6. DISCUSSION

In the current world scenario, people are affected by many microbial diseases. Man has found out ways and means to identify and prevent spread of many diseases in the human population. The list of new diseases is steadily increasing and so there is a need to evolve new tools and protocols to study them.

There has been an endless struggle for existence between human and the microbial populations. The mankind tries to eradicate the disease causing pathogenic microorganisms completely. On the other hand, the microorganisms have the potential to adopt, evolve, survive and multiply with fast changing environment. In these days, the microorganisms evolve new ways and means to resist the antibiotics. Some microorganisms even consume the antibiotics as growth factors. The emergence of new drug resistant strains poses a life threat to the existence of human race. There is a need to continuously review and update the disease spread, control measures and curative protocols.

Designing a universal protocol to control and cure a disease is a difficult task because, the susceptibility of an organism to a microbial disease depends not only on the immune status or vaccination, but also on the race, individual, age, gender, heredity, nutritional habits, locality, living conditions and the nature of the species.

As international travel to developing countries increases more people seek medical advice concerning food and water borne diseases including typhoid fever (Engels et al., 1998). Prevention of typhoid fever in high - risk groups (travelers to endemic areas, laboratory workers and household contacts of typhoid carriers) should rely primarily on prevention of exposure. However, immunization is an important adjunct. The decision to immunize against typhoid fever should be individualized, taking into account the benefits versus the risk of possible adverse reactions (Woodruff et al., 1991).

Typhoid vaccine is generally recommended for children traveling to most parts of Asia, Africa and Latin America. Although a more severe disease outbreak
in children and travelers, typhoid affects them 100 fold more than hepatitis-A (Rao, 1991). One estimate states that the risk of acquiring typhoid fever in travelers is 1 in 3000 people per month for adult travelers to the Indian subcontinent, Northern and Western Africa and Peru, with a 10 fold decrease in travelers visiting other developing countries (Steffen, 1987).

Children visiting these highly endemic areas such as the Indian subcontinent and particularly children returning to their parent’s country of origin for prolonged stays, have a higher risk of acquiring *S. typhi* (Wahdan et al., 1982). Children have the greatest overall incidence of this disease and once infected, have a 1-3% chance of becoming chronic carriers. The antibodies gained through breast feeding are likely to be effective against typhoid and hence breast feeding and proper food and water precautions should be encouraged (CDC, 2001).

6.1 Incidence of asymptomatic typhoid carriers

Poor personal hygiene and inadequate food handling can potentiate the transmission of *S. typhi*. Several food products kept at room temperature were found to favour the growth of *Salmonella* species. The food handlers prominently play a role in disseminating typhoid bacilli through different food products and water (Lin et al., 1988; Senthilkumar and Prabakaran, 2005). It was suggested that a periodic survey of samples from food handlers and food stuffs should be made and proper sanitation methods should be followed in hotels and restaurants to avoid food contamination and spread of *Salmonella sp* (Sasikumar et al., 2005). Similarly, the present work is also carried out to screen the typhoid asymptomatic carriers with respect to different socio economic status among the food handlers in Salem and Namakkal Districts, Tamilnadu, India.

Typhoid fever is widespread in all the parts of India. Transmission of typhoid fever is primarily through the asymptomatic carriers - those who work as the food handlers in hotels and restaurants. *Salmonella* can stay alive in an asymptomatic carrier in human gall bladder for years together. An antibiotic treatment of the carriers is often ineffectual against *S. typhi* due to the emergence
of drug resistant strains. Therefore, the multi-drug resistant *S. typhi* has recently turned out to be a center of attention in our country.

Outbreaks of typhoid fever from restaurants through the contaminated food by infected asymptomatic food handlers have also been reported from United States (Taylor *et al.*, 1984). Royan *et al.* (1989) have reported that in United States, out of 1013 cases of typhoid fever from 1975-1985, 28% were related to outbreak. The source of exposure was unknown in most cases, but 21% were associated with newly discovered carriers, 9% with previously identified carriers and 3% with exposure to laboratories.

