Chapter-5

DISCUSSION

Salmonella infections have remained a significant worldwide public health concern that affect livestock, children and adults not only in developing countries but also in the industrialized world (Bulgin *et al*., 1982). Despite tremendous progress made in respect of molecular characterization and epidemiological studies on Salmonellosis, a large number of questions regarding pathogenicity and epidemiology of *Salmonella* have still remained unanswered. Salmonellosis in human is endemic in India. The rate of food borne Salmonellosis in India is 6 per 1000 (Kumar *et al*., 2009). Among the typhoidal serovars of *Salmonella*, the most common serovars from human are *S*. Typhi (73%) and *S*. Paratyphi A (24%), while *S*. Worthington (28.2%) and *S*. Typhimurium (22.5%) are the most prevalent non-typhoidal serovars in India. In animals, *S*. Typhimurium is the commonest serovar followed by Weltevreden (Kumar *et al*., 2009). Currently there are more than 2579 *Salmonella* serovars distributed throughout the world (Seyfarth *et al*., 2003). Some of these serovars, *viz*. *S*. Gallinarum, *S*. Pullorum and *S*. Typhi are highly host-specific, the majority are un-adapted and can cause infection in a wide variety of animal hosts (Gupta and Verma,1993), contributing to its interspecies transmissibility and pathogenicity.

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, especially inactivated ones, are in use all over the world to control *Salmonella* infections. The outer membrane proteins of *Salmonella* are also known to have a significant

role in eliciting immune responses (Meenakshi *et al*., 1999). *Salmonella* OMPs have been thoroughly investigated as potential vaccine candidates, virulence factors, and diagnostic antigens (Isibasi *et al*., 1988). The 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 (Dehghani *et al*., 2012).

Considering the veterinary and public health importance of salmonellosis, the present study was undertaken to isolate and identify *Salmonella* from diverse sources and to develop a multiplex PCR assay for identification of common clinical serovars of *Salmonella*. Attempt was also made to clone and express the 15 kDa InvH surface protein of *Salmonella* Typhimurium in *E. coli* and to evaluate immuno-potential of the recombinant protein in mice.

5.1. Isolation

A total of 495 samples collected from different sources were examined for isolation of *Salmonella,* of which 76 (15.35%) were found to be positive for *Salmonella* (Table 4.1). The isolation rate was the highest in poultry (21.36%) followed by pig (15.9%), wild bird (14.85%), human (11.76%) and cattle (10%). The isolated *Salmonella* strains belonged to ten different serotypes namely *S*. Typhi, *S*. Typhimurium, *S*. Newport, *S*. Dublin, *S*. Enteritidis, *S*. Gallinarum, *S*. Litchfield, *S*. Weltevreden, *S*. Choleraesuis and *S*. Kentucky. In the present study, *S*. Weltevreden was found to be the most predominant serotype, irrespective of the host species. This was in agreement with Kumar *et al*. (2009) who reported *S*. Weltevreden to be one of the five serovars isolated most frequently in India.

In the present study, out of 103 cloacal swabs/faecal samples/intestinal content from poultry examined, 22 (21.36%) yielded *Salmonella*, which was in accordance with Saikia (2001), who reported recovery of *Salmonella* from cloacal swabs of 20.27 percent poultry. Kumar and Sawney (1971) reported the rate of recovery of *Salmonella* from poultry to be 14.54 percent. Similarly, *Salmonella* was recovered from 14.75 percent diarrhoeic poultry by Sarmah (2003) and 14.7 percent by Murugkar *et al*. (2005) in the north-eastern region of India. However, Saikia and Patgiri (1986) recorded a slightly lower prevalence (10.66%) of *Salmonella* in poultry. On the other hand, Shivhare *et al*. (2000) reported 7 per cent prevalence of *Salmonella* in poultry in India. Esteban *et al*. (2008) however, reported the prevalence of *Salmonella* as low as 2.9 percent in poultry. On the contrary, there were reports of recovery of *Salmonella* from as high as 50 per cent (AMM, 2003) and 36.1 per cent (Ishihara *et al*., 2009) samples from poultry. The moderate to high level of prevalence of *Salmonella* infection recorded in this region might be attributed to favourable climatic conditions like higher humidity, heavy rainfall and to the fact that poultry is one of the major reservoirs of *Salmonella* (Saikia *et al*., 2001).

