Chapter-1

INTRODUCTION

Salmonellae, the gram-negative bacilli of the family *Enterobacteriaceae*, are widely distributed in nature and cause diseases ranging from gastroenteritis to typhoid fever. *Salmonella enterica* subsp. *enterica* is one of the leading causative organisms of zoonotic food-borne diseases worldwide (Toboldt *et al.,* 2013). It is believed that epidemics caused by this organism may have significantly affected the history of mankind till date, whilst the effect of *Salmonella* infections are considered to augment economic burden in both developing as well as industrialized nations (Cunha *et al*., 2004).

Salmonellae usually occur as short rods measuring 2-4 μm in length and 0.5 μm in width. Occasionally, they develop into longer pleomorphic forms or very short cocobacilli after prolonged culture in laboratory media. With the exception of *Salmonella* Pullorum and *Salmonella* Gallinarum, all strains of other serotypes normally possess peritrichous flagella and are actively motile. Many serotypes are also known to develop fimbriae. Capsule formation has sometimes been observed and is associated more particularly with mucoid strains (*Salmonella* Typhi). Spore formation in *Salmonella*, however, has not been reported.

Salmonella infection in human and animals primarily causes self-limiting gastrointestinal infections with mild to moderate symptoms like fever, abdominal cramps and diarrhoea. Death may occur in cases of bacteremia or enteric fever (typhoid and paratyphoid), often characterized by severe headaches and high fever but no diarrheoa. The most common mode of transmission for the majority of *Salmonella* infections in humans is through consumption of contaminated foodstuff and water. Non-typhoidal *Salmonella* infection is an important food-borne infection with an estimated incidence of 1.3 billion

cases and 3 million deaths all over the world (Thong *et al*., 1995). *Salmonella* is of special concern in the developing world because of poor hygienic conditions that favour its proliferation (Nagappa *et al.,* 2007) contributed by low socio-economic status of majority of the people in these countries. At the same time, it is usually difficult to evaluate the status of salmonellosis due to lack of a perfect coordinated epidemiological surveillance system (Oosterom, 1991; Acha and Szyfres, 2001). In India, salmonellosis is considered to be hyper-endemic and its importance as a potential zoonosis of economic significance needs no explanation (Rahman, 2002). In spite of this, there have been very limited case studies of non-typhoidal salmonellosis in the entire country. Over 235 serovars have so far been reported in India (Singh, 2005) and this has been increasing at a constant rate. Humans acquire *Salmonella* infection mostly from the contaminated food such as pork, beef, poultry meat and eggs (Linam and Gerber, 2007), and fruits and vegetables (Pui *et al*., 2011). It is presumed that under-reporting of cases of salmonellosis and the prevalence of other diseases considered to be of high priority may have overshadowed the problem of salmonellosis in India.

The first member of this group to be studied was the typhoid bacillus, observed in human tissues by Eberth in 1880 and cultured by Gaffkey in 1884. Afterwards, Salmon and Smith reported an organism (*Bacillus cholerae suis*) which they isolated from diseased pigs. Similar isolations were also made in the subsequent years from a wide variety of human and animal sources. The name *Salmonella* was given in the memory of Salmon for his initial work on the organism.

Salmonella bongori and *Salmonella enterica* are the two species that comprise the genus *Salmonella*. The species *Salmonella enterica* is further divided into six subspecies,

namely, *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIB), *houtenae* (IV) and *indica* (VI), while *S. bongori* has not been subdivided further into subspecies. *Salmonella enterica* subsp. *enterica* strains are of the greatest clinical relevance and are typically isolated from humans and warm blooded animals. Serological classification of *Salmonella* strains based upon properties of various surface polysaccharide (O) and flagellar (H) antigens is considered as the reference method for epidemiologic surveillance. This method involves the characterization of over 150 unique O and H antigens to produce an antigenic formula that can be scored using the Kauffman-White scheme to determine a serovar for an isolate (Grimont *et al*., 2000). Currently there are more than 2579 *Salmonella* serovars reportedly prevalent in the world (Seyfarth *et al*., 2003). A few of them are highly host-specific, while majority of them are unadaptable to changing environmental factors and can cause infection in a wide variety of animal species. Among the top 10 *Salmonella* serovars, *S*. Typhimurium, *S.* Enteritidis, *S.* Anatum, *S.* Bareilly, *S.* Dublin, *S.* Newport, *S.* Paratyphi B and *S.* Weltevreden are responsible for human and animal ailments throughout the world (Verma *et al.*, 2001).

Salmonella Enteritidis and *S.* Typhimurium are the two serovars most frequently associated with human food poisoning (Oliveira *et al*., 2002). Among the two, *S*. Typhimurium is most commonly associated with enteric infection in man and animals (Rahman, 2002). Serovar *S*. Typhimurium has a diverse host range, which includes human, cattle, pig, sheep, horse, rodent and birds (Townsend *et al*., 2001). *Salmonella* Typhimurium can cause a more invasive infection in immunocompromised patients such as those with AIDS, certain cancers and under chemotherapy, which can be life threatening (Gulig and Doyle, 1993). It is also recognized asthe most common serovar of *Salmonella* causing cardiovascular, bone and joint infections (Kalpana *et al*., 1998).

