Chapter-2

REVIEW OF LITERATURE

The genus *Salmonella* owes its name to D.E. Salmon, a veterinary bacteriologist, who with Theobald Smith isolated and described the "hog cholera bacillus" for the first time (Salmon and Smith, 1886) but subsequently the organism was found to be only a secondary invader in hog cholera (Swine fever) and named as *Salmonella* Choleraesius.

Currently there are more than 2,600 *Salmonella* serovars prevalent in the world (Suez *et al*., 2013). A few of them are highly host-specific, while majority of them are un-adapted and can cause infection in a wide variety of animal species (Gupta and Verma, 1993). They are the primary cause of diarrhoeal diseases all over the world (Gianella, 1980). Newborn and young animals commonly suffer from enteric infections within 15 days of their birth (Kaura *et al*., 1980).

Enteritis associated with *Salmonella* infection is primarily caused by an enterotoxin, which characteristically induces a temporary reversible functional defect in epithelial cells involving water and electrolyte transport without causing any structural damage (Craig, 1972). Pathogenesis of *Salmonella* is a complex and multifactorial phenomenon. These species interact with ileal mucosa and disrupt normal intestinal function, which results in fluid secretion and enteritis (Wood *et al*., 1998). Enteric disorders that occur at organized animal farms account for 10-30 per cent of annual mortality in our country and are thus responsible for great economic loss (Kaura *et al*., 1980).

Salmonellosis is one of the most common food-borne diseases in humans. The Center for Disease Control (CDC) has estimated that non-typhoidal *Salmonella* caused 10, 27,561 cases of food-borne illnesses (11% of total food-borne illnesses), 19,336 cases of

hospitalization (35% of total food-borne illnesses resulting in hospitalization) and 378 deaths (28% of total food-borne illness deaths) in the United States (CDC, 2011). The data highlighted the influence of socioeconomic factors in disease, as invasive NTS (non-typhoidal salmonellosis) incidence was significantly higher in children from slum populations. Human immunodeficiency virus (HIV)/AIDS patients in Africa are another high-risk population for contracting NTS infections. In India, Salmonellosis is hyper endemic and causes heavy economic losses every year (Rahman, 2002). Of the 2435 reported serovars of *Salmonella*, 209 have been reported from India (Verma *et al.*, 2001). Although all serotypes must be considered potential human pathogens, only a limited number of serotypes are attributed to be the cause of infection in humans and animals. In addition, some specific clones of *S. enterica* have been very dominant in one or more host species and have been able to spread worldwide (Galan, 1998).

2.1. Microbiological Characteristics

The genus *Salmonella* belongs to the family *Enterobacteriaceae*; they are gram-negative, aerobic or facultatively anaerobic and non-spore forming bacilli that are generally motile with peritrichous flagella except *Salmonella* Gallinarum and *Salmonella* Pullorum (both being biovars). These organisms are non-lactose fermenters, oxidase negative, Methyl-Red test negative and urease negative but citrate positive (Sleigh and Duguid, 1989).

Salmonella usually occurs as short rods, measuring 0.7-1.5 μm in length and 2-5 μm in width. Occasionally, they develop into longer pleomorphic forms or very short coco-bacilli after prolonged culture on laboratory media. Capsule formation has sometimes been observed and is associated more particularly with mucoid strains (*S.* Typhi). Salmonellae

are non- or slow lactose fermenters with some strains fermenting it rapidly. The members of the genus can produce H_2S from thiosulphate but they are unable to hydrolyze urea and deaminate phenylalanine, while they do not ferment adonitol, sucrose, salicin and 2-ketogluconate. These biochemical properties can be used for proper identification of the organism (Bisping *et al*., 1988; Schwartz, 1999 and Grimont *et al*., 2000). Some *Salmonella* strains produce a biofilm, which is a matrix of complex carbohydrates, cellulose and protein. The ability to produce biofilm is an indicator of dimorphism, which is the ability of a single genome to produce multiple phenotypes in response to different environmental conditions (Anonymous, 2008).

2.2. Taxonomy and Nomenclature

The genus *Salmonella* consists broadly of two species- *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further subdivided into six subspecies, viz. *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIB), *houtenae* (IV) and *indica* (VI), while *Salmonella bongori* has no subspecies. *Salmonella enterica* subspecies *enterica* serovars are of the greatest clinical relevance, and are typically isolated from humans and warm-blooded animals. *Salmonella* comprises two sets of antigens, *viz.* somatic or 'O' antigen and flagellar or 'H' antigen. In some species, V_i surface antigen may also be seen. These antigenic structures can be detected by serological tests. Strains of *Salmonella* are classified into serovars on the basis of extensive diversity of lipopolysaccharide (LPS) antigens (O) and flagellar protein antigens (H) in accordance with the Kauffmann-White scheme (OIE, 2008).

2.2.1. Kauffman and White Classification Scheme

To develop immunity in the host against a particular pathogen, it is essential to know the antigenic components of that particular pathogen. The antigenic components of *Salmonella* have been explored with great interest with a view to understand the pathogenesis of the disease. The main antigenic components of *Salmonella* have been identified as capsular material, lipopolysaccharide and various membrane proteins. The antigenic classification system of various *Salmonella* serotypes used today is a result accumulated from many years of studies on antibody interactions with surface antigens of *Salmonella* organisms established by Kauffman and White almost a century ago.

All antigenic formulae of recognized *Salmonella* serotypes are listed in a document called the Kauffmann-White scheme. The World Health Organization Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (WHO Collaborating Centre) is responsible for updating the scheme. The terms “serotype” and “serovar” are both frequently used, but according to the Rules of the *Bacteriological Code* (1990 Revision) established by the Judicial Commission of the International Committee on the Systematics of Prokaryotes, the term serovar is preferred to the term serotype. Thus “serovar” is used in the Kauffmann-White scheme (Su *et al*., 2015).

The Kauffman and White classification scheme is a system that classifies the genus *Salmonella* into serovars, based on surface antigens. First the "O" antigen type is determined based on oligosaccharides associated with lipopolysaccharide. Then the "H" antigen is determined based on flagellar proteins. Since *Salmonella* typically exhibits phase variation between two motile phenotypes, different "H" antigens may be expressed. *Salmonella* that can express only one "H" antigen phase consequently have motile and non-motile phenotypes, and are termed monophasic, whilst isolates that lack any "H" antigen expression are termed non-motile. Pathogenic strains of *S.* Typhi, *S.* Paratyphi C and *S.*

Dublin carry the "Vi" antigen (Vi for **vi**rulence), which is a special subtype of the K antigen of the capsule (from the German *Kapsel*). Agglutination by antibodies specific for the various O antigens is employed to group salmonellae into the six serogroups: A, B, C1, C2, D and E (Anonymous, 2015).

Gibson *et al.* (2006) showed that *Salmonella* produces an O-antigen capsule co-regulated with the fimbria and cellulose-associated extracellular matrix. Structural analysis of purified *Salmonella* extracellular polysaccharides yielded predominantly a repeating oligosaccharide unit similar to that of *Salmonella enterica* serovar Enteritidis lipopolysaccharide O antigen with some modifications.

Secreted proteins are of major importance for the pathogenesis of infectious diseases caused by *Salmonella enterica.* A remarkable large number of fimbrial and non-fimbrial adhesions are present in *Salmonella*, which mediate biofilm formation and contact to host cells. Secreted proteins are also involved in host cell invasion and intracellular proliferation which are the two hallmarks of *Salmonella* pathogenesis. Andrade *et al.* (1998) isolated Omp-28 from *Salmonella* Typhi outer membrane and antibodies against Omp-28 were detected by ELISA in 43% of 28 sera from typhoid fever convalescent patients. They suggested that it should be used in further studies of animal protection against the disease caused by these pathogenic bacteria.

Bacterial lipoproteins have been shown to perform various roles, including nutrient uptake, signal transduction, adhesion, conjugation and sporulation and participate in antibiotic resistance, transport (such as ABC transporter systems) and extracytoplasmic folding of proteins (Alloing *et al.,* 1994; Lampen *et al.,* 1984; Mathiopoules *et al.,* 1991; Perego *et al.,* 1991 and Sutcliffe *et al.,* 1995). In the case of pathogens, lipoproteins have

been shown to play a direct role in virulence-associated functions such as colonization, invasion, evasion of host defense, and immunomodulation (Hutchings *et al.,* 2009; Jenkinson *et al.,* 1994 and Khandavilli *et al.,* 2008). In Gram-negative bacteria, such as *Salmonella*, *Escherichia coli* and *Shigella*, two of the three lipoprotein biosynthetic enzymes appear to be essential for viability (Gan *et al*., 1993, Gupta *et al* 1993, Paitan *et al*., 1999, Tjalsma *et al*., 1999), while in some Gram-positive bacteria, they have been shown to be dispensable (Leskela *et al.,* 1999). Consequently, mutations in enzymes involved in the pathway of lipoprotein processing are lethal in Gram-negative bacteria. Operon *inv JICBAEGFH* of *Salmonella* is associated with virulence, invasion and adhesion to the host. There has been a significant increase in the number of proteins reported to be lipoproteins, directly from biochemical studies, and these are predicted to be lipoproteins indirectly from sequenced genomes. Further study of this group of bacterial proteins will contribute to a better understanding of their roles and mechanisms of action, supporting their use in the development of counter measures against bacterial pathogens.

2.3. Pathogenecity Islands of *Salmonella*

Pathogenicity islands (PAIs), as termed in 1990, are a distinct class of genomic islands acquired by microorganisms through horizontal gene transfer. They carry functional genes, such as integrases, transposases, or part of insertion sequences, for enabling insertion into host DNA.

Pathogenesis of *Salmonella* is host-specific. While serotypes like *S*. Typhi and *S*. Paratyphi A (typhoidal serotypes) is highly host-adapted to man, the serotypes like *S*. Typhimurium (non-typhoidal serotypes) affect a wide range of animals. In humans, the infection by the former leads to potentially fatal typhoidal or enteric fever while an infection by the latter, the condition leads to a self-limiting gastroenteritis condition. Whether the

differences between the different disease outcomes are due to genetic differences between the two groups remains 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 the ability of the macrophage to control bacterial replication (Barrow *et al*., 1994). Although high gastric pH reduces the infectious dose, *Salmonella* has an adaptive acid tolerance response which helps to survive in low pH condition of the stomach (Garcia-del Portillo *et al*., 1993).