Also, the present study revealed that 4% of the individuals from restaurants, 4% of the individuals from I class hotels, 12% of the individuals from II class hotels, 23% of the individuals from the road side hotels, 17% of the individuals from road side vendors, 14% of the individuals from fruit juice stall, 7% of the individuals from milk men, 5% of the individuals from slaughter houses, 1% of the individuals from chicken stalls and 1% of the individuals from fried fish stalls were found to be typhoid asymptomatic carriers. The workers in roadside and second class hotels live relatively at a very poor hygienic condition as compared to the first class hotel workers. The results of the samples collected indicate that the roadside hotel workers and second class hotel workers were predominant carriers of typhoid bacilli. From this present investigation it is revealed that the incidence of typhoid asymptomatic carrier state has a bearing on the basis of socio economic status of the individuals. There is a high probability of spreading the typhoid bacilli to the susceptible persons through the food handlers in road side hotels and second class hotels. They pose a problem to human race as reservoirs of *S. typhi*, *S. paratyphi* A and *S. paratyphi* B

The susceptibility to carry the typhoid germs also varies with gender and age. Women exceed men as carriers by a ratio of 3:1. Carrier state occurs more commonly in elders (Hornick, 1970). In contrast, the present investigation revealed that among the food handlers, men can serve as more potential carriers of typhoid germs than women.
The series of over 9,000 cases reported in London during 1871-1894 represented clinical enteric fever in urban communities of all ages and the data shows that maximum susceptibility is found in people between the ages of 15 – 25 years (Goodall and Washbourn, 1896). Similarly, the present investigation of typhoid asymptomatic carrier state was found to be in people of the age group 15 – 45 years. Of the people who were suffering from typhoid fever during their lifetime, some were got cured, while a few continued to harvest and excrete typhoid bacilli in their faeces. Based on the life history of such persons, they were found to be chronic carriers. This study has gained support from other studies in terms of the results.

In hotels, the cooks and waiters could easily contaminate water and foodstuffs while handling them. Often their nail bits remain wet and dirty and their poor sanitation after defaecation gives a favourable environment to the growth and spread of the bacteria. Their palms too carry many enteric pathogens. Washing their palms just with pure water is inadequate to keep them sterile. In addition, food stuffs would favour the growth of S. typhi. The improper disposal of stools after defecation could also lead to severe water pollution. Particularly at the time of flood, it would lead to a large scale outbreak of typhoid that creates public health problems of severe magnitude.

6.2 Antibiotic sensitivity of Salmonella spp and resistance pattern of representative S. typhi isolates

Cephalosporins and ciprofloxacin were used in most of the areas to treat the typhoid carriers. Drug resistant strains of S. typhi might have originated by mutation and spread through conjugation and other modes like genetic material transfer among the bacteria. The plasmids and chromosomes confer the antibiotic resistance of the bacterial strains. The patterns of typhoid asymptomatic carriers and the patterns of antibiotic resistance of S. typhi isolates from the carriers were documented and studied in this present work. The mechanism of drug resistant
gene transfer among bacterial population by conjugation was also investigated in this study.

The rapid emergence and spread of the resistant S. typhi were reported in Vietnam (Smith et al., 1994; Vinh et al., 1996; White et al., 1996), Southern India (Shanahan et al., 1998) and Tajikistan (Murdoch et al., 1998) and their continued selection under antibiotic pressure raises scenario of the reemergence of untreatable typhoid.

In India, 91.3% of S. typhi was isolated between July 1990 and March 1991 from patients of pyrexia with multiple drug resistance (Agarwal et al., 1992). Multi drug resistant S. typhi has spread too many parts of India, causing severe therapeutic problems. Salmonella bacteremia was detected in 134 patients out of the 305 clinically suspected cases of enteric fever at Kasturba Hospital in Manipal between 1990 and June 1991. Out of 134 cases, S. typhi was isolated from 102 cases. Out of the 102 S. typhi isolates, 80 (78.4%) were resistant to ampicillin, chloramphenicol and trimethoprim which are the conventional antibiotics used to treat typhoid fever (Suganthi et al., 1993).