Out of 60 diarrhoeic pig stool samples examined, 14 (23.33%) showed presense of *Salmonella* (Table 4.1). Of these, 4 (28.6%) belonged to serovar *S*. Choleraesius, 5 (35.7%) *S*. Typhimurium and 5 (35.7%) *S*. Weltevreden. Padungtod and Kaneene (2006) earlier reported the prevalence of *Salmonella* in diseased pigs in farms, slaughterhouse and pork markets 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 which also supports the present findings. A number of workers reported isolation of *S*. Choleraesuis from pigs showing enteric disorder (Ghosh *et al*., 1992; Borah, 1994). Among the different serovars prevalent in Assam, *Salmonella* Weltevreden, *S.* Choleraesuis 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). All the serovars isolated from pigs are considered a hazard for public health by the European food safety authority (EFSA, 2006).

In the present study, 14 (16.47%) out of 34 diarrhoeic human stool samples examined showed the presence of *Salmonella* (Table 4.1). Out of those isolates, 4 (28.57%) belonged to the host-specific serovar *S*. Typhi and the rest belonged to non-host specific serovars *S*. Newport 2 (14.3%), *S*. Typhimurium 5 (35.7%) and *S*. Enteritidis 3 (21.43%). Globally about 70 per cent reports of salmonellosis in human usually come from China, India and Pakistan (Edelman, 1986). The threat for India has gained in hyper-endemic proportions calling for the need of urgent and extensive public health measures to contain it (Paul *et al.*, 1981). Isolation of *S*. Typhimurium from patients suffering from gastroenteritis has been reported by many workers (Sanyal *et al*., 1974; Sharma 1982). The magnitude of involvement of various serovars of *Salmonella* in human illness seems to vary with the changes in the geographical location.

None of the 14 faecal samples from apparently healthy cattle was found to be positive for *Salmonella*, while 7 (12.5%) out of 56 faecal samples from diarrhoeic cattle yielded the organism. Isolation of *Salmonella* from faeces of cattle has been reported by several workers (Nagaratnam and Ratnatunga, 1971; Dasgupta, 1974; Martel *et al*., 1980). Saikia (2001) could isolate *Salmonella* from 9.23 per cent of diarrhoeic calves in Assam, while Borah (2012) reported isolation of *Salmonella* from 6.34 per cent of bovine faecal samples. Murugkar *et al*. (2005) reported isolation of *Salmonella* from 9.6 percent rectal

swabs of cattle from Assam, Meghalaya and Arunachal Pradesh. The percentage of recovery of the organism from faecal samples of diarrhoeic cattle observed in the present study was slightly higher, which might be attributed to lesser sample size. Contrary to the lower prevalence recorded in India, Alam *et al*. (2009) reported recovery of *Salmonella* from 73.7 per cent samples from diarrhoeic cattle in Kansas, USA. Similarly, a study conducted by Mohammad *et al*. (2010) in Iran revealed recovery of *Salmonella* from 65.60 per cent of beef samples.

5.2. Serotyping of *Salmonella*

Serotyping of the 76 *Salmonella* isolates recovered in the present study from different sources revealed that 23 (30.26%) of them belonged to the serovar *S.* Weltevreden, 15 (19.74%) to *S.* Enteritidis, 10 (13.2%) to *S.* Typhimurium, 7 (9.21%) to *S.* Newport, 5 (6.6%) to *S.* Dublin, 4 (5.26%) each to serovars Typhi, Gallinarum and Choleraesius, and 2 (2.63%) each to serovars Kentucky and Litchfield (Table 4.2). There were several reports of isolation of *S.* Weltevreden from human, animals (Bhattacharyya *et al*., 1991; Borah, 1994; Ponce *et al*., 2007; Baisheng *et al*., 2017). *Salmonella* Weltevreden as the predominant serovar recovered from man and animals as was reported by Bhowmick *et al*. (2010) and Ortenzio *et al*. (2007), which also support the present findings. Saikia (2001) and Sarmah (2003) also isolated *Salmonella* belonging to serovars *S.* Typhimurium and *S.* Enteritidis in Assam.

The widespread prevalence of non-host specific serotypes of *Salmonella* poses a grave concern both for public health and livestock production. This indicates in clear terms that the serotypes are easily transmissible between their host species, thereby making the control of salmonellosis a difficult proposition.

5.3. Detection of Virulence Genes in *Salmonella*

The present study describes the development of a simple and rapid multiplex PCR (m-PCR) assay for simultaneous detection of seven major virulence genes of *Salmonella* (*inv*A, *inv*H, *stn*, *sop*B, *sop*E, *sef*C and *pef*A). Except for *inv*H, *stn* and *sop*B, there was variation in the presence of the other four genes in the standard and field isolates of *Salmonella*. Since *invH*, *stn* and *sopB* genes were found in all the isolates, they can be used as alternative target genes for direct detection of *Salmonella* from biological sources.

