells leads to uptake of the bacterium via macropinocytosis (ruffling) (Francis *et al*., 1993). TTSS-1 effectors also elicit the inflammation and fluid secretion associated with gastroenteritis in susceptible host species (Galyov *et al*., 1997; Watson *et al*., 1998; Tsolis *et al*., 1999; Lee *et al*., 2000 and Zhang *et al*., 2003).

To track *Salmonella* infections and disrupt epidemic spread, many nations have established extensive surveillance systems. However, global estimates are difficult to calculate because many countries, particularly developing countries, have insufficient surveillance data. Typing to the strain level has been an important tool in surveillance and outbreak investigation of *Salmonella* infections. Most of these surveillance projects rely on traditional (phenotypic) methods like serotyping, phage typing and biotyping, which provide a limited means of distinguishing epidemic from endemic or sporadic isolates. Nowadays, phenotypic methods are either replaced or complemented by more sensitive and discriminative molecular techniques. Typing schemes based on variation in particular Deoxyribo Nucleic Acid (DNA) sequences are digital and the same results could be achieved wherever the test is performed. Sequence based typing schemes can also be considered as genetic classification schemes (Liebana, 2002 and Winokur, 2003).

1.1. The genus *Salmonella*

1.1.1. Classification and taxonomy

The classification of *Salmonella* is complex because the organisms are a continuum rather than a defined species. *Salmonella* and *E. coli* might have originally diverged from a common ancestor 120-160 million years ago (Ochman and Wilson, 1987). *Salmonella* was diverged from *E. coli* approx. 100 – 160 million years ago and acquired the ability to invade host cells (Ochman and Wilson, 1987; Lawrence and Ochman, 1997 and Eichelberg *et al*., 1999). Currently, the genus *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica* based on the DNA relatedness. A number of biochemical and molecular technology are used to differentiate *Salmonella enterica* into six subspecies, namely, enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI) (Crosa *et al*., 1973; Reeves *et al*., 1989 and Grimont and Weill, 2007). *S. bongori* was originally designated subspecies V, prior its categorization as a separate species. Subspecies I strains are usually isolated from humans and warm-blooded animals (Porwollik *et al*., 2004 and Grimont and Weill *et al*., 2007). The other subspecies usually originate from cold-blooded animals and the environment (Bertrand *et al*., 2008).

Since 1968, the genus *Salmonella* contains a large number of serotypes, it have been further sub classified into serovars based on the surface antigens. Nomenclature of *Salmonella* is mainly based on the presence of somatic (O), flagellar (H) and capsular (Vi) antigens (Quinn *et al*., 2002 and Heyndrickx *et al*., 2005). The vast majority of serovars are found in subspecies enterica (I), it comprises over 2500 known serovars and each year new serovars are being reported and it account for >99.9% of known human and animal infection (Selander *et al*., 1996).

From clinical perspective, pathogenic *Salmonella* serovars were traditionally divided into two groups Typhoidal and non-Typhoidal group. The typhoidal group included the serovars like Typhi, Paratyphi A, Paratyphi B, Paratyphi C and Sendi which cause enteric fever. The second group usually referred to as the non-typhoidal *Salmonella* contains all remaining serovars of sub-species I.

1.1.2. Nomenclature

To avoid confusion between serovars and species, the serovar name is not italicized and starts with a capital letter. When cited the first time in a report, the genus name is given followed by the word "serotype" (or the abbreviation "ser.") and then the serotype name, e.g., *Salmonella* serotype or ser. Choleraesuis. Afterward the name may be shortened with the genus name followed directly by the serotype name, e.g., *Salmonella* Choleraesuis or *S*. Choleraesuis (Popoff *et al*., 2004). Because the type species name, *enterica*, was not approved before 2005, serotype names are used directly after the genus name without mention of the species. Following official approval of "*enterica*" as the type species name, further amendment to include the species name in the *Salmonella* nomenclature of the Centre for Disease Control (CDC) may be expected.

For those designated by their antigenic formulae, the subspecies name is written in Roman letters (not italicized) followed by their antigenic formulae, including O (somatic) antigens, H (flagellar) antigens (phase 1), and H antigens (phase 2, if present). A colon is used between each antigen, e.g., *Salmonella* serotype II 39:z10:z6. For serotypes in *S. bongori* (which previously belonged to subgenus V), V is still used for consistency, e.g., S. V 13,22:z35:– (Popoff *et al*., 2004).