Prior to 1986 S. typhi isolates were sensitive to all the antimicrobials tested by disc diffusion method. Most of the common multi drug resistance was against ampicillin, chloramphenicol, co-trimoxazole and tetracycline. The withdrawal of chloramphenicol due to high level resistance during 1990 - 1994, has led to reemergence of 43-93 % chloramphenicol mutants during 1995 - 1999. Two S. typhi isolates in 1995 and one in 1999 depicted resistance to ciprofloxacin. Susceptibility of S. typhi isolates to third generation cephalosporins ranged between 87-100%. A gradual increase in the time period in mean minimum inhibitory concentration of ciprofloxacin was observed (Kumar et al., 2002). The present study has also revealed that the antibiotic sensitivity test by Kirby-Bauer disc diffusion method shows a characteristic antibiotic resistant pattern of the representative strains Viz. SS 11, SS 20, SS 32, SS 42, SS 54 and SS 61 showing resistance of 9.09%, 36.36%, 45.45%, 54.55%, 54.55%, 36.36% respectively. Of the total isolates, 16.66%, 83.33%, 33.33%, 33.33%, 50.00%, 16.66%, 33.33%,
83.33%, 16.66%, 16.66%, 83.33% were resistant to O, T, A, Am, Ak, Cf, Co, R, Na, G, C respectively (Table 5.6; Figure 5.6). The above observation indicates that the strains were least resistant to O, Cf, G and Na, while highly resistant to T, R and C.

The isolates were not equal in developing antibiotic resistance. Some isolates of *S. typhi* showed resistance to chloramphenicol, ampicillin, streptomycin and tetracycline, while sensitive to gentamycin. Mirza and Hart (1993) have also observed similar results. In 1990, ciprofloxacin is an alternative to chloramphenicol for the treatment of typhoid fever patients. In 1995, four (1.7%) of 237 isolates of *S. typhi* in the UK showed reduced sensitivity to ciprofloxacin (Threlfall *et al.*, 1999).

Of 54 isolates of *Salmonella*, 94% were *S. typhi* and 6% were *S. paratyphi* A serotype. Of the 30 isolates, *S. typhi* subjected to phage typing, 2 phage types E1 23(77%) and A 7 (23%) were found to be prevalent at Nagpur. Multi drug resistance was observed in 12 (22%) strains of *S. typhi*. Thirty five (68%) isolates were sensitive to chloramphenicol, ampicillin, gentamycin, co-trimaxazole, cefataxime and ciprofloxacin. Resistance to two antibiotics was observed in four (8%) strains. Cefotaxime resistance was observed in one isolate and gentamycin resistance in two, while none of the isolates were found to be ciprofloxacin resistant (Chande *et al.*, 2002).

### 6.3 Plasmid profile analysis of the selected representative *S. typhi* isolates

The Plasmid digest band patterns of strains exhibiting resistance to trimethoprim, streptomycin, sulfonamide, tetracycline, kanamycin and nalidixic acid revealed a common 128 KDa Plasmid and 2 plasmids of 3.9KDa and 2.4KDa (Miko *et al.*, 2002). The problem of drug resistance is due to acquisition of plasmid encoding inactivating beta - lactamase and chloramphenicol acetyl transferase. In this case, ceftriaxone or fluozoquinolone can be used as a therapeutic agent. Ciprofloxacin is effective in treatment of carrier state (Campos *et al.*, 1990).
Of *S. typhi* strains isolated from typhoid asymptomatic carriers, 66.8% were multi-drug resistant. The plasmids of these representative strains were isolated. The plasmid profile was constructed by agarose gel electrophoresis for further examinations. The molecular weight of the plasmids of the representative strains was found to be ~120 Kb plasmids. Similarly, Karmaker *et al.* (1991) isolated two distinct groups of *S. typhi* from cases of typhoid in Calcutta (1889-1990). One strain was antibiotic sensitive and the other multi-drug resistant. Plasmid of 120 Kb and 14 Kb were identified amongst the multidrug resistant isolates of *S. typhi*.

Threlfall *et al.* (1997) have suggested ciprofloxacin as an alternative to Chloramphenicol for the treatment of typhoid fever. They have isolated the strains of *S. typhi* Vi-phage type E1 with plasmid encoded resistance to chloramphenicol, amikacin, ampicillin etc, and chromosome encoded resistance to nalidixic acid and ciprofloxacin. In this present study also, ciprofloxacin resistant *S. typhi* strain was screened and identified.