The investigation on molecular mechanisms of virulence in *Salmonella* has shown that pathogenic salmonellae are different from their non-pathogenic counterparts due to the presence of different virulence genes, often organized in so-called clusters referred to as 'pathogenicity islands'. *Salmonella* infections exhibit a complex pathogenesis in which numerous virulence genes are involved (Hensel, 2004). These genes are clustered within *Salmonella* pathogenicity islands (SPI) and 21 such islands (SPI-1 to SPI-21) have been reported to date (Suez *et al*., 2013). However, there are variations in respect of occurrence of these SPIs including individual genes among different serovars (Sabbagh *et al.,* 2010). The type III secretion system (T3SS) proteins encoded by two *Salmonella* pathogenicity islands (SPIs) are associated with the pathogenicity of *Salmonella* at the molecular level (Kaur *et al*., 2012). Besides, some virulence genes not located on SPIs such as chromosomally-encoded *stn (Salmonella* enterotoxin gene), *phoP/Q* (two component global regulators) and *iroB* also play important roles in the virulence of *Salmonella.* Some other genes such as *inv*, *sef*, etc. are involved in adhesion and invasion (Clouthier *et al.,* 1994; Galan *et al.,* 1998), while others are associated with the survival in the host system, *viz. mgtC5* or in the actual manifestation of pathogenic processes, *viz*. *sop*, *stn*, *pip* A, B and D (Wood *et al.,* 1998; Chopra *et al.,* 1999; Wallis *et al.,* 2000). Some *Salmonella* serotypes also harbour plasmids of varying sizes that carry genes which are responsible for virulence. Serovars like *S.* Typhimurium also harbour self-transmissible virulence plasmids (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; Rotger *et al.,* 1999). These virulence factors are of great diagnostic importance and serotyping is done on the basis of presence or absence of specific virulence factors as some virulence-profiles are observed to be serotype-specific.

Salmonellosis reflects the outcome of a combination of humoral and cellular immune responses of the host in response to virulence determinants of the invasive pathogen. The adhesion-dependant attachment of salmonellae to lumenal epithelial cells and M cells of the Payer's patches helps in internalization of the pathogen by a receptor-mediated endocytosis process. Cytotoxin localized in the bacterial cell wall facilitates entry of the organism into the epithelial layer. Cytoplasmic translocation of the infected endosome to the basal epithelial membrane culminates in the release of salmonellae into the lamina propria. During this invasive process, *Salmonella* secretes a heat-labile enterotoxin that induces a net efflux of water and electrolytes into the intestinal lumen (Chu *et al.,* 2006). The internalized pathogen is taken up by antigen presenting cells thereby activating both B and T cell lymphocytes. Some bacteria escapes this barrier and enters into the blood causing bacteremia, following which secondary infection arises in liver, bone marrow, gall bladder, Payer's patches, etc.

Several methods have been developed to detect the presence of potentially low levels of *Salmonella* in food and clinical samples. These methods include culture-based techniques (FDA,1992), DNA-based methods (Fitts *et al*., 1983,1985; Fluit *et al*., 1993; Kwang *et al*., 1996; McElroy *et al*., 1996;), bacteriophage-based assays (Hirsh *et al*., 1983,1984; Goodridge *et al*., 2002), immunoassays (Mattingly *et al*., 1984; Ibrahim *et al*., 1986; Hadfield *et al*., 1987), and biosensors (Ye *et al*., 1997; Bokken *et al*., 2003; Kramer *et al*., 2004; Taitt *et al*., 2004). Standard culture methods for *Salmonella* detection involve multiple-steps which are labour- intensive and time-consuming, taking 4 to 5 days for detection and confirmation (FDA, 1992). Furthermore, it has been reported that some of the routinely used selective enrichment broths are inhibitory towards many *Salmonella* strains

(Van der Zee, 1994). The DNA-based and bacteriophage-based methods have shown considerable success by reducing the detection time to 2-3 days, yet none of them have been recognized as a rapid, specific, and user-friendly technique for routine use in screening food and clinical samples. Various PCR assays have also been developed for the detection of different gene-encoded virulence factors, *viz*. simplex PCR for *Salmonella* enterotoxin (*stn*) (Prager *et al.,* 1995), *Salmonella* enteritidis fimbriae (*sef*) and plasmid encoded fimbriae (*pef*) (Rahman *et al.,*2000). Similarly, use of multiplex PCR as a tool for pathogen detection in clinical and environmental samples has been recommended by many workers (Mason *et al*, 2001; Wang *et al*., 2002; Lee *et al*., 2003).