In 2006 ASM "Instructions to Authors" indicated that, for the species, "*Salmonella enterica*" is used at first mention and "*S. enterica*" thereafter; for the subspecies, "*Salmonella enterica* subsp. arizonae" is used first, and "*S. enterica* subsp. arizonae" thereafter. Serovar names should be in Roman type with the first letter capitalized, e.g., *Salmonella enterica* serovar Typhimurium. After the first use, the serovar may be used without a species name, e.g., *Salmonella* Typhimurium.

1.1.3. *Salmonella enterica* subspecies enterica

Currently, 1,547 serovars make up subspecies I, by far the largest in *Salmonella enterica* (Guibourdenche *et al*., 2009). The virulence of specific strains of *Salmonella* in humans and animals is frequently serovar specific. These serovars differ widely in the range of hosts they can infect and in the nature of disease that may cause. This

difference is referred to as serovar-host specificity. Based on the host ranges, *S. enterica* subspecies enterica I classified into two groups viz., broad-host range serovars and host restricted serovars.

1.1.4. Broad-host-range serovars

Some *Salmonella* serovars produce different diseases in different hosts. *S.* Typhimurium and *S.* Enteritidis is a typical broad-host-range pathogen, *S.* Typhimurium is most frequently associated with disease in a number of animal species, including cattle, pigs, horses, poultry, rodents and sheep (Fedorka-Cray and Gray, 2000 and House, 2000; Poppe, 2000; Wray, 2000 and Davies *et al*., 2004). In addition, *S.* Typhimurium often causes gastroenteritis in humans and a disease similar to enteric fever in inbred mice (Bakken and Vogelsang, 1950).

1.1.5. Host restricted serovars

Some serovars have a restricted in their host range and are predominantly associated with severe systemic disease in a single host species, like *S.* Typhi and Paratyphi A (humans), *S.* Gallinarum (poultry), *S.* Cholerasuis (pigs), *S.* Abortusovis (sheep) and *S.* Dublin (cattle) (Rabsch *et al*., 2002).

1.1.6. Typhoidal *Salmonella*

Salmonella enterica serovars Typhi and Paratyphi A are closely related bacteria that cause typhoid and paratyphoid fever. *S.* Typhi can establish febrile systemic infection in the form of enteric fever, in some individuals colonisation may be asymptomatic leading to a chronically persistent carrier state. They were first described by Eberth and Koch in 1880 and first cultured by Gaffki in 1884 (D'Aoust, 1989). It can survive for days in groundwater or seawater and for months in contaminated eggs and frozen oysters (Elsarnagawy, 1978; Nishio *et al*., 1981; Cho and Kim, 1999 and Wait and Sobsey, 2004). The infectious dose varies between 10^3-10^6 organisms given orally (Hornick, 1970). Transmission route of the infection occurs by ingestion of food or water contaminated with faeces. In addition to causing typhoid fever, infection occasionally results in long-term carriage of the bacteria in the human gall bladder (Gonzalez-Escobedo *et al*., 2011 and Geoffrey and Gunn, 2013). These carriers remain

healthy themselves, but can unwittingly spread typhoid to those around them, some famous examples being 'Typhoid Mary', who infected at least 50 people (The New York Times, 1938) and 'Mr N The Milker', who spread typhoid to more than 200 people over 16 years (Mortimer, 1999). Tracing the sources of typhoid outbreaks – usually human carriers or contaminated water sources - is a sleuthing exercise that has kept doctors and scientists busy from the 19th century to the present day. However direct transmission is hard to prove, as epidemiologically unrelated Typhi isolates are often so similar as to look identical using most typing techniques (Browning *et al*., 1995; Swaminathan *et al*., 2001; Quintaes *et al*., 2002; Tatavarthy *et al*., 2012 and Ozdemir and Acar, 2014). The incidence of typhoid fever decreased dramatically in the developed world during the twentieth century as sanitation improved but remains high in developing countries where access to clean water is poor. Still, several typhoid cases are stated in developed countries every year, it often associated with travel to areas where the disease is more common, such as India, South Asia, South America and parts of Africa (Merieux Foudation, 2007). Vaccines against typhoid were developed by the British army in the late 19th century and remained in use until the 1980s. Safer and more effective vaccines were developed in the 1980s and currently two are licensed for use (Wright and Leishman, 1900; Cantlie, 1974 and Hardy, 2000).