Salmonella virulence factors, such as adhesion, invasion, and toxin genes are clustered in certain areas of the chromosome known as "*Salmonella* pathogenicity islands (SPI)" and 23 such islands (SPI-1 to SPI-23) have been reported to date (Suez *et al*., 2013). The size of the islands ranges from 10-200 kb. However, there are variations in respect of occurrence of these SPIs including individual genes among different serovars (Sabbagh *et al.*, 2010). Some genes such as *inv*, *sef*, etc. are involved in adhesion and invasion (Cloutheir *et al.*, 1994 and Galan *et al.*, 1998), while others are associated with the survival in the host system- *mgtC5* or in the actual manifestation of pathogenic processes, *viz*. *sop*, *stn*, *pip A, B, D* etc (Chopra *et al.*, 1999; Wood *et al.*, 1998 and Wallis *et al.*, 2000). Serovars like *S.* Typhimurium also harbour self-transmissible virulence plasmid (pSLT) carrying virulence genes such as the *spv* operon, involved in intramacrophage survival and the plasmid-encoded fimbriae (*pef*) fimbrial operon (Ahmer *et al.*, 1999 and Rotger *et al.*, 1999).

The two hallmarks of *Salmonella* pathogenesis are- i) host invasion and ii) intracellular proliferation. *Salmonella* bears type III protein secretion system for host cell epithelial invasion.

2.3.1. *Salmonella* Pathogenicity Island 1

Infection of *Salmonella* involves internalization, a process induced by the bacteria themselves and referred to as bacteria-mediated endocytosis. Microscopic studies reveal that *Salmonella* has the ability to enter the M cell of intestine through bacteria-mediated endocytosis process (Francis *et al.*, 1992). Shortly after bacteria adhere to the apical epithelial surface using various fimbrial adhesins, they disrupt the normal epithelial brush border to induce the membrane ruffling that engulf the organisms (Takeuchi, 1967; Baumler *et al.*, 1996). In this process, bacteria use the type three secretion system (T3SS), a supramolecular needle like structure encoded by *Salmonella* Pathogenicity Island 1 (SPI-1) (Kubori *et al*., 1998).

2.3.2. Construction of SPI-1 coded T3SS

The SPI-1 is a 40 kb region in the *Salmonella* genome, located at centrosome 63 and flanked by *fhlA* and *mutS* genes (Mills *et al.*, 1995). It is believed that SPI-1 was acquired very early during the evolution of *Salmonella* spp. at the point of divergence of *Salmonella* and *Escherichia* spp.from a common ancestor (Li *et al.*, 1995; Baumler, 1997; Galan, 1999). Using electron microscopy, Kubori *et al*., (1998) have shown that this needle apparatus is multi-ringed with acylindrical base that is anchored to both the inner and outer membranes and a slender, hollow, needle-like barrel projecting outward from the outer membrane. For proper assembly of the needle complex, SPI-1 contains a group of genes present in *inv, spa, prg* and *org* operons called as SPI-1 associated T3SS apparatus protein (Eichelberg *et al.*, 1994; Collazo *et al.*, 1995; Jones and Falkow, 1996; Fu and Galan, 1998a, b). The *prg HIJK* operon is mainly responsible for construction of SPI-1 coded T3SS. The proteins PrgH and PrgK are known to be the major base components of needle structure while PrgI and PrgJ are part of the external needle structure. The construction of T3SS is controlled by the operon *inv JICBAEGFH*. While *InvJ* controls the length of the

T3SS, *InvA* and *InvC* control assembly of T3SS and release of effector proteins (Eichelberg *et al.*, 1994) and *InvI* regulates expression of PrgI (Sukhan *et al.*, 2001). *InvH* increases the T3SS assembly process and a mutation in the gene reduces the normal expression of several genes like *prgH*, *prgK*, *prgI*, *invG* and *sipC* (Sukhan *et al.*, 2001). The outer ring structure, composed of InvG and InvH is assembled in the outer membrane and connected to the inner ring structure and is stabilized with the aid of the regulatory protein InvJ. Another operon found to be involved with the T3SS apparatus is the *orgABC*. Initially, it was identified as a single operon (*orgA*); later Klein *et al.* (2000) split this region into two ORFs, *orgA* and *orgB* along with identification of another ORF, *orgC*, overlapping the *orgB* gene. The proteins OrgA and OrgB are involved in invasion and secretion of other apparatus proteins, e.g. protein PrgI (Klein *et al.*, 2000; Sukhan *et al.*, 2001). The *spa* locus (*spaOPQR*) has also been shown to be part of the T3SS apparatus and is essential for host invasion and secretion of proteins like InvJ, SipB and SipC (Collazo and Galán, 1997). The SPI-1 secreted effectors, SopE and SopE2 act as guanine nucleotide-exchange-factors (GEFs) for the small GTPases Cdc42 and Rac (Thomson *et al.*, 2004). Recent work suggests that SopE can also activate RalA, a GTPase that is required for assembly of the exocyst (Nichols and Casanova, 2010) and has been implicated in Fc gamma receptor- mediated phagocytosis (Corrotte *et al.*, 2010). The other effector proteins of the operon *sopABCDE* help in immune cell recruitment, fluid secretion, cytoskeleton rearrangement and neutrophil recruitment.

In contrast to the type II secretion system, translocation via the T3SS occurs independent of an N-terminal conserved *sec*-sequence that is cleaved after secretion. The T3SS is an ATP – dependent system. The T3SS are restricted to Gram-negative bacteria and are present in a number of different species, where they perform distinct functions ranging from antiphagocytic and cytotoxic effects on host cells (Ysc/Yop system of *Yersinia* spp.),

invasion of host cells (*S. enterica* SPI-1 system, *Shigella* spp. Mxi/Spa system), intracellular pathogenesis (*S. enterica* SPI-2 system, *Chlamydia* spp. T3SS) to the establishment of symbiotic relationships such as that observed for the insect endosymbiont *Sodalis glossinidius* and the plant symbiont *Rhizobium* spp. *Salmonella enterica* possesses two distinct T3SS with roles in different phases of pathogenesis. Although *S. enterica* was the first example of a pathogen employing two T3SS, genome sequencing revealed that multiple T3SS also occur in other species such as *Yersinia* spp., *Vibrio parahaemolyticus* and *Burkholderia pseudomallei* (Pallen *et al.*, 2003).

2.3.3. Virulence genes present in other *Salmonella* Pathogenicity Islands

SPI-2 is inserted next to the *valV* tRNA gene and encodes a second T3SS, which is involved in intracellular survival (Shea *et al.*, 1996; Hensel *et al.*, 1998). Three ORFs (STY1735, STY1739 and STY1742) are pseudogenes in *S.* Typhi. These ORFs, however, are not part of the T3SS, but part of a tetrathionate reductase complex.

SPI-3 is inserted next to the *selC* tRNA gene, is involved in intracellular survival and encodes a magnesium transporter (Blanc-Potard & Groisman, 1997). SPI-3 is divided into three regions (Blanc-Potard *et al.*, 1999; Amavisit *et al.*, 2003). First region contains many pseudogenes in *S.* Typhi: STY4024 (*cigR*), STY4027 (*marT*), STY4030 (*misL*), STY4034, STY4035 and STY4037. A few more pseudogenes in *S.* Typhi are found in the second and third portions of SPI-3, including STY4012, STY4007 and STY4003.

SPI-4 carries genes *(spi4R* and *spi4D)* that are predicted to encode a type I secretion system.

SPI-5 encodes at least six genes, *pipD*, *sigD/sopB*, *sigE*, *pipB*, *pipC* and *pipA* all of which contribute to enteropathogenesis as assessed in a calf model of infection (Wood *et al*., 1998). SPI-5 encodes the effector proteins for both T3SS encoded by SPI-I and SPI-2 (Steele-Mortimer *et al*., 2000; Zhou *et al*., 2001).

SPI-6 is inserted adjacent to *aspV* tRNA gene and contain the *saf* and *tcf* gene cluster for fimbriae.

The important virulence factor encoded by SPI-7 is the Vi antigen, a capsular exopolysaccharide (Pickard *et al*., 2003). Vi expression is associated with a cluster of 10 genes located in the *S*. Typhi chromosome, known as the *viaB* operon (Liu and Sanderson, 1995), regulated by two component regulator systems, *rcsBrcsC* (Houng *et al*., 1992) and *ompR-envZ* (Pickard *et al*., 1994).

SPI-8 encodes two bacteriocm pseudogenes and a degenerate integrase; notably, genes conferring immunity to the bacteriocins remain intact.

The putative virulence factor of SPI-9 includes type I secretion system and large repeats in toxins (RTX) like protein.

On SPI-10, the *prpZ* locus encoding for proteins with homology to eukaryotic-type Ser/Thr protein phosphatase and kinases has been found to promote survival in macrophages (Faucher *et al.,* 2008).

SPI-11 carries *pagC* and *pagD* genes that encodes for macrophage survival and serum resistance (Harris *et al.,* 2006).

SPI-12 is inserted next to the *proL* tRNA gene of *S*. Typhi and *S*. Choleraesuis. The only virulence factor present in SPI-12 is SspH2. This is a secreted effector protein of T3SS that influences the rate of actin polymerization inside the infected cells (Miao *et al*., 2003).

SPI-13 is located next to the *phe V* tRNA gene and of the 18 genes present in the island, only 3 are associated with virulence viz., *gacD*, *gtrA* and *gtrB* and have been shown to be responsible for the virulence of *S*. Gallinarum in one day old chick infection model (Shah *et al*., 2005).

SPI-14 carries two genes viz., *gpiAB*, which also have been found associated with the virulence of *S*. Gallinarum in one day old chicks (Shah *et al*., 2005).

SPI-15 is a 6.5 kb island of five ORFs encoding hypothetical proteins, is inserted near the *glyU* tRNA gene in *S*. Typhi and is absent in *S*. Typhimurium (Vernikos & Parkhill, 2006).

SPI-16 is found in *S*. Typhimurium and *S*. Typhi inserted next to an *argU* tRNA site, and encodes five or seven ORFs, respectively, four of which are pseudogenes in *S*. Typhi. The three remaining ORFs show a high level of identity with P22 phage genes involved in seroconversion (Vernikos & Parkhill, 2006) and were suggested to mediate O-antigen glycosylation (Mavris *et al.*, 1997; Guan *et al.*, 1999) and cell surface variation (Allison & Verma, 2000; Bogomolnaya *et al.*, 2008). These ORFs (genes *yfdH, rfbI* and STM0557) were required for the intestinal persistence of *S*. Typhimurium in mice (Bogomolnaya *et al.*, 2008).