Robertson *et al.* (2002) have analysed 48 clinical *S. typhi* strains for antibiotic resistance pattern by disc diffusion method. The susceptibility of each isolate to ampicillin, chloramphenicol, streptomycin, tetracycline and trimethoprim was examined. Five isolates were resistant to all these antibiotics including ampicillin, chloramphenicol, and trimethoprim. The first line antibiotics in the treatment of typhoid fever were resisted by 10 isolates. Thirty-four isolates were resistant to at least one of the antibiotics tested. Incompatibility group Inc HI conjugable plasmids were found in 62% of these isolates. In 90% of the strains, these conjugable plasmids conferred multiple drug resistance. Additionally, 14 strains contained transformable plasmids. Out of 14, 6 of them expressed multiple drug resistance.
6.4 Transfer of drug resistance through conjugation by representative *S.typhi* isolates

In *S.typhi* isolates, several varieties of plasmids were detected. All the multi drug resistant *S.typhi* isolates carried a 140 Kb plasmid. Several isolates had an additional 80 to 90 Kb plasmid. It was confirmed that the large 140 Kb Inc HI incompatibility group plasmids were responsible for the expression of multiple drug resistance. The common and stable 80 - 90 Kb plasmid does not appear to be involved in drug resistance (Wain *et al.*, 1999).

Experiments on bacterial conjugation showed that the resistance to chloramphenicol, tetracycline and trimethoprim in the case of strain 2504 and to chloramphenicol, tetracycline, trimethoprim and kanamycin in the case of strain in 2496) was transferable at 22°C in linkage group on the 128 MDa plasmids. However, those strains carrying only an 88 MDa plasmid had lost trimethoprim, streptomycin, sulfonamide and tetracycline resistance (Miko *et al.*, 2002).

In the present study, the ability of *S.typhi* to transfer drug resistance to *E.coli* CSH57 was tested. For this study 6 F* S. typhi* isolates were used as a donor and F* E.coli* CSH57 strain was used as a recipient which does not have the plasmid. The plasmids were transferred by 4 strains (66.66%) (SS 20, SS 32, SS 42, SS 54) through conjugation and 2 strains (SS 11, SS 61) did not show the transfer of plasmid. All the 4 representative strains bearing conjugable plasmids showed multi drug resistance. In contrast, the strain-1 showing single antibiotic resistance had no plasmid. The strain 6 has also no plasmid, but its resistance to ciprofloxacin was due to chromosomal DNA. This investigation demonstrates that the recipient strains acquire tetracycline and chloramphenicol resistance. *E.coli* CSH57 strain acquired the multi drug resistant ~120 Kb plasmid after the completion of conjugation process. Similarly, Karmaker *et al.* (1991) have done the conjugation experiments and demonstrated the transfer of a 120 Kb plasmid from *S.typhi* to *E.coli* and they concluded that the 120 Kb plasmid encoded chloramphenicol, amikacin, tetracycline resistance determinants.

Hirose *et al.* (2002) have observed that the mutations in the *gyrA*, *gyr B*, *par C* and *par E* genes of *S. typhi* and *S. paratyphi* A was responsible for
fluoroquinolone resistance. The sequence of the quinolone resistance determining region of the \textit{gyrA} gene in clinical isolates showed decreased susceptibilities to fluoroquinolones. This gene underwent a single mutation either at the Ser-83 or at the Asp-87 codon and no mutations were found in the \textit{gyr B}, \textit{par C} and \textit{par E} genes.

Evolutionists say that mutations are the source for the origin of new genetic materials and pointed out that it also develops antibiotic resistant bacteria from population of non-resistant bacteria. The resistance exists only in a small number of bacteria except for the occasional mutations. The non-resistant bacteria die, while the resistant bacteria survive and begin to multiply just about the time that the patient thinks he is getting better.

Resistance to the antibiotics develops through several ways, all of which are related to the bacteria's gene pool, which is the sum total of genetic material available in a specific strain or species of bacteria. Resistance does not come about by haphazard mutations. It is important that the original genome of the bacterial strain should undergo appropriate changes in order to code for resistance to antibiotics.

Drug resistance also emerges due to inappropriate handling of drugs and overdoses of antibiotics by the physicians and pharmacists. Nowadays, individuals take antibiotics on their own accord without the prescriptions of qualified physicians to cure their illness. Using the broad spectrum of antibiotics generates the drug resistance in bacteria and affects the normal microflora. Conjugation, transformation, transduction, mutation, environmental changes and evolution are also responsible for emergence of the drug resistance in bacterial strains.

This crisis can be overcome by health education programmes, administering correct dose and appropriate drugs to the patients, and then insisting the public not to take drugs on their own accord. In addition, antibiotics should be recommended after finding the antimicrobial sensitivity and resistance pattern. It could be suggested to take narrow spectrum antibiotics for treatment as drug
resistance might be minimized. Emergence of multidrug resistance would leave human kind at stake, because it would force them to find alternative drugs.