1.1.7. Non Typhoidal *Salmonella* (NTS)

Causative agent of NTS infection transmitted either from animal to human or from human to human causing infection. Some NTS serotypes are broad host-range pathogens capable of infecting more than one host species. The NTS species are freely present in the environment and reside in the gastrointestinal tracts of animals. Generally, it can cause a self-limiting gastroenteritis associated with abdominal pain, vomiting and inflammatory diarrhoea. Occasionally, it causes serious complications such as toxic megacolon, bowel perforation (Chao *et al*., 2000 and Chiu *et al*., 2002), meningitis, septic arthritis (Graham, 2002), osteomyelitis (Chi *et al*., 2001), endovasculitis, and septicaemia (Hohmann, 2001and Graham, 2002). The worldwide burden of non-typhoidal gastroenteritis estimated as 2.5 million cases of the disease and 4,100 deaths per year in Africa (Adagbada *et al*., 2014). The majority of human cases

of non-typhoidal salmonellosis are caused by a limited number of serovars, which may vary from country to country and over time (Hendriksen *et al*., 2011). However, NTS bacteraemia has emerged as a significant public health problem in sub-Saharan Africa, associated with Human Immunodeficiency Virus (HIV) in adults and malnutrition, anaemia, malaria and HIV in children. The mean infective dose to produce clinical or subclinical infection in human is 10^5-10^8 Salmonellae (McCullough and Eisele, 1951a; 1951b; 1951c; 1951d). The pathogenicity of NTS strains has been related to genes present in a high-molecular-mass virulence-associated plasmid and in the chromosomal SPI.

1.1.8. Morphological characteristics of *Salmonella*

Salmonella are Gram-negative, rod shaped, facultative anaerobic bacteria belonging to the family Enterobacteriaceae. The bacteria are non-spore forming with a size of 0.7-1.5 μm × 2.0-5.0 μm and producing colonies generally 2-4 mm in diameter (Le Minor, 1984). Structurally, most members of this genus are motile by peritrichous flagella, a few are non-flagellated variants. Most of them are possess long flagella, which direct their movement, acting as a propeller for swimming. They also covered with surface pili, which are short, hair like structures that are involved in cellular attachment. Like other Gram-negative bacteria, the outer membrane of the cell wall is composed of various structurally and functionally important molecules. One of these molecules is lipopolysaccharide (LPS), which is an important virulence factor for Gram-negative bacteria. One portion of LPS, the O-specific polysaccharide tail, contains sugar variations which are used to identify different *Salmonella* types. These 'O' or somatic antigens are heat stable and are exposed on the surface of the bacteria to the surface environment. Some capsulated *Salmonella* (*S*. Typhi and *S*. Paratyphi) also possess another surface polysaccharide, the Vi antigen, which is heat-labile and may provide the organism protection from phagocytosis (Todar, 2004 and CDC, 2005).

1.2. REVIEW OF LITERATURE

Food from unsafe or poor hygiene sources is a significant contributing factor for human food borne infection. It causes major public health problem with major economic and social impacts. Food borne pathogen such as *Salmonella*, *Vibrio*, *Listeria* etc., can enter into the food chain and transmitted to the human at any point of time. *Salmonella* have emerged as one of the important food borne pathogen. The ubiquity and persistence of *Salmonella* in food products have generated awareness worldwide, due to the risk of such pathogenic bacteria pose to human being as well as animal health. Although, distribution of *Salmonella* are well documented both from clinical and other sources, information on the respective *Salmonella* serovars and its nature of risk are scanty. Now a days, it is received much attention by the health care sector. In recent years number of publications has appeared and the important works among them are compiled here.

1.2.1. Background-historical

During early nineteenth century, the study of *Salmonella* began with Carl Eberth's first recognition of the organism through pathological examination of a contaminated spleen in 1880 (Edelman and Levine, 1986). This was followed by the successful isolation and cultivation of the "Bacillus typhosus" by Georg Gaffky in 1884, now referred to as *Salmonella enterica* serovar Typhi (D'Aoust, 1989). Later in 1885, two American veterinarians, Salmon and Smith isolated the bacterium causing hog cholera from infected pigs (Salmon and Smith, 1886). The genus was named *Salmonella* by Lignieres in 1900 was subsequently adopted in honour of Dr. Salmon (Le Minor, 1992). Over the decades following the pioneering work of Salmon and Smith, many other *Salmonella* were isolated from both animals and humans. Widal and others demonstrated that convalescent sera from typhoid fever patients caused the organism to stick together in large balls and lose their motility (Widal, 1896). Further investigations led to the isolation of other Salmonellae, it became a common practice to name each new isolate based on the disease it caused or the species of animal from which isolated. In early 20^{th} century, great advances occurred in the serological detection of its possession of a particular LPS or 'O' antigen and flagella or 'H' antigen

within *Salmonella* group. An antigenic scheme for the classification of Salmonellae was first proposed by White (1926) and subsequently expanded by Kauffmann (1941) into Kauffmann-White scheme, which currently includes more than 2540 serovars (Kauffmann, 1950; Miller *et al*., 2000 and Popoff and Le Minor, 2005).