SPI-17 encodes six ORFs inserted next to an *argW* tRNA site and is absent in *S*. Typhimurium, but present in *S*. Typhi (Vernikos & Parkhill, 2006). Seroconversion genes

homologous to P22 phage are present and showed high homology to genes of SPI-16, including a putative lipopolysaccharide modification acyltransferase. Most of these genes (four) are pseudogenes in *S*. Typhi.

SPI-18 was identified in *S*. Typhi harbouring only two ORFs: STY1498 and STY1499 (Fuentes *et al.*, 2008). The gene *clyA* (STY1498), also known as *hlyE* or *sheA* is found in *E. coli* and *S. enterica* serovars Typhi and Paratyphi A (del Castillo *et al.*, 1997; Green & Baldwin, 1997; Oscarsson *et al.*, 1999, 2002). *It* is important for invasion of human epithelial cells *in vitro*, with its heterologous expression in *S*. Typhimurium leading to colonization of deep organs in a murine model (Fuentes *et al.*, 2008). The encoded product of the gene *taiA* (STY1499) is an invasin that increases bacterial uptake by human macrophages (Faucher *et al.*, 2009).

SPI-19 is present in subset of serotypes belonging to *S*. Dublin, *S*.Weltevreden, *S*.Agona, *S*.Gallinarum and *S*. Enteritidis. It encodes two ORFs for Hcp-1 and Hcp-2 proteins. Hcp-1 is a putative operon that includes most of the T6SS function and Hcp-2 is found upstream of a VgrG homolog.

SPI-20 is located adjacent to *aspV* tRNA gene having 28 ORFs with seventeen of them involved in T6SS function.

SPI-21 locus is located adjacent to *thrW* tRNA gene and encodes a T6SS in the genome of serotype IIIa 62:z4, z23. It has 57 ORFs, 20 of which encode T6SS function. In addition SPI-21 includes 4 ORFs encoding putative colicin/pyocin immunity proteins.

2.4. Plasmid-Encoded Virulence

In addition to the virulence factors associated with the SPI-1 and SPI-2 T3SS, some factors such as virulence or antimicrobial resistance can be found on plasmids (Rotger and Casadesus, 1999; Sheppard *et al.*, 2003). Strains from many serovars lack virulence plasmids; however, some of the most important serovars for human health, including *S.* Typhimurium, *S.* Enteritidis, and *S.*Choleraesuis are known to harbour virulence plasmids (Lu *et al.*, 1999; Villa and Carattoli, 2005; Yu *et al.*, 2006). These virulence plasmids have a genetic locus called *Salmonella* plasmid virulence, which contains *spvRABCD* genes. The *spv* genes appear to be important for bacterial multiplication within host cells during extra-intestinal infections (Guiney *et al.*, 1995).

Additional virulence genes located on virulence plasmids include those encoding fimbriae (*pef-BACDI*) and serum resistance (*traT*) (Rotger and Casadesus, 1999). Although most virulence plasmids are not self-transmissible, some appear to contain a full concert of transfer *(tra)* genes that allow the plasmids to be transferred to additional strains by conjugation potentially increasing the virulence of the recipients (Ahmer *et al.*, 1999). Due to their conservation among members of a particular serovar, virulence plasmids provide a significant advantage to the strains harboring these plasmids (Foley and Lynne, 2008).

2.5. Epidemiology of *Salmonella*

The epidemiology of *Salmonella* spp. associated infections varies widely depending on the type of *Salmonella* serovar involved. While enteric fever, caused by *S.* Typhi and *S.* Paratyphi, generally leads to a severe and life-threatening disease that primarily affects communities in underserved nations, non-typhoidal salmonelloses tend to be self-limiting and to affect the community worldwide (Hardy *et al*., 2004). It is found that more than 60% of all *Salmonella* strains identified and 99% of the serovars responsible for

disease in warm-blooded animals are members of the subspecies I. The other *Salmonella* subspecies, in particular subspecies IIIa (Arizona) and *S. bongori*, are associated with disease in cold-blooded organisms and are occasionally responsible for systemic diseases in humans (Chan *et al*., 2002).

Salmonellosis in human is endemic in India. The rate of foodborne salmonellosis in India is 6 per 1000. Till today, 205 serotypes of *Salmonell*a have been reported from different parts of the country (Kumar *et al*., 2009).

Salmonella Typhimurium is the most common cause of human salmonellosis in the United States and is among the top five most detected pathogens for each major food animal species (CDC, 2006). Serologically, *S*. Typhimurium is a member of the serogroup B, with serovars such as *S*. Heidelberg, *S*. Derby, and *S*. Agona (CDC, 2006). A 4-year review of FoodNet data indicated that *S*. Typhimurium is one of the top two most commonly isolated serovars from humans with salmonellosis and accounts for nearly 50% of death due to salmonellosis (Kennedy *et al.*, 2004; Perch *et al.*, 2004). Even though *S*. Typhimurium is one of the most common serovars associated with salmonellosis, it has a disproportionately high mortality rate for the proportion of infections it causes. Among 238 cases of *Salmonella* Typhimurium outbreaks reported from 1996 to 2005, 28 outbreaks were due to consumption of chicken meat and 47 from egg, indicating poultry as the major source of *S*. Typhimurium outbreaks compared to other food items (Greig and Ravel, 2009).

2.6 Incidence and prevalence

Human:

It is estimated that enteric fever causes 2, 00,000 deaths and 22 millions of illness per year worldwide predominantly in low-income nations (Crump *et al*., 2004). The

incidence of enteric fever, however, varies subsequently between countries. High incidence estimates were calculated in south-central Asia and south-east Asia while low incidence was reported in Europe, Australia and New Zealand and North America. In US, the incidence of *S.* Typhi infection is low and, for the most part, it was related to travel, whereby, US travelers returning from developing countries, or foreigners traveling to the US (Nguyen *et al*., 2009) contracted diseases due to S.Typhi.

The incidence of enteric fever, however, varies substantially among countries. Among the Asian countries the annual incidence of *S.* Typhi was higher in Pakistan (451.7 cases per 100,000) and India (214.2 cases per 100,000) compared with Vietnam and China (21.3 and 15.3 cases per 100,000, respectively) (Ochiai *et al*., 2008).

In India, many investigators isolated *Salmonella* from different sources. Salmonellosis is hyper-endemic in India and is a cause for heavy economic losses every year (Rahman, 2002). During 2001–2005, a total of 3079 isolates were received and identified at the National *Salmonella* and *Escherichia* Centre (NSEC), Central Research Institute, Kasauli, India. Out of these, 2098 isolates were from humans, 250 from animals, and 726 from meat, vegetables, seafood and the environment. *Salmonella* isolates were distributed among 35 different *Salmonella* serovars. The most common serovars from humans were *Salmonella* Typhi (73%) and *Salmonella* Paratyphi A (24%) among typhoidal serovars and *Salmonella* Worthington (28.2%) and *Salmonella* Typhimurium (poultry 41.4% and human 43%) among non-typhoidal serovars (Kumar *et al.*, 2009). *S*. Typhi and *S.* Paratyphi have only humans as a reservoir and the route of transmission includes ingestion of contaminated food and water with patient's and carrier's faeces (Crump *et al*., 2008). The main route of *S.* Paratyphi transmission is believed to be associated with

consumption of street vendor's food (Crump *et al*., 2010). *S.* Typhi has been a major global problem during most of the 20th century while *S.* Paratyphi was limited to a smaller proportion of enteric fever cases (Fangtham *et al*., 2008). *S.* Weltevreden was found to have zoonotic significance in India and has been constantly recorded, and is one of the five serovars isolated most frequently (Kumar *et al*., 2009).

Cases of enteric fever in endemic areas are generally more frequent in infants, pre-school age and school age children than in adults (Kariuki *et al*., 2008). In the last decade the annual incidence among children aged 2-5 years was around 27 per 100,000 in Vietnam and China, and around 450 per 100,000 in Pakistan and India, with an incidence of bacteremia in children less 2 years of age of 443.1 per 100,000 child years (Ochiai *et al*., 2008; Owais *et al*., 2010).

It is estimated that 93.8 million cases of gastroenteritis due to *Salmonella* spp. occur worldwide leading to 1, 55,000 deaths each year (Majowicz *et al*., 2010). According to Salm-Surv, a WHO supported food-borne disease surveillance network data from 2001 to 2005, S. Enteritidis was the most common serotype world-wide (65% of the isolates), followed by S. Typhimurium (12%) and S. Newport (4%) (Galanis *et al*., 2006).

The most recent outbreak leading to significant concern among health authorities, consumers and farms owners, was the January 2010 multistate outbreak that lasted one entire year. This outbreak caused by *S.* Enteritidis-contaminated eggs affected 16 states and resulted in an estimated number of 1939 cases (CDC- 2010). The estimated 380 million contaminated chicken eggs shipped across the US led to a massive egg recall recommended by the US Food and Drug Administration to prevent further spread of the infection.

Farm animals are the major reservoir for non typhoidal Salmonellosis in industrialized countries with transmission by their contaminated product. Non Typhoidal Salmonellosis are naturally found in chicken, ducklings, sheep, goats, pigs, reptiles, amphibians, birds, pet rodents, dogs, cats and in variety of wild animals making infection control a challenge to public health authorities (Wacheck *et al*., 2010; Dione *et al*., 2011).

Cattle:

Nagaratnam and Ratnatunga (1971) isolated 79 strains of *Salmonella* from 815 rectal swabs of cattle. The isolated serotypes were *S*. Typhimurium (65), *S.* Enteritidis (7), *S*. Stanley (3) and *S*. Bareilly (4). Isolation of *Salmonella* from 38 of 1479 beef samples was reported by Dasgupta (1974) in Calcutta. The isolates belongs to eight serotypes, viz. *S.* Typhimurium, *S*. Newport, *S.* Dublin, *S.* Anatum, *S*. Stanley, *S.* Butantum, *S*. Virchow and *S*. Kiambu.

Garg and Sharma (1979) examined rectal swabs from 464 calves, of which 92 were suffering from diarrhea and other intestinal disorders. Seventy two strains of *Salmonella* were isolated, of which 32 were *S.* Typhimurium and 23 *S.* Dublin. Similarly, Martel *et al*. (1980) could isolate *S.* Typhimurium from eight outbreak of blood stained diarrhea in newborn calves on Charolais farms.

Farid *et al*. (1987) reported isolation of *Salmonella* from 7 of 100 dead animals, 30 of 100 slaughtered animals and 10 of 200 diarrhoeic animals with an overall incidence of 4.17 percent. The serotypes isolated were *S.* Typhimurium (11), *S.* Dublin (6), *S.* Bovismorbificans (4), *S.* Derby (1) and *S.* Entertidis (1).