Drug resistance in asymptomatic typhoid carrier state has become focused by scientists and researchers. Since the source is asymptomatic carrier it might be difficult in treating them and eventually patients also. Normal microflora bearing plasmids coding antibiotic resistance can be transferred to sensitive bacteria in the human body (in-vivo). It would be hard mission, if normal flora becomes resistant to wide range of antibiotics. Ultimately in-vivo transfer of resistant genes pose hazard to human and animal kind as frequency to meet resistant and non resistant bacteria. Consequently, at a standstill having setback of drug resistance is being emerged by both in-vivo and in-vitro gene transfer.

6.5 Live Attenuated typhoid vaccines

Expression of the porin is co-ordinately regulated at the level of transcription. This involves an environmental sensor, a histidine kinase inner membrane protein and a cytoplasmic transcriptional activator, OmpR. S.typhimurium strains harbouring stable mutations in OmpR are attenuated both orally and parenterally makes excellent single dose oral vaccines (Dorman et al., 1989). Interestingly, further studies have revealed that an OmpC and OmpF double mutant does not mimic the behavior of an OmpR mutant. Although attenuated to a similar level orally, it exhibited little attenuation when given intravenously (Chatfield et al., 1991). This suggests that other OmpR regulator genes may play a role in the early stages of the infection process.

Efficacy of live attenuated Salmonella vaccines delivered by the mucosal route is limited by the close interference from mucosal flora of the alimentary tract. In a mouse model, the total antibody response towards lipopolysaccharide of S.typhi was significantly enhanced at day 21 post immunization with live attenuated S.typhi (Ty21a) when ampicillin was concomitantly administered and lethal dose of mice in the ampicillin and control groups immunized with Ty21a after wild type S.typhi challenge on day 24 was 4x10^7 and 1x10^7 respectively (Woo et al., 2000).
Natural infections with *S. typhi* and vaccination with live oral typhoid vaccines elicit poor Vi antibody responses even though it is known that Vi is a good immunogenic substance and can be an effective vaccine when given as a subunit preparation. However, higher level of Vi antibody is detected in individuals who become chronic carriers (Tacket *et al.*, 2000).

It may be that the level of expression of this Vi polysaccharide is down regulated after the organism reaches its favored host environmental niche, an intracellular environment such as macrophages, because the organism for its longer survival requires protection against the actions of complement mediated system. This may be an explanation for the poor Vi antibody responses elicited as only low doses of this antigen are presented to the immune system.

In *in-vitro*, it was demonstrated Vi antigen was essential for the survival of *S. typhi* in human serum possibly because of Vi reducing the rate of complement activation by alternative pathway, thereby reducing complement mediated killing and opsonization. This may be linked to the fact that Vi antigen was found to decrease the level of fixation of the C3b component of the complement.

As knowledge of *Salmonella* pathogenesis has increased, so as to have the availability of other attenuating lesions. In particular, attention has been focused on the role of global regulators in virulence. When a pathogen enters the host it has to respond quickly to different host compartments which will in turn exert different stresses and demands on the cell. The pathogen must be able to sense these different environmental conditions and co-ordinately control the expression of genes whose products are required under certain conditions but not others.

A more rational approach to attenuation would be targeted to microbial components and genes known to be important for their virulence. An attenuated typhoid fever vaccine was developed on this principle, aiming at a conditional elimination of the bacterial ‘O’ antigen (outer most part of the cell surface lipopolysaccharide molecule), which resulted in good attenuation of *S. typhimurium* in the mouse model of *Salmonellosis* (Germanier and Furer, 1971).
6.5.1 Amplification of OmpR and EnvZ genes of S.typhi

A key theme in the coordinate regulation of different classes of bacterial genes is in response to environmental stimuli in the involvement of the family of two compound systems of OmpR and EnvZ genes (Albright et al 1989; Stock et al., 1983). In this present study PCR primers specific for the region of OmpR – EnvZ outside the deleted region was used to amplify the chromosomal DNA from S. typhi isolates. The amplified product was compared with DNA standard molecular marker to detect the molecular weight. All the five S. typhi isolates SS1 to SS5 gave rise to ~799 bp for OmpR and ~1000 bp for EnvZ PCR products, indicating the presence of the wild type gene encoding for the virulence of the isolates. The results obtained in the present study were similar to those of Pickard et al. (1994).