1.2.2. Isolation of *Salmonella enterica* serovars from different food and clinical sources

1.2.2.1. Indian scenario

In India, the occurrence and distribution of *Salmonella* from different food samples were extensively studied by various research groups (Mahajan *et al*., 1998; Murugkar *et al*., 2005; Shabarinath *et al*., 2007; Lingathurai and Vellathurai, 2010; Suresh *et al*., 2011; Rajagopal and Mini, 2013; Singh *et al*., 2013; Sushila Dahiya *et al*., 2013; Kumar *et al*., 2014 and Saravanan *et al*., 2015). Similarly, *Salmonella* were also reported from clinical samples by many Indian researchers (Sinha *et al*., 1999; Rodrigues *et al*., 2003; Bhan *et al*., 2005; Ochiai *et al*., 2008; Sur *et al*., 2006 and Priyanka Jain *et al*., 2015). In India, *S.* Typhi is one of the most common causative agents for enteric fever. Usually, the disease is observed in urban area of the country and the outbreak was observed throughout the year (Bhan *et al*., 2005 and Sur *et al*., 2006). Some studies showed a peak of the disease from July to September. Similarly, the prevalence of invasive disease causing NTS bacteraemia has raised in many African and Asian countries, the infection is relatively unknown in India. Few studies have reported on NTS serovars from various places in India, where *S.* Senftenberg, *S.* Agona, *S.* Typhimurium and *S.* Enteritidis were predominantly reported serovars (Menezes *et al*., 2010; Taneja *et al*., 2014 and Saravanan *et al*., 2015).

1.2.2.2. International scenario

Worldwide, extensive series of studies have been going on *Salmonella* research which includes surveillance, nomenclature, serovar types, ecological distribution, antibiotic resistance, genetic variation etc., from different sources. Prevalence of *Salmonella* in different food, water and clinical sources with serovars type have been documented well (Refsum *et al*., 2002; Botteldoorn *et al*., 2004; Touron *et al*., 2005;

Van *et al*., 2007; Molla *et al*., 2010; Brichta-Harhay *et al*., 2011; Rabie *et al*., 2012; Jing Lai *et al*., 2014 and Bell *et al*., 2015). Worldwide, *S.* Enteritidis and *S.* Typhimurium are considered as a leading cause of *Salmonella* foodborne infection to human and these serovars are mainly transmitted through consumption of poultry products (Keller *et al*., 1997; Guard-Petter, 2001; Braden et al., 2002; CDC, 2003; Ridha *et al*., 2007; Zerrin *et al*., 2007; Antoine *et al*., 2008; Bacci *et al*., 2012 and Tortajada-Genaro *et al*., 2015). Also, some other serovars such as *S.* Newport, *S.* Heidelberg, *S.* Choleraesuis and *S.* Javiana are also frequently reported by many research groups (Chiu *et al*., 2002; Botteldoorn *et al*., 2004; Yan *et al*., 2005; Hernandez *et al*., 2013; Byrne *et al*., 2014 and Rothrock *et al*., 2015).

1.2.3. Molecular characterization of *Salmonella enterica*

To track *Salmonella* infections and disrupt epidemic spread, many nations have established extensive surveillance systems. Typing to the strain level has been an important tool in surveillance and outbreak investigation of *Salmonella* infections. Most of these surveillance projects rely on traditional (phenotypic) methods like serotyping, phage and biotyping, which provides a limited means of distinguishing epidemic from endemic or sporadic isolates (Doyle, 2001; Biswas, 2005; Sen *et al*., 2007; Aimey *et al*., 2013; Philbey *et al*., 2014 and Feasey *et al*., 2015). Usually, isolation and identification of *Salmonella* cultures take 4–7 days; this is a problem for diagnosis and treatment. In addition, sensitivity of the cultures can be affected by antibiotic treatment, inadequate sampling, variations of bacteraemia and a small number of viable organisms in faeces (Jordan *et al*., 2009). The rapid detection of microbial pathogens is critical factor since people's lives may depend on it. The problem with the conventional method is laborious, time consuming, and can not differentiate within serovars (Nashwa *et al*., 2009). It also depends on the availability of hundreds of antisera, needs highly trained personnel, consumes high volumes of reagents, and a minimum of three days is required to identify a serotype (Alvarez *et al*., 2004; Cai *et al*., 2005 and Yoshida *et al*., 2007). Hence, National molecular subtyping network for foodborne bacterial disease surveillance (PulseNetUS) has been illustrating the effectiveness of molecular methods as a surveillance tool since 1996 (Swaminathan