Losineger *et al*. (1997) recovered *Salmonella* from 38 (38.0%) of the 100 feedlots, 52 (26%) of the 200 pens and 273 (5.5%) of the 4977 faecal samples collected from two pens of a dairy farm. In a study conducted by Abouzeed *et al.* (2000), the prevalence of *Salmonella* in beef cattle was 4.6% (11/240). The rate was significantly higher in fasted cattle (7.46%), than in non-fasted cattle (0.94%). The prevalence rate in chickens was 32.5 per cent (39/120). Saikia (2001) isolated six strains of *S.* Typhimurium from 65 rectal swabs of diarrhoeic calves.

In Europe, *Salmonella* enteric subspecies enteric Dublin is one of the most prevalent *Salmonella* serovars isolated from cattle (Wray and Davies, 2000). In Neatherland, *Salmonella* Dublin accounted for 53 % of the *Salmonella* isolates from cattle obtained between 1993 and 2000 (van Duijkeren *et al*., 2002). Bovine *Salmonella* Dublin infections may result in subclinical excretion, latent carriership, or in clinical disease causing enteritidis, septicemia, meningitis, abortion, osteomyelitis, arthritis, terminal dry gangrene, depressed milk yield or pneumonia (Quinn *et al*., 1994; Wray and Davies, 2000). Although *Salmonella* Dublin is adapted to cattle (Quinn *et al.,* 1994), it may occasionally infect other animal species including humans. In humans, these infections can result in severe invasive disease (Brackelsberg *et al*., 1997) and often associated with the consumption of contaminated dairy products (Vaillant *et al*., 1996; Jacobs *et al*., 2002).

Sarmah (2003) isolated 11 *Salmonella* from 129 rectal swabs from diarrhoeic calves in Assam. Serovars identified included *S.* Typhimurium and *S.* Enteritidis. Mahajan *et al*. (2003) reported isolation of 6.5 per cent *Salmonella* belonging to serovar *S*. Enteritidis from diarrhoeic neonatal calves. *Salmonella* was isolated in faecal samples from 1026 (4.9%) of 20,089 cows by Fossler *et al*. (2005) in Minnesota, Wisconsin, Michigan, and

New York. The prevalence of *Salmonella* was demonstrated in 1.4 per cent out of 144 in cattle in Tanzania (Kusiluka *et al*., 2005).

Padungtod and Kaneene (2006) conducted a study on the epidemiology of *Salmonella* in chickens, pigs and dairy cows in northern Thailand during 2000-2003. The prevalence of *Salmonella* in chickens at the farm, slaughterhouse and chicken meat at the market were 4 per cent, 9 per cent and 57 per cent, respectively. In pigs, the prevalence at the farm, slaughter house and pork at the market were 6 per cent, 28 per cent and 29 per cent, respectively. The prevalence of *Salmonella* in dairy cows was 3 per cent. Vo *et al.* (2006) reported *Salmonella* Typhimurium, *S.* Anatum, *S.* Weltervreden, *S*. Emek and *S.* Rissien as the most prevalent serovars recorded from 56 human and 241 faeces, carcasses and meat samples of animal origin in Vietnam.

Cumming *et al*. (2010) screened herds for presence of *Salmonella* by either environmental or faecal culture from cattle. Among the 57 enrolled herds, 44 (77%) yielded *Salmonella*. The serovar recovered was *Salmonella* Cerro. *Salmonella* was isolated from 124 out of 189 beef samples in a study conducted by Mohammad *et al*. (2010) in Iran during the year 2006-2007. *Salmonella* Thompson was the dominant serovar of *Salmonella* followed by *S*. Hadar.

Poultry:

A variety of investigations of outbreaks and sporadic cases have indicated that food vehicles identified as the most common source of *Salmonella* infections are poultry and poultry by-products, including raw and uncooked eggs (Harrison *et al*., 1994 and Hennessy *et al*., 2004). *Salmonella* cause asymptomatic intestinal infections in birds but acute outbreaks exhibiting clinical disease along with high levels of mortality occur in

chicks younger than 2 weeks old (Duchet-Suchaux *et al*., 1995). Egg shells can be contaminated with *Salmonella* as a result of intestinal passage and the ability to penetrate into the avian egg (Parry *et al*., 2002). Pullorum disease, for example, is caused by *S.* Pullorum and is spread from an infected parent bird via the egg to the chicken. While clinical signs are variable and non-specific, the outcome is an excessive number of dead-in-shell chicks and deaths shortly after hatching. *Salmonella* can be highly invasive in laying hens leading to systemic infections that can potentially be deposited in the internal contents of eggs by trans-ovarian transmission following colonization of the intestinal tract (Humphrey *et al*., 1989 and Woodward *et al*., 2005). *S.* Enteritidis, in particular, has shown a greater ability to colonize the vaginal epithelium of laying hens compared to other serotypes (Mizumoto *et al*., 2005). Birds that are asymptomatic carriers may facilitate the spread of disease infections among flock (Duchet-Suchaux *et al*., 1997 and Gast *et al*., 1998).

Many outbreaks of *Salmonella* infections has been reported worldwide and the serovars encountered frequently were *S.* Typhimurium, *S*. Enteritidis, *S.* Gallinarum, *S*. Pullorum, *S.* Newport, *S*. Cerro, *S.* Montevideo, *S*. Agona and *S.* Dublin (Konrad *et al*., 1994; Abdel, 2004). Although *S*. Gallinarum (Rao *et al*., 1952) and *S.* Pullorum (Das *et al*., 1959) are the major pathogen of poultry with enteric infection during the last two and half decades, numerous outbreaks of avian Salmonellosis have been reported throughout the world.

An outbreak of *S*. Bareilly infection was reported by Kapoor *et al*., (1980) which resulted in 92.5 per cent mortality in chicken and 22 per cent in quail chicken. In Slovakia, Simko (1984) reported isolation of 19 serotypes of *Salmonella* from apparently healthy

fowls comprising serotypes of *S*. Gallinarum (65%), *S*. Typhimurium (4%), *S*. Bareilly (7%), *S*. Enteritidis (2%) and *S*. Agona (2%).

Saikia and Patgiri (1986) reported isolation of *Salmonella* from 16 out of 150 dead poultry in Assam and the serotypes isolated were *S*. Chester and *Salmonella* 4,12:e,h:-. Rahman *et al*., (1997) recorded an outbreak of Salmonellosis in a flock of 3000 broiler chicks in Assam with an average mortality of 36 per cent. The isolated serovars were *S*. Gallinarum and *S*. Indiana, both from dead and ailing birds. An outbreak of Salmonellosis in ducklings was reported by Rahman *et al*. (1999). Five strains of *S*. Enteritidis were isolated from dead birds.

In Madhya Pradesh, Shivhare *et al*. (2000) isolated *Salmonella* from 35 (7%) out of 500 specimens collected from poultry birds and carcasses. The most common serotypes being *S*. Typhimurium (71.2%), *S*. Gallinarum (20%) and *S*. Pullorum (8.8%).

In Assam, Arunachal Pradesh and Meghalaya, *S*. Typhimurium (40.38%) was found to be the most predominant isolate followed by *S*. Enteritidis (30.77%), *S*. Gallinarum (21.15%), *S*. Paratyphi B (5.77%) and *S*. Newport (1.92%) among the breeder flocks in organized poultry farms (Bhattacharya, 2000). Saikia *et al*., (2001) isolated 15 strains of *Salmonella* belonging to *S*. enterica serovar Typhimurium and Enteritidis from 80 samples of poultry, consisting of 72 cloacal swabs from diseased birds and eight organ samples from dead birds in Assam.

Veling *et al*. (2002) in a study on 47 cases of diarrhoea in a farm experienced a clinical outbreak of salmonellosis, in which serovar Typhimurium phage type 401 and 506 (definitive type 104, DT104) were the most frequently isolated phage types (13 isolates).

Ahmed *et al.* (2008) reported the sero-prevalence of *Salmonella* in poultry (45.9%) and also opined that the rate of prevalence decreases with advancement of age of the birds. The cultural prevalence among sero-positive birds was 71% and in sero-negative birds, it was 59%. In dead birds, the cultural prevalence in liver was 64% and from cloacal swabs was 57 per cent. A total 160 isolates were characterized, among which 64.2% were *S.* Pullorum, 22.3% were *S.* Gallinarum and 13.5% were *S*. Paratyphi.

Sahar and Nagwa (2009) studied prevalence of *Salmonella* in chicken in Giza, Kafr El-Sheikh and Dakahlea and Sharika Governorates from faecal samples of diarrhoeic as well as apparently healthy birds and reported the rate of recovery was 17.5 and 3.4 per cent, respectively. The serovars were *S.* Enteritidis (7.3% and 1.7%), *S.* Pullorum (5.1% and 0.8%), *S.* Typhimurium (2.9% and 0.8%) and *S*. Gallinarum (2.2% and)%) from diarrhoeic and apparently healthy chicken, respectively. A total of 155 faecal samples from broilers were examined for the presence of *Salmonella* in Japan during 1999 by Ishihara *et al*. (2009) and they recorded 36.1 per cent *Salmonella*. The most predominant serovar was serovar Infantis.

Betancor *et al*. (2010) conducted a nationwide survey over 2 years that included the testing of sera from 5,751 birds and 12,400 eggs. Serological evidence of infection with *Salmonella* was found in 24.4 per cent of the birds. *Salmonella* were recovered from 58 of 620 pools made up of 20 eggs each, demonstrating presence of *Salmonella* at least 1 in every 214 eggs. The serotypes included *S.* Entertidis, *S*. Derby, *S*. Gallinarum, *S*. Enteritidis and *S.* Panama.

Pig:

Salmonella Choleraesuis was the first *Salmonella* serotype isolated from pigs (Salmon and Smith, 1886). Infection of pigs with the swine adapted serotypes Typhisuis and Choleraesuis usually result in swine typhoid, characterized by severe systemic disease that is often fatal and has been a subject of intense research (Gray *et al*., 1996; Lichtensteiger and Vimr, 2003; Chiu *et al*., 2004, 2005; Ku *et al*., 2005; Nishio *et al*., 2005; Zhao *et al*., 2006).