Fortunately, vaccination is considered as an effective tool to control diseases caused by *Salmonella* spp. (Dosmorov *et al*., 1991). Presently, in poultry production units, both killed and attenuated live vaccines are commonly used; although the efficacy of their action could prove successful to a limited extent only (Timms *et al*., 1990). Killed vaccines fail to elicit both cell-mediated and secretory IgA mediated immune responses, which are vital for long term protection including protection of mucosal surfaces (Lax *et al*., 1995). Moreover, attenuated live *Salmonella* vaccine bear the risk of reversion into their original pathogenic forms, especially in immuno-compromised individuals. Such vaccines can be hazardous due to residual virulence caused by insufficient attenuation. Development of vaccines based on non-toxic components of such pathogens has led researchers to extensively study the complex antigenic forms in *Salmonella*. In recent years, adjuvants are being used as essential components of new subunit vaccines that boost the immune responses that protect immunity in the vaccinated individual enabling the host to restrict a range of therapeutic problems including serious infections, cancer, neurological and addictive disorders etc. This has led to recent advances in vaccine development and in

particular, the increasing use of recombinant subunit and synthetic vaccines, where the need of adjuvants are more acute. It is assumed that the use of well-defined recombinant proteins in combination with appropriate adjuvant is more likely to overcome many limitations and provide species or strain specificity. Additionally, antibiotic therapy or prophylaxis is increasingly coming under close scrutiny, largely because of the fear of increased levels of resistance in food-borne human pathogens (Threlfall *et al*., 1998). One major advantage of the genus *Salmonella* is the latent condition whereby it can enter and hide inside cells of the body and can turn resistant to antibiotics. Such characteristic features in bacteria like *Salmonella* is possible due to the presence of bacterial outer membrane proteins (OMPs), that are ubiquitously found in the outer membranes of gram negative bacteria and possibly even *Archaea* (Nikaido, 2003). OMPs primarily consists of phospholipids, lipopolysaccharide, and proteins and account for approximately 50% of the outer membrane mass (Koebnik *et al*., 2000). OMPs include integral membrane proteins as well as lipoproteins that are anchored to the outer membrane via N-terminally attached lipids and are essential for maintaining the integrity and selective permeability of bacterial membranes (Lin *et al*., 2002). Additionally, the productions of OMPs are often regulated by environmental cues that play important roles in bacterial pathogenesis by enhancing the adaptability of bacterial pathogens to diverse environmental conditions. They are extremely immunogenic and could be effective candidates for vaccine and diagnostic development owing to their exposed epitopes on the cell surface. Some of them also serve as adhesins and hence play an important role in virulence mechanism (Ebanks *et al*., 2005; Khushiramani *et al*., 2008). The outer membrane proteins of *Salmonella* are also known to have a significant role in eliciting immune responses (Meenakshi *et al*., 1999). They have been considered potential candidates for conferring protection against the disease typhoid. *Salmonella* OMPs have been thoroughly investigated as potential vaccine candidates,

virulence factors, and diagnostic antigens (Isibasi *et al*., 1988). Likewise, an outer membrane lipoprotein, invH, an integral part of the *Salmonella* Pathogenicity Island 2 (SPI-2) of the Type III Secretion System (T3SS) serves as an adhesin molecule for the entry of the bacteria into epithelial cells of the host, and has been targeted for vaccine development. Except for *S. enterica* subsp. *arizonae*, the invasive outer membrane protein InvH is present in all *Salmonella* strains (Altmeyer *et al*., 1993). It was also found to be immunogenic against *Salmonella* Enteritidis (Dehghani *et al*., 2012) and is therefore expected to provide convincing solutions to genus-specific vaccine development. Recent progress in molecular biology techniques provide an opportunity of developing new, subunit vaccines from well-defined repertoire of immunogenic proteins like OMPs and facilitating the enhancement of immune responses in hosts to combat *Salmonella* infections. It has been presumed that preparation and characterization of recombinant variants of this major immunogenic protein (InvH) of *S.* Typhimurium can assist towards the development of potential vaccines with considerable usefulness for the control of *Salmonella* infections in animals and humans and hence has also been deliberated as a potential candidate for future drug development.

Better technological possibilities combined with increased knowledge in related fields such as immunology and molecular biology is envisaged to provide options for new vaccination strategies (Nasser *et al.,* 2002; Charles *et al.,* 2010). It is pertinent from all the available literature that vaccination is the only best suited explanation to fulfill the requirements of practical anti- *Salmonella* intervention without much associated risks compared to other control measures.

With the aforementioned facts in view, the present study was undertaken with the following objectives:

1. To isolate and identify *Salmonella* from man, animals and birds.

2. To develop a rapid multiplex PCR assay for detection of certain major virulence genes of *Salmonella enterica*.

3. To clone, express and purify the *invH* gene encoding a 15-kDa protein of outer membrane of *Salmonella enterica* serovar Typhimurium

4. To study the immunogenic potential and protective efficacy of the purified recombinant protein.