et al., 2001; Malkawi and Gharaibeh, 2003). Nowadays, phenotypic methods are either replaced or complemented by wide variety of methods which are commercially available is sensitive and discriminative immunological as well as molecular biological techniques for *Salmonella* detection and identification. These includes, electrical conductance/impedance, antibody coated dipsticks, latex agglutination, Immune Magnetic Separation (IMS), Enzyme-Linked Immuno-Sorbent Assay (ELISA) (Bell and Kyriakides, 2002 and Molbak *et al*., 2006), gene probe PCR methods (Haque *et al*., 1999), Real Time-PCR (Malorny *et al*., 2004), quantitative PCR (Piknova *et al*., 2005 and Espy *et al*., 2006) or microarray analysis (Rasooly and Herold, 2008). Polymerase Chain Reaction (PCR) can be used as gold standard techniques to determine the invaluable tool for detection and it should be implemented to obtain a rapid (Prakash *et al*., 2005). It is possible, using molecular methods, to identify and distinguish between different *Salmonella* serovars within 4 h if a whole cell PCR is performed or 7 h if genomic DNA is to be extracted first. With gene specific PCR, it is also possible to specifically detect a pathogenic organism from a mixed bacterial culture (Chaudhry *et al*., 1997). Typing schemes are based on variation in particular DNA sequences are digital and the same results could be achieved wherever the test is performed. Now a days, sequence based typing schemes can also be considered as genetic classification schemes such as Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE), or other emerging genetic typing technologies such as Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) and Multilocus Sequence Typing (MLST) (Chansiripornchai *et al*., 2000; Liebana, 2002; Winokur, 2003 and Kruy *et al*., 2011).

1.2.4. Pulsed Field Gel Electrophoresis (PFGE) of whole chromosomal DNA

Among the other techniques, PFGE has been considered as the 'gold standard' molecular typing methods for a variety of clinically important bacteria. The technique was developed in 1984 and has since become the gold standard for bacterial subtyping. PFGE permit to analysis of bacterial DNA fragments over an order of magnitude larger than that of other conventional Restriction Enzyme Analysis (REA) (Arbeit, 2000). This is the primary subtyping method used by PulseNet, a network of

public health laboratories that perform for food borne disease organisms such as *Salmonella*, *E. coli* O157:H7, *Shigella* (Ribot *et al*., 2006), *Listeria monocytogenes* (Graves and Swaminathan, 2001), and *Campylobacter jejuni* (Ribot *et al*., 2001). PulseNet has also been responsible for detecting many food borne disease outbreaks (CDC, 2008; CDC, 2009 and Wendel *et al*., 2009), and has been extremely successful. The PFGE was adapted to *Salmonella* in the 1990s, demonstrated the capacity to identify strains at the origin of an outbreak and rapidly became very popular. Thong *et al*. (1995) used the PFGE method for the sub typing of *S*. Typhi strains isolated from several Southeast Asian countries and found that PFGE was a powerful technique for the analysis of *S*. Typhi strains. Thong and his colleagues (1996) also found that although considerable genetic diversity existed among *S*. Typhi strains, some PFGE patterns might be shared between isolates obtained from different countries, for example, Malaysia, Indonesia, and Thailand.

1.2.5. Virulence factors

The outcome of a *Salmonella* infection is determined by the status of the host and status of the bacterium. Status of the bacterium is determined by the so-called virulence factors (Van Asten and Van Dijk, 2005). The virulence factors of the bacteria are typically proteins or other molecules that are synthesized by enzymes. These proteins are coded for by genes in chromosomal DNA, bacteriophage DNA or plasmids. Certain bacteria employ mobile genetic elements and horizontal gene transfer (Alphons *et al*., 2005; Nogueira *et al*., 2009; Brown *et al*., 2014 and Dimitriu, 2015).