Singh *et al*. (1980) examined the intestinal contents of 463 pigs from Babugarh and Bareilly and found 2.55 per cent of the samples positive for *Salmonella* which included *S*. Typhimurium, *S.* Paratyphi B, *S*. Goverdhan, *S*. Welterveden, *S*. Senflenberg, *S*. Kirkee and *S*. Newport. Gupta *et al*. (1986) isolated *S*. Chester from rectal swabs of 46 diarrhoeic piglets. Jayarao *et al*. (1989) collected 200 faecal samples from pigs at a Budapest abattoir and isolated 96 *Salmonella* strains belonging to seven different serotypes that included *S.* Derby (70.8%), *S.* Typhimurium (8.33%), *S.* Bredney (4.16%), *S.* Panama (2.08%). Nakamura *et al*., (1989) recovered 26 (11%) strains of *Salmonella* from 236 pigs raised in farms and the most frequently encountered serotype was *S.* Paratyphi B. The other serotypes isolated were *S*. Agona, *S*. Amsterdam, *S.* London, *S.* Lexington, *S.* Welterveden, *S.* Typhimurium, *S*. Enteritidis and *S*. Chincol.

In addition to the non host adapted serotypes involved in pig salmonellosis i.e. *S*. Typhimurium (Lynn *et al*., 1972), *S*. Paratyphi B (Nakamura *et al*. 1989) or *S*. Enteritidis (Rahman *et al*., 2001), a number of workers have reported isolation of *S*. Choleraesuis from pigs showing enteric disorder (Ghosh *et al*., 1992; Borah, 1994). Bole Hribovek (1994) reported isolation of *Salmonella* from pigs and classified them into 21 serovars and the common serotypes isolated were *S*. Choleraesuis, *S.* Heidelberg, *S.* Typhimurium, *S.* Derby, *S.* Enteritidis and *S.* Infantis.

Among the different serovars prevalent in Assam, *Salmonella* Weltevreden, *S.* Choleraesuis, *S.* Paratyphi B and *S.* Typhimurium from pigs were reported by different workers (Bhattacharyya *et al*., 1991; Borah, 1994). Other *Salmonella* serotypes, *viz*. *S.* Chester, *S.* Enteritidis, *S.* Gallinarum, *S.* Typhimurium, *S.* Newport and *S.* Indiana were also isolated from Assam from different sources (Saikia and Patgiri, 1986; Rahman *et al*., 1997; Bhattacharyya, 2000).

Narayanswamy *et al*. (1996) isolated six strains of *Salmonella* which included two serotypes viz. *S*. Choleraesuis and *S.* Enteritidis from 230 samples from slaughter house and various piggery units in Bangalore. From a total of 13,468 caecal contents of pigs, 30 different serotypes of *Salmonella* enteric were isolated from 832 pigs in Denmark. The predominant serotype was *S.* Typhimurium, which included 536 (64.4%) of the isolates (Baggesen *et al*., 1996; Davies *et al*. (1998) reported that prevalence of *Salmonella* on gilt developmental farms was 3.4 per cent, whereas on breeder farms the prevalence was between 18 and 22 per cent.

Asai *et al*. (2001) screened faecal sample of 887 pigs from 235 farms and found *Salmonella* in 84 samples (9.5%) from 45 farms (19.1%). Higher prevalence was found in fattening pigs (17.3%), weaned pigs (12.4%) and suckling pigs (4.5%) than in sows (4.2%). Isolation rate of *S*. Typhimurium was higher in weaned and fattening pigs than in the others.

Rahman *et al*. (2001) reported isolation of *Salmonella* Enteritidis from an outbreak among captive pigmy hog (Sus salvanius) in Assam. Saikia (2001) isolated 10.63 per cent of *Salmonella* from rectal swabs of 94 diarrhoeic piglets. Barber *et al*. (2002) found that 1.4 to 3.1 per cent of swine on the farms they sampled were positive for *Salmonella*. A study conducted by Gebreyes and Altier (2002) at multiple sites in North Carolina, a total of

7,452 samples from pig were examined for Salmonellosis. The study revealed the presence of 484 *Salmonella* including 156 *Salmonella* serovar Typhimurium and 328 *Salmonella* serovar Typhimurium var. Copenhagen. Sarmah (2003) reported the isolation of 30 (14.08%) *Salmonella* strains from 213 rectal swabs of diarrhoeic piglets in Assam.

Farm- level and pig – level prevalence of *Salmonella* were reported to be 35.5 per cent and 2.2 per cent in 1998-1999, and 35.7 per cent and 3.3 per cent in 2004-2005 respectively. The predominant serotypes identified were Agona (28.4%), Typhimurium (17.9%) and Infantis (16.4%) in 1998-1999 and Typhimurium (32.5%), Anatum (24.6%) and Infantis (13.5%) in 2004-2005.

A study conducted by Padungtod and Kaneene (2006) on diseases of pigs reported prevalence of *Salmonella* in farm, slaughterhouse and pork at the market to be 6 per cent, 28 per cent and 29 per cent respectively in the Chiangmai and Ampo on provinces of Northern Thailand during the period of 2000 to 2003. Bonde and Sorensen (2007) reported that prevalence of *Salmonella* in 1609 faecal samples from pigs was 0.87 per cent. Futagawa-Saito *et al*. (2008) studied the prevalence of *Salmonella* in healthy pigs and collected faecal samples from 6771 pigs of 73 farms during 1998-1999 and 2004-2005.

During 1950s and 1960s, *Salmonella* Choleraesuis, including variant Kunzendorf, was the predominant serotype isolated from pigs worldwide (Fedorka-Cray *et al*., 2000). At present, *Salmonella* Choleraesuis is still highly prevalent in North America and Asia, but is found rarely in Australia and Western Europe countries (Wilcock and Schwartz, 1992; Fedorka-Cray *et al*., 2000; Chiu *et al*., 2004; Davies *et al*., 2004; Chang *et al*., 2005; Nollet *et al*., 2006). Pigs can be infected by several *Salmonella* serotypes and the occurance of these serotypes is also partly geographically determined (Fedorka- Cray *et al*., 2000;

Loynachan *et al*., 2004). All serotype isolated from pigs are considered a hazard for public health by the European food safety authority (EFSA, 2006). The public health risk of *Salmonella* infection from consumption of contaminated pork depends on multiple factors including the level of infection in the pig herd (Hill *et al*., 2003; Nollet *et al*., 2005), hygiene during carcass processing in the slaughter house (Broch *et al*., 1996), meat storage and distribution conditions (Mann *et al*., 2004) and occasionally through the handling of undercooked pork by the consumer (Hill *et al*., 2003).

In pigs, the most common serotypes are *S.* Typhimurium and *S*. Derby (Letellier *et al*., 1999; Davies *et al*., 2004; Gebreyes *et al*., 2004; Nollet *et al*., 2004; Valdezate *et al*., 2005; Rostagno *et al*., 2007). Pigs usually get infected through oral intake of the organism. After infection, animals can become carriers in the tonsils, the intestines and the gut – associated lymphoid tissue (Wood *et al*., 1989; Fedorka-Cray *et al*., 2000). Most of the time, carriers are not excreting the bacteria but under stressful conditions, re-shedding may occur. In this way carriers are a permanent potential source of infection for other animals, including humans. Stress factor may occur during fattening period but also prior to slaughter, for instance during transport to the slaughter house or during the stay in the lairage (Isaacson *et al*., 1999; Seidler *et al*., 2001; Rostagno *et al*., 2010).

Birds:

Salmonella appears to be a relatively common pathogen among pigeons and doves, but the prevalence seems to differ by serotype and habitat (Tizard, 2004). While a variety of serotypes have been isolated from pigeons and doves, *S.* Typhimurium var. Copenhagen phage types 2 and 99 are the most commonly isolated subtypes (Pasmans *et al.*, 2003). Intriguingly, *S.* Typhimurium isolates from pigeons differ biochemically and antigenically from other Typhimurium isolates, likely indicating host adaptation of these

Typhimurium subtypes to pigeons (Faddoul and Fellows, 1965; Pasmans *et al.*, 2003; Tizard, 2004; Pedersen *et al*. 2006) compared *Salmonella* prevalence in wild pigeons from urban areas and dairy farms in Colorado and detected *Salmonella* in approximately 8% of samples from dairy-exposed pigeons but not in samples from pigeons in urban areas. However, the isolation of various *Salmonella* serotypes from wild pigeons in urban areas in Japan has also been reported, indicating a potential risk for human health (Tanaka *et al.*, 2005).

2.7. Characteristics of *Salmonella* genome

Salmonella is comprised of a large circular chromosome consisting of approximately 4.8 mega bases (Mb). Extra-chromosomal DNA can be present in the form of plasmids of various sizes. Several *Salmonella* whole genome sequencing projects are ongoing worldwide. The Sanger Centre, UK, sequenced a number of *Salmonella* serovars, e.g. *S. enterica* serovars Typhi (Parkhill *et al*., 2001), Typhimurium (McClelland *et al*., 2001; Cooke *et al*., 2008), Enteritidis and Gallinarum (Thomson *et al*., 2008); simultaneously certain other groups sequenced *S. enterica* serovar Choleraesuis (Haneda *et al*., 2001). By 2008 (Benson *et al*., 2008), the genomic sequence for 26 *Salmonella* strains were available online.The plasticity of the genome describes the dynamic character of the genomic organization and enables the bacteria to adapt towards selection pressures. The structure of bacterial genomes consists of an endo-genome, the core of genes and an individual set of accessory elements the exo-genome (Mushegian and Koonin, 1996). In many cases insertion/ deletion differences can be detected in isolates of the same serovar that include other DNA elements such as transposons, retroposons, prophages, pathogenicity islands, plasmids that are putatively acquired or lost by horizontal gene

transfer. Horizontal gene transfer is believed to be a major contributor to *Salmonella* evolution (Porwollik and McClelland, 2003).

2.8 Genotypic Diversity Study of *Salmonella* by Multiplex PCR

The rapid identification of disease causing organism is of utmost importance to develop control or remedial measures. Therefore, PCR-based techniques find greater application over conventional methods. Among the DNA based molecular methods, multiplex PCR is a well-authenticated method for rapid unambiguous identification of organisms. It is an alteration variant of PCR in which several segments of target DNA are amplified simultaneously in a single PCR reaction to conserve template DNA and to minimize the time and cost (Fierer and Guiney, 2001; Alvarez *et al*., 2004; Khoo *et al*., 2009). Attempts have been made by various workers to develop multiplex PCR assays for detection of important virulence genes of *Salmonella* isolated from different sources. Optimization of multiplex PCR using different sets of primers is complicated with regard to annealing temperatures, combination and concentration of primers and maintenance of high specificity.