5.3.1. Screening of *Salmonella* Isolates for Virulence Genes

The chromosomally located *invA* gene codes for a protein in the inner membrane of bacteria which is necessary for invasion to epithelial cells (Drawin and Miller, 1999). The *invA* gene of *Salmonella* contains a sequence unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications (Rahn *et al*., 1992). In recent years, most investigators (Guo *et al*., 1999; Ferretti *et al*., 2001; Schneder *et al*., 2002) tried to establish a method, which can reduce the periods of *Salmonella* identification procedures from various samples. Amplification of *invA* gene now has been recognized as an international standard for detection of *Salmonella* genus (Malorny *et al*., 2003). *Salmonella* specific PCR with primers for *invA* is said to be a rapid, sensitive, and specific method for detection of *Salmonella* in many clinical samples (Lampel *et al*., 2000). In this study, *inv*H gene could also be detected in all the *Salmonella* isolates irrespective of serovars. This finding was similar to that of Pucciarelli *et al.* (2003) who detected *invH* gene in all the tested serovars of *Salmonella* from humans and animals. Various workers also revealed that *invH* is highly conserved in *Salmonella enterica* serotypes and mutation of this gene reduces *Salmonella* induced enteritis in cattle (Watson *et al*., 1997).

The *stn* gene in *Salmonella* is one of the chromosomally encoded genes that codes for production of enterotoxin. It has been proposed as a putative virulence factor and the cause of diarrhoea (Clouthier *et al*., 1994). Observations from the present study indicated that the *stn* gene is univeraslly present in all *Salmonella* isolates, irrespective of the serovars, which was in agreement with Prager *et al.* (1995), Rahman (1999) and Murugkar *et al.* (2003). The 750 bp *stn* gene encodes a 29 kDa *Salmonella* specific enterotoxin protein (Chopra *et al.,* 1994a, b). The *stn* gene is highly conserved in *Salmonella enterica* serotypes (Murugkar *et al.,* 2003; Riyaz- Ul- Hassan *et al.,* 2004). Chopra *et al*. (1999) also reported detection of *stn* in all the strains of *S. enterica*, but not in *S. bongori*.

Salmonella outer proteins (Sop) are the effector molecules of type-III secretion system (TTSS) which are involved in the early stage of *Salmonella* infection. These proteins are encoded by *sop* genes and several polymorphisms of these genes have been identified, *sop*A–*sop*E (Wood *et al*., 1998). Among the different types of Sop proteins (SopB, a 60 kDa protein), initially identified in the culture filtrate of *S.* Dublin was found to be associated with *Salmonella*-induced diarrhoea and gastroenteritis (Chopra *et al*., 1999). In the present study, *sop*B gene was detected in all the serovars of *Salmonella* tested, irrespective of host species and place of origin. This finding was similar to that of Rahman (2006), who detected *sop*B gene in all the tested serovars of *Salmonella* from humans and animals. In contrast to *sop*B, the *sop*E gene was found to be present in 15 of the 17 standard strains of *Salmonella* and was absent in *S.* Schwarzengrund and *S.* Typhimurium strains screened in the present study. The present findings were similar to those of Rahman *et al.* (2004, 2005), who also detected *sop*E gene in *S.* Enteritidis, *S.* Gallinarum and *S.*Virchow isolates by PCR. The product of this gene is known to play a role in *Salmonella* virulence although its absence from multiple invasive *Salmonella* isolates suggests that it is not

necessary for invasive manifestation in human (Suez *et al*., 2013). In the present study, presence of *pef*A gene was also evaluated, which encodes for the major portion of the *pef* operon. The *pef*A gene was detected in 3 of the 17 standard *Salmonella* strains, and in all the field isolates of *Salmonella* except serovars *S.* Typhi, *S.* Gallinarum and *S.* Kentucky. These findings were in agreement with those of Murugkar *et al.* (2003), who detected the *pef* gene in *S.* Enteritidis, *S.* Paratyphi B and *S.* Typhimurium isolates from humans, animals and birds. However, they found it to be absent in *S.* Bareily isolates. They also recorded variation among isolates of the same serovar which coroborates with the present findings. Inter-serovar variation in the presence of *pef* gene was also recorded by Rahman *et al*. (2000). It was noteworthy that Muthu *et al*. (2014) could not detect *pef* gene in *S.* Typhi, *S.* Paratyphi A and *S.* Typhimurium from the samples they studied.