6.5.2 Phylogenetic analysis on the basis of OmpR gene of S.typhi

Fukushima et al. (2002) have done the phylogenetic analysis of about 200 strains of Salmonella, Shigella and E.coli that was carried out using the nucleotide sequence of DNA gyrase B (gyr B), which was determined by directly sequencing PCR fragments. The results established a new phylogenetic tree for the classification of Salmonella, Shigella and E.coli in which Salmonella formed a cluster separate from but closely related to Shigella and E.coli. In comparison with 16s rRNA analysis and the gyr B sequences indicated a greater evolutionary divergence for the bacteria. Thus, in screening for the presence of bacteria, the gyr B gene might be a useful tool for differentiating between closely related species of bacteria such as Salmonella spp, Shigella spp and E.coli.

In this present study OmpR gene sequence is the basis for the phylogenetic studies of S.typhi. OmpR gene sequence was used for finding dispersal pattern, diversity and evolutionary changes of strains of S.typhi over the world (Tree 5.1).Moreover, the evolutionary relationship results concluded that species S. typhi SS3 and SS5 was evolved from the species S.typhimurium (Fig. 5.5, 5.6). In addition the genus level tree concluded that species S.typhi is more similar to E.coli strains and also both of these come under the same family of
Enterobacteriaceae. It is clearly indicated that the genus *Salmonella* has evolved from the genus *Escherichia* and the family Enterobacteriaceae also suggests the same (Fig. 5.7, 5.8).

Before suggesting this present methodology, the evolutionary history, global diversity and population genetic structure of *S.typhi* were poorly understood. On the basis of results and evidences in the present study, it is revealed to the scientific community that these phylogenetic and comparative analyses may rectify problems that are mentioned above.

**6.5.3 Induction of mutation on OmpR genes of *S.typhi***

By demanding bacterial growth on sugars, too large to normally diffuse three porin channel alterations in the channel loop resulting in functionally large channels (Misra and Benson, 1988). On such alteration in the *OmpC* porin was an R74C substitution (*Omp C R 74 C* or *OmpC1 cys*) (Misra, 1993). The *OmpC* and *OmpF* porin genes are transcriptionally regulated by a classical two component signal transduction regulatory system consistent of the *Omp R-Env Z* proteins (Hall and Silhavy, 1981a; Hall and Silhavy, 1981b).

The *OmpR* gene of *S. typhi* is involved in the regulation of the synthesis of Vi capsular polysaccharide, the regulation may be due to osmolarity. Vi expression is very sensitive to changes with osmolarity of the growth medium. Thus, in this study *S. typhi* SS 3 and SS 5 were subjected to mutation to evolve a mutant. The isolates were grown on 0.1M, 0.2 M, 0.3 M, 0.4 M, osmolar concentration of NaCl, that yielded ~799 bp amplified product in SS-3 strain. But in case of SS-5 isolate grown in 0.1 to 0.3 M osmolar concentration the resulting was only ~799 bp amplified product. So the low osmolar concentration of NaCl was not yielding the mutant isolates. But 0.5 M and 0.6 M of NaCl yield mutant (SS 3) attenuated strain and 0.4, 0.5 M and 0.6 M of NaCl yield mutant (SS 5) attenuated strain which was indicated by ~282 bp amplified products in the PCR products. Thereby, resulting attenuated strains due to mutation were indicated by the deletion of ~517 bp of *OmpR* gene in the *S.typhi* SS 3 and SS 5 strains. Similarly, Pickard et al. (1994) had also observed the similar type of the results.
that deletion was found at mutants about 517 bp of OmpR gene, thereby, rendering the strain avirulent.

6.5.4 Expression of OmpR gene in S. typhi wild and mutant strains

*S. typhi* employs the same regulatory proteins to regulate Vi synthesis as *E. coli* to regulate colonic acid synthesis. The results reported here indicate that another two component system, OmpR-EnvZ possibly responding to a different environment signal is also involved in the regulation of Vi synthesis in *S. typhi*. Vi polysaccharide is considered to be important in virulence of *S. typhi*, since the majority of *S. typhi* strains isolated from the blood of patients with typhoid possess Vi antigen (Robbins and Robbins, 1984).