Liu *et al.* (2003) developed a multiplex PCR method (m-PCR) incorporating primers flanking three variable- number tandem repeat (VNTR) loci (arbitrarily labeled TR1, TR2 and TR3) in the CT18 strain of *Salmonella enterica* serovar Typhi and demonstrated that the m-PCR could be performed on crude cell lysates and that the VNTR banding profiles produced could be easily analysed by visual inspection after conventional agarose gel electrophoresis. The assay was highly discriminative in identifying 49 distinct VNTR profiles among 59 individual isolates. This m-PCR based VNTR profiling method provides a simple, rapid, reproducible and high-resolution molecular tool for the epidemiological analysis of *S. enterica* serovar Typhi strains.

Alvarez *et al*. (2004) reported the development of an m-PCR assay for *Salmonella* detection and epidemiological typing. Six sets of primers were designed to detect the major *Salmonella* serotypes and phage types in Spain. An internal amplification control was designed in order to detect PCR inhibition. Using this method, one can detect a specific band for DT104 and U302 phage types in *Salmonella* serotype Typhimurium.

Kim *et al*. (2006) developed an m-PCR method to differentiate between the most common clinical serotypes of *S. enterica* subsp. *enterica* encountered in the United States. Six genetic loci from *S.* Typhimurium and four from *S*. Typhi were used to create an assay consisting of two five-plex PCRs. The assays gave reproducible results with 30 different serotypes that represent the most common clinical isolates of *S. enterica* subsp. *enterica*. Of these, 22 serotypes gave unique amplification patterns compared with each other and the other eight serotypes grouped in to four pairs. These were further resolved by two additional PCRs. This assay could be easily performed on multiplex samples with final results in less than 5 hr and in conjugation with the pulsed-field gel electrophoresis; it formed a very robust method for molecular subtyping of *Salmonella enterica* subsp. *enteric* isolates.

Karami *et al*. (2007) developed an m-PCR for rapid detection of different *Salmonella enterica* serovars. The primers for three target genes, *tyv*, *prt* and *invA* were used for amplification of the genes by PCR. Simple DNA extraction method, rapid PCR cycles and rapid electrophoresis procedure with simple and very cheap buffer were used to separate the PCR products. The results showed that all reference and clinical isolates of *S. enterica* were accurately identified by this assay with no cross-reaction with other enterobacterial strains tested. These data indicated that the optimized rapid cycle m-PCR is a potentially valuable tool for rapid diagnosis of *S. enterica* using a conventional thermal cycler. This method reduced the reaction time of PCR from 3.5 hr to less than 1 hr.

Cardona-Castro *et al.* (2009) developed two m-PCR protocols to improve limitations of *Salmonell*a serotyping, using a strategy that identifies first the genes encoding serogroups (*rfbJ* and *wzx*) and according to the serogroup determined, a second m-PCR identifies serotype (*fliC, fljB, wcdB* and *sdf-I* sequence). Standardization and evaluation of both m-PCRs were carried out subsequently.

Leader *et al*. (2009) developed a high-throughput molecular assay to determine the most common clinical and non-human serovars of *S. enterica* in the United States. Sixteen genomic targets were identified based on their differential distribution among common serovars. Primers were designed to amplify regions of each of these targets in a single m-PCR while incorporating a 6-carboxyfluorescein-labeled universal primer to fluorescently label all amplicons. These labeled PCR products were separated using capillary electrophoresis, and a *Salmonella* multiplex assay for rapid typing (SMART) code was generated for each isolate, based upon the presence or absence of PCR products generated from each target gene. This high-throughput multiplex PCR assay allowed simple and accurate typing of the most prevalent clinical serovars of *Salmonella enterica* at a level comparable to that of conventional serotyping, but at a fraction of both cost and time.

Peterson *et al*. (2010) developed an m-PCR assay, which was capable of identifying 42 serovars by providing a valuable prediction of pathogenicity of the isolates by detecting the presence of virulence genes, *sseL, invA* and *spvC*. The gene *spvC* was the best predictor of pathogenicity. In a blind study, traditional serologic methods were correlated at 100 per cent similarity with the multiplex PCR-based method.

Ngan *et al.* (2010) developed an m-PCR assay for pan-*Salmonella* detection as well as for specific detection of serovars Typhi and Paratyphi A. The assay detected

members of the *Salmonella* genus by amplifying the Outer membrane Protein C (*omp C*). The presence of either *S.* Typhi or *S.* Paratyphi A was indicated by amplification of the putative regulatory protein gene *STY4220*, which was common to both serovars. The m-PCR was evaluated using 124 clinical and reference *Salmonella* serovars. *Salmonella* Typhi and *S.* Paratyphi A were detected at 100 per cent specificity and sensitivity. This m-PCR could prove to be a useful diagnostic tool for the detection and differentiation of serovars Typhi and Paratyphi A.

Thirumalai *et al.* (2011) developed a rapid and sensitive m-PCR based assay for the detection of *Salmonella enterica* serovars such as Typhi, Paratyphi A, Typhimurium, Enteritidis, Weltevreden, Bovismorbificians, Brunei, Arizonae and Infantis in shrimps within 4 hr of pre-enrichment. The *Salmonella* genus-specific gene *himA* was selected and 16S-23S internal transcribed spacer region was used as an internal amplification control (IAC). The genomic DNA was extracted by using boiling and centrifugation method. Sensitivity of the assay was tested by artificially inoculating the shrimp homogenate with viable cells of *Salmonella*. The m-PCR assay could detect up to five cells within 4 h of pre-enrichment. This assay provided specific, rapid and reliable results and allows for the cost-effective detection of serovars of *S. enterica* in one reaction tube in mixed bacterial communities that were prevalent in shrimp products.

2.9. Recombinant protein expression

Recombinant proteins have gained enormous importance for clinical applications. Nearly 30% of currently approved recombinant therapeutic proteins are produced in *Escherichia coli* (Huang *et al*., 2012). The main applications of recombinant proteins obtained by genetic engineering are in the medical therapeutic fields (e.g. production of

recombinant vaccines, and therapeutic proteins for human diseases), and medical diagnosis (e.g. antigen engineering for poly and monoclonal antibody production used in disease diagnosis). Other areas where recombinant proteins are commonly utilized include enzymes for food and fiber production, testing food for microbial contamination and veterinary medicine (Nilsson *et al*., 1992; Huang *et al*., 2012). Most proteins are expressed in extremely small amounts in their native cells and tissues, and it is only by recombinant techniques that it is possible to produce amounts greater enough for basic research or for practical use. Therefore, the expression of engineered proteins in efficient heterologous protein expression system is integral to the production and purification of many proteins of interest.

2.9.1. Recombinant Protein Production in *Escherichia coli*

Demands of the expanding biotechnology industry have driven different improvements in protein expression technology, which have been translated into the production of a spectrum of recombinant proteins in different systems for a wide variety of purposes. Most of the recombinant proteins are now-a-days produced either in bacteria, yeasts, engineered animal cell lines, hybridoma cells or even human cells. However, research continues on the development of alternative production systems, particularly in the use of transgenic animals or plants (Walsh, 2005; Desai *et al*., 2010). In the recent years, baculovirus and mammalian cell cultures have gained importance for the production of biopharmaceuticals due to the increasing needs of complex proteins and antibodies. An alternative to baculovirus or mammalian expression systems are yeasts, especially when large amounts of secreted protein are required (Porro *et al*., 2011). The use of transgenic

plants and animals as production vehicles may also play a role in applications requiring exceptional product volumes, but regulatory issues still remain to be addressed.

Even though the choice of expression system is progressively widening, *E. coli* is still the dominant host for recombinant protein production. It is used in many industrial fields to produce high value intermediates, detergents, nutraceuticals and pharmaceuticals, amongst others. It is the most popular choice when simple proteins are required, and significant advances have been made to overexpress complex proteins, hormones, interferons and interleukins in it (Walsh, 2005; Tripathi *et al*., 2009). The production of heterologous proteins or parts thereof in cytoplasmic compartments of *E. coli* offers multiple applications, for example, in diagnostics and vaccine development (Panda, 2004; Huang *et al*., 2012). Thus, bacterial expression systems are the preferred choice for production of many recombinant proteins. The reasons for this lie in the cost-effectiveness of bacteria, their well-characterized genetics, and the availability of many different bacterial expression systems. As a host for recombinant expression, *E. coli* is especially valued because of its rapid growth rate, capacity for continuous fermentation, low media costs and achievable high expression levels (Yin *et al*., 2007; Kamionka, 2011). Foreign proteins can be produced in *E. coli* in large amounts (5-50% of total protein). The major drawbacks of using *E. coli* for recombinant protein production are its lack of secretion systems for efficient release of proteins to the growth medium, limited ability to facilitate extensive disulfide-bond formation and other posttranslational modifications, inefficient cleavage of the amino terminal methionine which can result in lowered protein stability and increased immunogenicity, and occasional poor folding due to lack of specific molecular chaperones (Yin *et al*. 2007; Berkmen, 2012). Even though *E. coli* may not be useful for all foreign protein production, it has been successfully utilized to produce many functional human

proteins such as human growth hormone, proinsulin, interferon-gamma and antibody fragments (Schmidt, 2004; Tripathi *et al*., 2009).

2.10. *Escherichia coli* as a Host

Escherichia coli is a Gram-negative, facultatively anaerobic and non-sporulating bacterium that is commonly found in the lower intestine of warm-blooded animals. The morphology of the cells is rod-shaped, about 2 μm long and 0.5 μmin diameter. Most of the *E. coli* strains are harmless, but others can cause serious damage to humans. The harmless strains are part of the normal flora of the gastrointestinal tract, and can benefit their hosts by producing vitamin K2 and by preventing the establishment of pathogenic bacteria within the intestine (Bentley and Meganathan, 1982). However*, E. coli* can be easily grown outside the intestine as well, and it has become a model organism in biotechnology because of the simplicity of its genetics and easy manipulation. Till date, the most popular target for genetic manipulation in *E. coli* has been the modification of host cell metabolism to reduce acetate formation (Eiteman and Altman, 2005). Other genetic manipulations have been focused on improved protein folding (which may be achieved by overexpression of intracellular chaperons), or efficient disulfide bond formation (Berkmen, 2012). All these modifications have driven to the existence of various strains of *Escherichia coli* with different genotypes (genetic constitution) which are used as expression systems. In particular, *E. coli* K12 and *E. coli* B (e.g. BL21 or BLR) and their many derivatives are the most commonly used hosts (Sahdev *et al*., 2008; Tegel *et al*., 2010).