1.2.5.1. *Salmonella* Pathogenisity Islands (SPIs)

The majority of virulence genes of *Salmonella* are clustered in regions distributed over the chromosome called SPIs (Ochman and Groisman, 1996; Marcus *et al*., 2000; McClelland *et al*., 2001; Lavigne and Blanc-Potard, 2008; Sanchez-Jimenez *et al*., 2010 and Switt *et al*., 2012). The SPIs are of major importance for the virulence of *Salmonella enterica*. Hallmarks of *Salmonella* virulence, such as cell invasion, intracellular survival and the production of Vi antigens (capsule) are encoded by SPIs. Until recently more than 10 SPIs have been identified on the *Salmonella* chromosome, but SPI-1 and SPI-2 is necessary for invasion of intestinal epithelial cells

and induction of intestinal secretory and inflammatory response of *Salmonella* infections (Galyov *et al*., 1997; Hansen-Wester and Hensel, 2001; Lichtensteiger and Vimr, 2003; Hapfelmeier *et al*., 2004; Woo and Lee, 2006; Boyen *et al*., 2006a; 2006b; Muller *et al*., 2009; 2012 and Singer *et al*., 2014). These two SPIs are present in all pathogenic *Salmonella enterica* strains; it harbours two TTSS, TTSS1 and TTSS2, encoded on the pathogenicity islands SPI1 and SPI2, respectively. *Salmonella* lacking a functional SPI-1 TTSS are unable to invade epithelia cells and induce cytokine synthesis (Hobbie *et al*., 1997; Knodler *et al*., 2002; Kuhle and Hensel, 2004). TTSS are specialized virulence devices of *Salmonella enterica* that have evolved indirect translocation of bacterial virulence proteins into the host cell cytoplasm. TTSS are composed of several proteins that form a remarkable needle-like organelle in the bacterial envelope (Kubori *et al*., 2000; Galan, 2001; Deane *et al*., 2006; Galkin *et al*., 2011 and Feria *et al*., 2015).

1.2.5.2. Toxin

Enterotoxin may also play a role in *Salmonella* gastroenteritis. An enterotoxin antigenically similar to Cholera toxin also has been identified (Aguero *et al*., 1991). Flagella phase variation that is exploited by the majority of flagellated *Salmonella* might be related to escaping the host defence system (Asten and Dijk, 2005).

Detection and characterization of this various SPI encode virulence gene in *Salmonella enterica* studied by Miki *et al*. (2004); Baison-Olmo *et al*. (2012); Lahiri *et al*. (2014) and Kuang *et al*. (2015). Similarly detection of enterotoxin encoded *Stn* gene in *Salmonella enterica* were recorded from various part of the world (Murugkar *et al*., 2003; Riyaz-Ul-Hassan *et al*., 2004; Skwark *et al*., 2004; Soto *et al*., 2006; Baba *et al*., 2014 and Abdallah *et al*., 2014).

1.2.6. Trends of Drug Resistance in *Salmonella*

Since the beginning of the 1990s, *Salmonella* strains are showed resistant to a range of antimicrobials, including first-choice agents for the treatment of humans, which have emerged and are threatening to become a serious public health problem (Prevention, 1996). This resistance results from the indiscriminate use of antimicrobials

both in humans and animal husbandry. Now a days, multi-drug resistances by critically important antimicrobials are compounding the problem worldwide (Davis *et al*., 2002; Parry *et al*., 2003; Butayea *et al*., 2005; Akiba *et al*., 2007; Le Hello *et al*., 2011; Mueller-Doblies *et al*., 2013 and Katoh *et al*., 2015). Surveillance data demonstrated an obvious increase in overall antimicrobial resistance among *Salmonella* from 20%-30% in the early 1990s to as high as 70% in some countries at the turn of the century. The resistance rate, however, varies with different serotypes, antibiotics and in different geographical area of the world (Su *et al*., 2004; Mshana *et al*., 2013 and Wong *et al*., 2015).