Salmonella Enteritidis fimbriae 14 (SEF 14) is one of the major fimbriae of *Salmonella*, encoded by fimbrial operons *sef*A, *sef* B, *sef*C and *sef*D (Galan *et al*., 1998). *SefC* forms the largest component of the fimbriae and its presence indicates SEF adhesion. The *sefC* gene was detected in four of the 17 standard *Salmonella* strains belonging to *S.* Typhi, *S.* Enteritidis, *S.* Choleraesius and *S.* Idikan. The *sefC* gene showed variation in its presense within the same serovar amongst the field isolates. The present findings were similar to those of Rahman *et al.* (2000), who showed that except for strains of *S.* Enteritidis and *S.* Gallinarum, none of the other serovars, *viz*. *S.* Typhimurium, *S.* Newport, *S.* Kentucky, *S.* Weltevreden and *S.* Indiana harboured this gene. However, Murugkar *et al*. (2003) also detected this gene in the field isolates of *S.* Enteritidis of human and animal origin.

Presence of multiple virulence genes in different combinations in all the field isolates of *Salmonella* as revealed by the present study indicated their possible role as a pathogen in the host species.

5.3.2. Standardization of Multiplex PCR:

The reaction condition for m-PCR assay was optimized to ensure that all the target virulence gene sequences were satisfactorily amplified. The annealing temperature of 56◦C was selected because at this temperature adequate resolution of all the amplified products could be seen without any non-specific bands. The primer concentration was optimized after standardizing individual simplex PCRs for the studied genes and showed similar annealing temperatures without any non-specific amplification. The m-PCR also confirmed the virulence gene profile (Table 4.4) of the isolates without any non-specific bands (Figure 4.1). The m-PCR had a reasonably high level of sensitivity in spiked human stool samples and was able to detect as low as 10 organisms per gram of stool sample. Lim *et al.* (2003) reported that the sensitivity of m-PCR was 500 ng DNA of *S. enterica* serovar Typhimurium. Kapley *et al.* (2000) reported higher sensitivity of the PCR reaction with 102 cells per ml for amplification of *invA*, *lamB* and *ctxA* genes of *Salmonella* Typhi, *E. coli* and *Vibrio cholerae*. The present findings were similar to those of Pathmanathan *et al.* (2003), who found increased sensitivity of PCR for detection of *S.* Typhimurium in direct stool samples from 1200 cfu per PCR to 120 cfu per PCR and 1.2 cfu per PCR after 4 and 6 h pre-enrichment of stool samples, respectively. Further more, the results showed that DNA templates from all the *Salmonella* isolates yielded 942, 305, 470 and 543 bp amplicons for *invA*, *invH*, *sopB* and *stn* genes, while 254, 609 and 185 bp products (Figure 4.2) were obtained for *sopE*, *sef C* and *pefA*, respectively. No amplicons of corresponding sizes were detected in case of non-*Salmonella* strains, indicating the specificity of the assay. Therefore,

this assay demonstrated 100% specificity since all the desired amplicons were produced in *Salmonella* isolates, while no amplicons were produced from any of the non-*Salmonella* strains tested. All the field isolates of corresponding serovars from the same source were found to have the same virulence gene profile in the m-PCR assay. The m-PCR assay developed in the present study was able to concurrently amplify more than one common as well as uncommon virulence locus in a single reaction. The m-PCR assay developed in the present study provided a rapid and reliable detection method for detection of *Salmonella* isolates with pathogenic potential and to compare their virulence gene profiles.

5.4. Cloning and Expression of *inv*H Gene in Prokaryotic Host

In the present study, recombinant InvH (15 kDa) surface protein of *Salmonella* Typhimurium was successfully cloned into pET303CT/His vector and expressed in *Escherichia coli* BL21 cells. The expressed InvH surface protein was purified by Ni-NTA affinity chromatography under denaturing condition, which was then renatured by a multi-step dialysis procedure and was finally dissolved in phosphate buffered saline. The final yield was approximately 7 mg/ml of culture and the purity of the renatured recombinant protein was greater than 98 per cent as assessed by SDS-PAGE. These findings are in agreement with Kumar *et al.* (2017), Ghosh *et al.* (2011) and Hamid *et al.* (2008) who found similar results in yield and purity of OMP's of *S.* Typhi and *S.*Typhimurium respectively.