All these features allow *E. coli* to be one of the most competitive hosts for rapid and economical production of simple recombinant proteins, amino acid and metabolite production compared to other hosts.

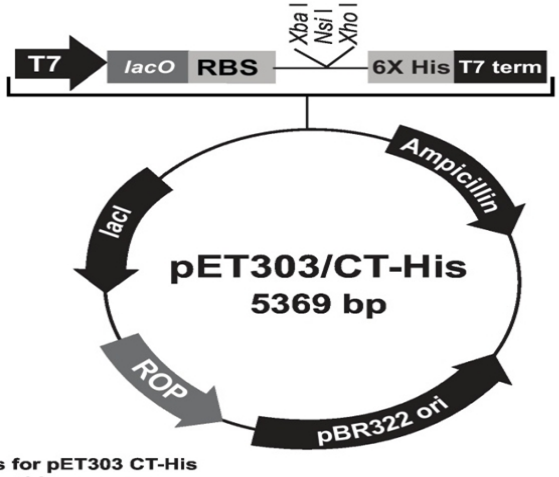
2.10.1. *E. coli* expression systems

Because of the vast knowledge about its genetics, biochemistry and molecular biology, *E. coli* is the system of first choice for expression of many heterologous proteins. Genetic manipulations are straight-forward, culture of *E. coli* are easily and inexpensively grown, and many foreign proteins are well-tolerated and may be expressed at high levels (Sambrook and Russell, 2001). *Escherichia coli* expression systems can be used for the production of recombinant proteins either intracellularly or extracellularly. Recombinantly expressed proteins can in principle be directed to four different locations namely the cytoplasm, the periplasm, the membrane fraction or the cultivation medium. Recombinant expression plasmids require a strong transcriptional promoter to control high-level gene expression. Promoter induction is either thermal or chemical and the most common inducer is the sugar molecule IPTG. The T5 promoter is specific for the RNA polymerase coded by T5 bacteriophage and a gene inserted downstream of this promoter will be expressed at a high level. Addition of IPTG to the growth medium switches on synthesis of the T5 RNA polymerase by triggering the release of tetrameric Lac I from the *lac* operator. This in turn leads to the transcription of the target gene from the T5 promoter, which is initiated by T5 RNA polymerase (Sorensen and Mortensen, 2005).

2.11 The pET Expression System

Expression systems are designed to produce many copies of a desired protein within a host cell. A number of central elements are essential in the design of recombinant expression systems (Baneyx, 1999; Jonasson *et al*., 2002). Expression is normally induced from a plasmid harboured by system compatible genetic background. The genetic elements

of the expression plasmid include origin of replication (ori), an antibiotic resistance marker, transcriptional promoters, translation initiation regions (TIRs) as well as transcriptional and translational terminators (Baneyx, 1999). Recombinant expression plasmids require a strong transcriptional promoter to control high-level gene expression. High-level gene expression of 6X His-tagged proteins in *E. coli* using pET 303 vectors is based on the T7 promoter transcription-translation system. The pET system was originally developed by Studier and colleagues and is derived from pBR322; it takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in *E. coli* from the T7 promoter (Studier and Moffatt, 1986; Rosenberg *et al.*, 1987; Studier *et al.*, 1990). Expression of recombinant proteins encoded by pET 303CT/His vector are rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG), which binds to the Lac repressor protein and inactivates it. Once the *lac* repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter, the transcripts produced are then translated into the recombinant protein (Stüber *et al*., 1990). IPTG has two advantages over lactose: first, its uptake is not dependent on the Lac permease (it diffuses through the inner membrane) and second, it cannot be cleaved by β-galactosidase preventing turn off of transcription (Glascock and Weickert, 1998; Schumann and Ferreira, 2004). The BL21 (DE3) *E. coli* strain is specifically designed for expression of genes regulated by the T7 promoter.



Comments for pET303 CT-His
5369 nucleotides

T7 promoter: bases 20-36
T7 promoter priming site: bases 20-39

Fig.1: Map and features of pET 303 CT His expression vector

(https://assets.thermofisher.com)

2.12. Purification Strategies for Recombinant Proteins

2.12.1. Refolding of Solubilized Recombinant Protein

Refolding and purification of bioactive protein is the major bio-processing parameter for efficient production of prophylactic, diagnostic or therapeutic proteins from *E. coli*. As level of expression for soluble proteins are in general low in *E. coli*, rarely such process is employed for large-scale production of therapeutic protein.

The soluble proteins in general are refolded to their native state after removing the chaotropic agents or other salts by dialyzing the proteins in buffers containing reducing and

oxidizing agents (Wang *et al*., 2006; Tan *et al*., 2010). Purification of the recombinant protein can be carried out either before renaturation in denaturing conditions or after refolding of the solubilized protein. Refolding followed by purification is generally preferable as some of the high molecular aggregates along with the contaminants can be co-purified in a single step. Dilution of the solubilized protein directly in to the renaturation buffer is the most commonly used method for small-scale refolding of recombinant proteins. However, the major limitation of dilution method is the problem of scale-up and low yield. Renaturation of bioactive protein with little aggregation has also been achieved by addition of low molecular weight additives (Kolaj *et al*., 2009). The most commonly used low molecular weight additives have been glycerol, L-arginine, low concentration of (1-2 M) urea or guanidine hydrochloride, and detergents. Among the additives, the positive effect of glycerol, L-Arginine/HCl in reducing aggregation have been demonstrated on various proteins like human gamma interferon (Babu *et al*., 2000; Wang *et al*., 2009) and for fibroblast growth factor (Alibolandi *et al*., 2011). Protein refolding can also be achieved by the use of diafiltration and dialysis using ultrafiltration membranes (Dasari *et al*., 2008). Huge volumes of solubilized protein material can be processed using such refolding procedures. It was found that use of chromatography results in higher yield of the refolded protein in comparison to dilution method. Affinity chromatography offers multiple advantages of buffer exchange, protein refolding and separation of pure protein.

2.12.2. Chromatographic Purification Strategies for Recombinant Proteins

Purification is an important step in the production of recombinant proteins. The characteristics of large scale purification schemes, such as conventional chromatography, have a significant impact on the final cost of production. It is often more efficient to use one

of the available tags to obtain the target protein. The purpose for which the protein will be used determines required degree for its purity and authenticity. Chromatographic methods (affinity, ion exchange, size exclusion and hydrophobic interaction) can be utilized either in traditional, low pressure or high performance liquid chromatography instrumentation.

2.12.2.1. Affinity Chromatography

Affinity chromatography together with recombinant DNA technology offers a simple and fast technique to purify proteins to high purity with a single purification step (Tan *et al*., 2010). Fusion can be made on either side or both sides of the target gene depending on specific application, but the majority of fusion proteins place the tag at the N-terminus of the protein (Nilsson *et al*., 1997). Although affinity chromatography can be used for laboratory scale purification, its utilization on a preparative scale can represent a major cost for the final protein product. Successful separation by affinity chromatography requires that a biospecific ligand is available, and that it is covalently attached to a chromatographic bed material. Due to the specificity of this recognition, it is often possible to obtain high purity of a protein sample (Arnou *et al*., 2006; Young *et al*., 2012). The packing material used, called the affinity matrix, must be inert and easily modified. Agarose is the most common substance used as a matrix, in spite of its relative high costs. The ligands, or affinity tails, that are inserted into the matrix can be genetically engineered to possess a specific affinity. In the process, the desired molecules adsorb to the ligands on the matrix until desorption is carried out, e.g. with a high salt concentration, a competition reaction (e.g. imidazole), strong chelating agents and/or low pH. To date, a large number of different fusion partners that range in size from one amino acid to whole proteins capable of selective interaction with ligand immobilized onto a chromatography matrix, have been

described (Nilsson *et al*., 1997: Young *et al*., 2012). Immobilized metal affinity chromatography (IMAC) systems have three basic components: an electron donor group, a solid support and a metal ion. The metal ion (usually Ni^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}) is restrained in a coordination complex where it still retains significant affinity towards macromolecules (Wang *et al*., 2009). The use of polyhistidine tags has been demonstrated in a wide range of host cells including *E. coli*, *S. cerevisiae*, insect cells as well as in mammalian cells. Affinity tags like Maltose Binding Protein (MBP), Calmodulin Binding Peptide (CBP), Glutathione-S-Transferase (GST), Histidine (His) and FLAG have been developed to achieve rapid expression and maximum purification of the recombinant proteins. His tag is a sequence of 6 histidine residues (vector pET303) that can bind nickel (Ni2+) resins via high affinity of imidazole to nickel. His tags have been known to improve folding of protein (White *et al.*, 2011). GST and His tags are top choices for large scale expression and purification due to high capacity and low cost (Lichty *et al*., 2005).

2.13. Outer Membrane Proteins (OMPs) of *Salmonella* and Immune Response

The outer membrane of Gram-negative bacteria is a complex structure with a major role of adaptation of the bacterium to various external environments while passively and selectively controlling influx and efflux of important solutes, peptides or proteins, nucleic acids, and other organic compounds such as lipids and polysaccharides. Outer membrane proteins (OMPs) and lipoproteins function in many capacities including membrane structure and stability, active and passive ion and solute transport, signal transduction, defense, and catalysis (Khalid *et al*., 2008). Most OMPs are surface exposed and therefore, are potentially important in interfacing bacteria with the mammalian host and its defenses, bacteriophages, and other bacteria or microorganisms (Krishnan and

Prasadarao, 2012). The membrane proteins also form channels or receptors that allow the transmission of signals and molecules across membranes. Thus, they act as entries and doorways that regulate the interactions between the two sides of the membrane. It is estimated that membrane proteins form at least 20% of the total proteins in prokaryotes. Kukreja (1989) studied immunoprotective efficacy of sonicated extract of *Salmonella* Gallinarum and reported that a better cell mediated immune response was evoked by subcutaneous injection as compared to oral route in vaccinated chicken. Meenakshi *et al.* (1999) studied the immunogenicity of a sonicated extract (SE) and of outer membrane proteins of *Salmonella* Enteritidis in birds of about 8 weeks of age and observed that adjuvanted OMP vaccines gave better protection than SE vaccines. OMP preparations extracted from a rough mutant of *S.* Typhimurium were shown to be good immunogens in mice and rabbits (Kuusi *et al.,* 1979). High titres of anti-porin antibodies were detected by using ELISA. Rabbits immunized with outer membrane proteins of *S.* Dublin also showed significant protection (66.66%) against oral challenge (Chaturvedi *et al.*, 1994). Bouzoubaa *et al.* (1987) observed that immunogenicity and protective efficacy of OMP from *S.* Gallinarum at different concentrations with or without mineral oil adjuvant against live challenge.