Some genetic factors may also involve in the antibiotic resistant mechanism, particularly integrons play a major role. Integrons are mobile genetic elements with the ability to capture genes by horizontal gene transfer, which encode one or more antimicrobial resistance genes (Paulsen *et al*., 1993; Guerra *et al*., 2000; Winokur, 2003; Michael *et al*., 2004; Mathew *et al*., 2007; Ruekit *et al*., 2014 and Mattiello *et al*., 2015). It is one of the most recently characterized systems that facilitate horizontal gene transfer, it encoding antibiotic resistance gene by site-specific recombination (Collis *et al*., 2002; Daly and Fanning, 2004 and Tseng *et al*., 2014). There are at least three classes of integrons are identified based upon the type of integrase gene they possess. Among the three classes, class 1 integrons are most prevalent in clinical strains, being found in many different organisms (Collis *et al*., 1998). Many studies have also identified and characterized class 1 integron of *Salmonella* from various sources (Kim *et al*., 2007; Murphy *et al.,* 2007; Rayamajhi *et al*., 2008; Zhang *et al*., 2009; Melendez *et al*,. 2010 and Deekshit *et al*., 2012)

In India, antibiotic resistance among *S*. Typhi has been reported since 1960, and the first outbreak of multidrug resistant *S*. Typhi (MDR-ST) was reported in Calicut (Agarwal, 1962). After that, the frequency of drug resistant strains of *Salmonella* is being increased (Sheorey *et al*., 1993; Madhulika *et al*., 2004; Gaind *et al*., 2006; Kumar *et al*., 2007; Zaki and Karande, 2011; Deekshit *et al*., 2012; Gupta *et al*., 2014 and Mehla and Ramana, 2015). Moreover larger outbreaks of Multidrug Resistant (MDR) *Salmonella* and transferrable genetic elements were also reported from India

(Das and Bhattacharya, 2000; Chande *et al*., 2002; Saha *et al*., 2002; Rodrigues *et al*., 2003; Chowta and Chowta, 2005; Walia *et al*., 2005; Nagshetty *et al*., 2009; Mandal *et al*., 2011 and Dutta *et al*., 2014). Likewise the occurrence and distribution of multidrug resistance strain and its transferrable genetic element from clinical and environmental sources were reported throughout the world (Sandvang *et al*., 1998; Mirza *et al*., 2000; Mills-Rovertson *et al*., 2002; Parry *et al*., 2002; Angulo *et al*., 2004; Wain and Kidgell, 2004; Doublet *et al*., 2005; Kariuki *et al*., 2005; Butaye *et al*., 2006; Akinyemi *et al*., 2007; Dagnra *et al*., 2007; Kariuki *et al*., 2010; Gross *et al*., 2011; Lunguya *et al*., 2012 and Baltazar *et al*., 2015).

1.2.7. Stress response of *Salmonella*

Salmonella encounters and survives various stresses such as high to low temperature, acidic to basic pH, high to low osmolarity, various types of oxidative stress and a variety of anti-microbial compounds encountered during its journey from the environment to food and to the animal host. During stress condition the bacteria present in different state such as viable and culturable, injured, dormant, Viable But Non Culturable (VBNC) and dead (Kell *et al*., 1998). The VBNC state, has been extensively studied in some pathogenic bacteria (Oliver, 2005). The VBNC state of bacteria were characterized by an inability of the cells to grow on culture media, even though they are still viable and maintain a detectable metabolic activity, this state is reversible upon return of favourable conditions (Yamamoto, 2000). It has first been discovered in *V. cholerae* and *Escherichia coli* by Xu *et al*. (1982) and they reported that the cells could enter a dormant state called as VBNC state. However, only limited reports are available in India (Saroj *et al*., 2009; Senoh *et al*., 2010; Senoh *et al*., 2012 and Ramamurthy *et al*., 2014; Senoh *et al*., 2014 and Baskaran, 2015). But, worldwide many researchers including clinicians turned their attention toward this VBNC studies in order to know the existence of virulence potential even after entering into dormant state and it was proved by most research groups (Xu *et al*., 1982; Kell *et al*., 1998; Mizunoe *et al*., 2000; Saux *et al*., 2002; Baffone *et al*., 2003; Bates and Oliver 2004; Oliver *et al*., 2005; Asakura *et al*., 2006; Alam *et al*., 2007; Seeligmann *et al*., 2008; Lai *et al*., 2009; Oliver *et al*., 2010; Su *et al*., 2013; Li *et al*., 2014 and Casabianca *et al*., 2015). Also, resuscitation of VBNC cells to culturable state using different

substrate, different temperatures, salinities etc., were also carried out by various research groups. (Whitesides and Oliver, 1997; Reissbrodt *et al*., 2002; Armada *et al*., 2003; Gupte *et al*., 2003; Nara Figueroa and Lionello, 2004; Liao and Fett, 2005; Vora *et al*., 2005; Faruque *et al*., 2006; Sung *et al*., 2006; Coutard *et al*., 2007; Amel *et al*., 2008; Falcioni *et al*., 2008; Amel *et al*., 2010; Ali *et al*., 2012; Morishige *et al*., 2013; Vezzulli *et al*., 2014 and Fernandez-Delgado *et al*., 2015).