5.5. Evaluation of Immuno-Potency of Recombinant InvH Protein In Mice

Two groups of mice were taken for analyzing immune potential of r-invH with FCA and bacterin vaccine with FCA. Blood samples of tested mice were collected on 7, 14, 21, 28, 35 and 42 days post primary immunization. Booster doses were administered on the

14th day post-primary vaccination. Both the immunized groups (r-invH with FCA and bacterin with FCA) showed the maximum mean antibody titre on 21st day post-primary vaccination with significant ($p < 0.01$) difference between them. The mean anti-r (InvH) IgG response in serum of mice was significantly higher on 21st day post-immunization (16.69 ± 0.16), which gradually declined till 42nd day (3.99 ± 0.01). In the group injected with bacterin vaccine, there was a significant decline in IgG response ($P<0.05$) after 21st day (9.77± 0.14) till 42nd day (3.53 ± 0.01). The bacterin vaccine could not elicit good antibody response as compared to recombinant invH vaccine. It might be due to the fact that there is targeted immune response towards specific microbial epitopes present in the purified outer membrane proteins, while in case of whole cell bacterin vaccines, many of the antigenic determinants are not properly exposed due to various membrane components including lipopolysaccharides (Sharma *et al*., 2013). The difference in antibody titre between the two groups also appeared statistically significant ($P < 0.01$). These results were in agreement with Dehghani *et al.* (2012) who also found significant rise in antibody titre with purified InvH recombinant protein.

The invH mutation has been reported to significantly reduce the secretory and inflammatory responses and it was shown to have important role in immune response (Marcus *et al*., 2000). Researches on mutation in invH gene confirmed that mutants contain reduced number of needle complexes suggesting that invH may modulate organelle assembly (Sukhan *et al*., 2001). To determine the efficacy of recombinant invH protein in conferring protection to mice against infection by homologous (*S.* Typhimurium) and heterologous (*S.* Enteritidis and *S.* Gallinarum) virulent strains of *Salmonella* two weeks after the last booster dose, the mice of the immunized (r- invH with FCA and Bacterin with FCA vaccinated) groups and the non-immunized control (PBS inoculated) group were

challenged with respective lethal dose (100 x LD_{50}) of *Salmonella* through the oral route. All the mice in the control group died after 72 hours of infection. The group of mice immunised with (r-invH+ FCA) showed complete protection (100%) against *Salmonella* Typhimurium and *Salmonella* Enteritidis infections, while protection was 90 percent against *Salmonella* Gallinarum. These results corroborated with those of Dehghani *et al.* (2012), who reported 100 per cent protection conferred by r-invH + FCA against *Salmonella* Enteritidis. However, no reference could be traced regarding use of the same preparation as a vaccine against *Salmonella* Typhimurium and *Salmonella* Gallinarum for comparing with the present findings. The bacterin vaccinated group showed a protection of 60 per cent against all the three serovars under study, *viz*. *S*. Typhimurium, *S*. Enteritidis and *S*. Gallinarum. This finding on the protective efficacy of *Salmonella* bacterin vaccine was similar to that of Dhanda *et al*. (1955) on *S*. Abortusequi, Nicholas and Andrews (1991) on *S*. Typhimurium, Liberal (1989) on *S*. Dublin, Mohrah and Zaki (1995) on *S*. Gallinarum, and Barbour *et al*. (2001) on *S*. Enteritidis.

During the studies, *Salmonella* could be re-isolated from liver, spleen, intestinal lymph nodes and faecal samples of all the mice in the control group. The identity of the challenged strains was re-confirmed by genus-specific PCR. On the other hand, bacteria-free status of spleen and intestinal lymph node and spleen of the immunized mice supported earlier findings that mutations in invH reduce invasion by *Salmonella* strains into eukaryotic cells (Watson *et al*., 1998). The ability to enter cells of the intestinal epithelium is an essential step in the pathogenic life cycle of *Salmonella*. This organism passes through the intestinal epithelium in membrane-bound vesicles, reaching the lamina propria and cells of the reticuloendothelial system (Altmeyer *et al*., 1993; Kim, 2003). In the present study, liver and spleen from infected mice did not show any tissue damage. Depending upon the

infecting serovar or the infected host, *Salmonella* may penetrate to deeper tissues such as liver and spleen or may remain localized in the lamina propria of the intestinal epithelium (Mittrucker *et al*., 2000).

On the basis of the findings of the present study, it could be concluded that recombinant InvH protein could elicit antibody response in mice. Immunization with rInvH protein could confer protection against infection with *S. enterica* serovar Typhimurium. The recombinant InvH protein may be used as a vaccine candidate as well as a diagnostic antigen for detecting *Salmonella* infections. However, this needs further study particularly in respect of the kinetics of antibody response involving larger number of experimental animals and application of the recombinant protein as a diagnostic antigen for detection of *Salmonella* infection in animals and human.