Bacterial lipoproteins perform many roles, as signal transduction, nutrient uptake, adhesion, conjugation, sporulation, and participate in antibiotic resistance, transport (such as ABC transporter systems) and extracytoplasmic folding of proteins. Lipoprotein has been reported as a vaccine candidate against many pathogenic bacteria, they play a direct role in virulence-associated functions, such as colonization, invasion, evasion of host defense, and immunomodulation against pathogens (Kovacs et al., 2011). Moreover, lipoprotein as a vaccine candidate is expected to have relatively low production costs and high efficacy

compared with many other three vaccines. invH is an outer membrane lipoprotein of Salmonella enterica which play an important role in bacterial adhesion and virulence. Studies have shown that invH outer membrane lipoprotein of S.enterica with an apparent molecular mass of 15kDa is highly immunogenic, evokes humoral and cell mediated immune responses, and confers 100% protection to immunized mice against challenge with high doses of S. Enteritidis (Dehghani et al., 2012). In a study it is shown that invH of S. Typhi can provoke cell mediated as well as humoral immunity and can be proven a promising vaccine candidate against typhoid (Dehghani et al., 2014). Also, in a study it was observed that this 15kDa lipoprotein is antigenic in nature and antibody against this protein can also recognize epitopes on intact S. Typhi cells conferring it a potential candidate to produce vaccine against typhoid (Kumar et al., 2017).

2.14. Vaccination against Salmonellosis

Many efforts have been made to find effective vaccines against *Salmonella* infections in livestock and currently there is demand for a vaccine to control *Salmonella* infections associated with human food poisoning, in particular, those caused by *S.* Enteritidis (Feberwee *et al*., 2001). However, due to the complicated pathogenesis of *Salmonella* infection, no significant breakthrough has been achieved (Chiu *et al*., 2004).Vaccines to control *Salmonella* infections, especially inactivated vaccines, is in use all over the world. In recent years, increasing numbers of live vaccines have been developed but most of them are not yet authorized. Vaccination can play an important role in intervening against *Salmonella* in high-prevalence herds (Springer *et al*., 2001; Haesebrouck *et al*., 2004). Vaccines can be divided into two broad categories: active and passive. An active vaccine is intended to stimulate the body's immune system to produce specific antibodies (humoral response), cellular immune responses (e.g. cytotoxic T-

lymphocytes), or both, with the aim of protecting against or eliminating a pathogen. A passive vaccine is a preparation of antibodies that is protective against a pathogen or disease and is administered before, at or around the time of known or potential exposure. Active vaccines can in turn be divided in to three main categories, killed vaccines, live attenuated vaccines and subunit vaccines. In poultry production units, both attenuated and inactivated bacteria-based vaccines are commonly used (Methner *et al.*, 2001; Woodward *et al.*, 2001).

Initial work on development of *Salmonella* vaccine started in late nineteenth century with attenuated vaccine (Wright and Sample, 1997) for typhoid infection in human beings. Later, Smith (1956) developed 9R and 9S strains of *S. enterica* sp. *enterica* serovar Gallinarum (*S*. Gallinarum) for control of fowl typhoid. Subsequently, killed vaccines were used successfully with confidence of safety to stamp out salmonellosis from equines. Later, many different *Salmonella* serovars were used to produce killed bacterins for veterinary use such as *S*. Typhimurium (Nicholas and Andrews, 1991) *S*. Dublin (Liberal, 1989), *S*. Gallinarum (Mohrah and Zaki, 1995) and *S*. Enteritidis (Barbour *et al.*, 2001). In India, the most successful killed vaccine was made from formalin killed; alum precipitated *S*. Abortusequi (Dhanda *et al*., 1955) which was found to be superior to earlier vaccines yeilding up to 86% protection in mice as compared to heat-killed phenolized vaccine which could protect only 50% of the vaccinated population.

Chrome alum (Siddiqui, 1968), potash alum, Freund's complete and Incomplete adjuvant (Gupta *et al*., 1987) alhydrogel (Ghosh, 1989), mineral oil (Mohrah and Zaki, 1995), have been used in different *Salmonella* vaccines. For inactivating *Salmonella* in killed vaccine agents, heat (Nicholas and Andrews, 1991), β-propiolactone (BPL),

glutaraldehyde (Kataria,1978) and formaldehyde (Ghosh,1989) have been tried in order to preserve the antigenicity and increase the efficacy of vaccines.

Poor performance of the killed vaccines forced researchers in 1980s to develop other types of *Salmonella* vaccines employing sub-cellular components of *Salmonella* and as a result several subunit vaccines came into being. The development of subunit vaccines is presently the main strategy being evaluated for prevention of infectious diseases. Common sub-cellular components of *Salmonella* used for development of vaccines are: outer membrane proteins (OMPs), porins, toxins and ribosomal fractions. The epitopes recognized by neutralizing antibodies are usually found in just one or a few proteins present on the surface of the pathogenic organism. Isolation of the genes encoding such epitope-carrying protein immunogens and their expression in heterologous hosts form the basis of recombinant-subunit-vaccine development (Hansson *et al.*, 2000). Such vaccines tried in different animals had variable success.

There are currently three types of licensed vaccines for *S.* Typhi, but none for the other serovars (Tennant *et al*., 2015). The oldest of these vaccines is live attenuated *S.* Typhi Ty21a, which was constructed by chemical mutagenesis and is unable to synthesize galactose due to a mutation in the *galE* gene (Germanier *et al*., 1975). The vaccine is currently formulated as enteric-coated capsules which are taken orally on alternate days until three (in most world regions) or four (in the United States) doses have been received. This vaccine is highly efficacious against typhoid fever and shows some cross-protection against *Salmonella* Paratyphi B, but not against *Salmonella* Paratyphi A (Black *et al*., 1990; Levine *et al*., 2007). There are also two vaccines that target the Vi capsule polysaccharide: a polysaccharide-only vaccine and a conjugate vaccine. However, these are only effective

against *Salmonella* Typhi and show no efficacy against other *Salmonella* serovars. As such, there is a need for additional vaccines to protect against paratyphoidal and nontyphoidal *Salmonella* infection.

Recombinant strategies are of great interest in the development of new vaccines and in addition, for the improvement of existing vaccines. Research pertaining to this innovative approach to vaccination has focused on the evaluation of purified proteins as immunogens (Vindurampulle *et al.*, 2004). The potential advantages of recombinant vaccines are their safety, the potential abilities to target the vaccines to the site where immunity is required, and to differentiate vaccinated animals from the infected ones through the right selection of the components. In this regard, Hamid *et al.* (2008) worked on a 49 kDa outer membrane protein of *Salmonella* Typhimurium which successfully conferred protection against Typhoid. Agarwal *et al.* (2010) worked on the outer membrane protein C (OmpC) of *Salmonella* and have found it to be a potential immunogen to be produced as vaccine. Ghosh *et al.* (2011) found adhesin protein (T2544) of *Salmonella* to be a possible vaccine candidate against infections caused by different serovars of *Salmonella*. Anwar *et al*. (2012) worked on recombinant flagellin (fliC) and found it to be a prospective candidate vaccine against typhoidal strains. Dehghani *et al,* (2012) successfully worked on recombinant LPS vaccine of *Salmonella* Enteritidis against typhoid fever. Kaur *et al*. (2013) worked on a 49kDa OMP of *S*.Typhi and found it to be a potential candidate vaccine against typhoid fever. Kumar *et al.* (2017) reported that 31kDa recombinant lipoprotein is a suitable immunogen against *S*.Typhi.

All of the *Salmonella* vaccines currently in development will need to be evaluated for safety and immunogenicity in preclinical studies. The design, selection and production

of recombinant subunit vaccines will be the basis of modern vaccinology. Various strategies to administer the subunit vaccine, as a protein immunogen, via a live delivery vehicle or as a nucleic acid construct are being studied.

2.15. Adjuvant

Adjuvants are substances that, when used in combination with vaccine antigens, induce a stronger and more efficious respose to the vaccine as compared to that induced by vaccine alone (Corradin and Giudice, 2005). Different types of adjuvants have been recognized but not much is known about their modes of action.

2.15.1. Mechanism of Action of adjuvants

Adjuvants trigger events that appear to come from one or the combination of several of the following effects known as the Depot effect. It is a known fact that antigens in solution are quickly removed by neutrophils and macrophages, but subsequently, they are unable to prime naive T cells. Therefore, following the antigen's disappearance, the immune response is hardly detectable. The most widely used adjuvants, such as oil-emulsions and antigen-absorbing aluminium salts, may retain antigen at the injection site, from where it is released in minute quantities over a prolonged period of time. These compounds mainly stimulate the production of antibodies by the induction of Th2-lymphocytes. In the case of use of alum, the mechanism of action seems to be due, at least in part, to the formation of a depot of free alum that would induce the recruitment and activation of immune cells to the site of inoculation. However, this "favourable" local inflammation may derive in a granulome, or even eosinophilia (Gupta *et al*., 1995). Also, allergic reactions might occur after a reimmunization by these adjuvants.

2.15.2. Effect on Antigen presenting cells (APC):

Enhancement of an immune response through an adjuvant may be attributed to the improved delivery of antigens into the draining lymph nodes. This may be achieved by facilitating the antigen uptake by APCs, or by increasing the influx of APCs into the injection site. Whichever is the case, the result is the same: an effective priming of specific T cells derived from an increase in the provision of antigen-loaded APCs, promoting the activation state of APCs by upregulating costimulatory signals or MHC expression. This results in the corresponding cytokine release, enhancing the speed, magnitude and duration of the specific immune response.

2.15.3. Nonspecific immunostimulanting effect:

Some agents can stimulate the non-specific component of the immune system. Numerous microorganisms contain "alert signals", the so called "microbial or pathogen associated molecular patterns" (MAMPs or PAMPs), not present in mammalian cells. These structures activate immune cells through interaction with specific receptors (toll like receptors, TLRs). Some examples are: lipopolisaccharide (LPS), monophosphoryl lipid A (MPL), flagellin, lipoproteins, muramil dipeptide (MDP); trehalose dimycolate (TDM), or CpG DNA, among others (Roth *et al*., 2005; Salman *et al*., 2006). Besides, the special chemical nature of some polymers used in the formulation of vaccine delivery systems may also be recognized as scavenger ligands for the APCs (Kim *et al*., 2002; Yamamoto *et al*., 2002).