1.2.8. Biofilm formation of *Salmonella*

Salmonella biofilms are encountered on many biotic and abiotic surfaces. Several reports have demonstrated the ability of *Salmonella* strains to form biofilms on abiotic as well as food contact surfaces outside the host. It causes major concern for the food processing industry and has been the subject of significant studies in recent years (Gough and Dodd, 1998; Joseph *et al*., 2001; Annous *et al*., 2005; Lapidot *et al*., 2006; Oliveira *et al*., 2006; Manijeh *et al*., 2008; Chia *et al*., 2009; Pui *et al*., 2011 and Wang *et al*., 2014), which are commonly encountered in farms, slaughter houses, food processing industry and kitchens. It is one of the important factor for survival of the bacteria on surface environment (Bressler *et al*., 2009). In most environments, microorganisms are able to adhere to a surface, producing a matrix of extracellular polymeric sub-stances (EPS) mainly composed of exopolysaccharides, proteins and nucleic acids (Costerton *et al*., 1995; Branda *et al*., 2005; Hoiby *et al*., 2010). The use of chemical sanitizers is generally unable to eliminate most biofilm-associated bacteria (Nett *et al*., 2008; Smith and Hunter, 2008; Wong *et al*., 2010). *Salmonella* biofilms in inside of the host have recently been implicated as the cause of chronic *Salmonella* infection in humans as well as it playing role in the development of asymptomatic career state (Prouty *et al*., 2002; Crawford *et al*., 2008; Crawford *et al*., 2010b; Gopinath *et al*., 2012 and Chelvam *et al*., 2014). But *S*. Typhi and *S*. Typhimurium are having bile resistant properties (Van Velkinburgh *et al*., 1999). However, very few research groups are studied about the *Salmonella* biofilm formation in human gallstone (Prouty *et al*., 2002; Prouty and Gunn, 2003; Crawford *et al*., 2008 and Crawford *et al*., 2010; Gonzalez-Escobedo and Gunn, 2013 and Marshall *et al*., 2014).

1.3. OBJECTIVES OF THE STUDY

Salmonellosis is a major public health problem in developing countries, where inadequate sanitation system and poor hygiene are prevailing. *Salmonella* is one of the leading food and waterborne pathogen and infect the individuals through ingestion of contaminated foods. Estimation of the real impact of salmonellosis in many developing countries is difficult, because the clinical picture is confusing with other febrile and diarrhoeal illnesses. In addition, the disease is under estimated, because there are no sufficient bacteriological laboratories for culturing in most areas of developing countries and there is no proper surveillance system available. These factors are believed to result in many cases going undiagnosed. In recent years increasing resistance of *Salmonella* species to commonly used antimicrobial drugs has become a matter of concern. Since the kind of serotypes and resistance rate of the isolates may vary greatly in different geographical areas and with time, continuous surveillance must be undertaken both nationally and locally in order to develop national and local guidelines for antibiotic treatment. Identification studies should go to the level of serotypes, so that comparison with serotypes isolated from animals/food products locally or elsewhere will be possible and these data can be used for tracing of possible source of human infection. Molecular study is very important to analyse the relatedness among the isolates. Realising the importance of this aspect in mind the present study was therefore undertaken to isolate and characterize the *Salmonella enterica* from various sources.

- To determine the magnitude of salmonellosis in human with suspected case of typhoid fever and diarrhoeal illness admitted to government K.A.P.Viswanatham Medical College & Hospital, Tiruchirappalli, TamilNadu. And also to determine the prevalence of *Salmonella enterica* serovar in different food samples.

- To identify the antigenic nature of the *Salmonella enterica* isolates from food and clinical samples by serological technique.

- To determine the antibiotic resistant pattern of the *Salmonella* isolates by both phenotypic and genotypic method.

- To detect the presence and distribution of various virulence genes in *Salmonella enterica* serovars (both food and clinical samples) by PCR identification method.

- To determine the genetic variation among the *Salmonella* isolated from various sources by PFGE molecular typing method.

- To know the survival strategy of different serovars of *Salmonella enterica* strains under different stress conditions.

- To study the ability of *Salmonella* from biofilm in tissue substrate.