The *in-vivo* relevance of these findings to the mechanisms of host defense and pathogenesis of *S. typhi* still remain speculative and nothing is known about the regulation of Vi synthesis *in-vivo*. However, the finding that Vi expression in *S. typhi* is regulated by members of the family of two component system which are known to be important in controlling gene expression *in-vivo* coupled with what is known about its role in virulence suggesting that there is a need to regulate Vi antigen in different host microenvironment so that for *S. typhi* expresses full virulence.

Expression of the OmpF genes in *S. typhi* is under the control of EnvZ and OmpR, a two-component signal transduction system encoded by the OmpB (OmpR-EnvZ) locus. Interestingly, a shift in osmolarity only affects OmpF expression, and OmpC levels remain constant (Majumder *et al.*, 1999). The outer membrane of *S. enteritidis* generally contains three major outer membrane proteins OmpC (36 KDa), OmpF (35 KDa) and OmpA (33 KDa). OmpC and OmpF are encoding for porin formation, whereas OmpA has no pore forming properties (Lugtenberg and Van Alphen, 1983).

In this present study, further more the OmpR expression was studied and analysed by SDS PAGE. The wild isolates showed the outer membrane proteins viz. Omp C, Omp F and OmpA were found to be ~36 KDa, ~35 KDa, and ~33 KDa respectively. However, the mutant strains showed only a single band of
OmpA which is not having pore forming properties. Thus, in case of the mutants expression of OmpC and OmpF was down regulated. Similar types of the results were observed in the wild and mutant strains of S.typhi in the studies of Pickard et al. (1994).

From these results, it may be concluded that when the isolate is grown in more than 0.4M osmolar concentrations, it yields the mutant strains. The OmpR gene of S. typhi is involved in the regulation of the synthesis of the Vi capsular polysaccharide such the regulation may be osmolarity, Vi expression is very sensitive to changes in osmolarity of the growth medium.

It is well known that live attenuated vaccines elicit potent cell mediated responses and can be very effective vaccines. It was also demonstrated that OmpR mutants of S. typhimurium are excellent vaccines in mice. There are now some evidences to prove that, if Salmonella vaccine to be effective, it must have the ability to elicit opsonizing antibody against the bacterial cell surface as well as eliciting cell mediated immunity (Mastroeni et al., 1993).

The outer membrane protein OmpC of S.typhi has been subject to attenuation for the development of candidate vaccines against typhoid fever. This has been motivated both because of the protein surface location and due to its ability to elicit protective immunity in animal models (Isibasi et al. 1988; Isibasi et al., 1992).

6.5.5 Phenotypic characterization and Immunodiffusion analysis of sonicates of S.typhi mutants

Tartera and Metcalf (1993) reported the effect of changes in the osmolarity of the growth medium on the synthesis of Vi polysaccharide in S.typhi. Pickard et al., (1994) have reported that LB agar containing a range of NaCl concentrations (0.0 to 0.7 M) was prepared to mutate S.typhi. Conclusion proposed was that CVD 908 no longer agglutinated with Vi antiserum when grown in medium containing 0.5M NaCl and only weakly agglutinated at concentration of 0.4 M NaCl. However, at NaCl concentration of 0.4 M NaCl, all cultures agglutinated with Vi antiserum to the same degree, and 09 agglutination was not detectable without
boiling the sample first. It was therefore concluded that Vi synthesis in *S.typhi* can be regulated by external osmolarity. *S.typhi* CVD 906 was weakly agglutinated with Vi antiserum, even at NaCl concentrations below 0.4M. At 0.3 M NaCl, the CVD 906 culture was weakly Vi positive, but had a strong 09 agglutination without boiling.

In this study, phenotypic test was carried out to identify the mutant strains which were not agglutinated with Vi antiserum. It is possible that the lack of agglutination of *OmpR* mutants with antiserum was due to a defect in the transport of Vi polysaccharide to the surface of the organism and that Vi was accumulating intracellularly. To investigate this possibility counter current immunoelectrophoresis was performed on the bacterial sonicates with Vi antiserum. The sonicate mutant failed to produce any line of precipitation. This indicates that mutant strains were down regulated in the expression of porin coding *OmpF* and *OmpC*. Thereby, resulting mutant was presented with lack of Vi polysaccharide antigen.

6.5.6 **Assessment of virulence in mice using wild and mutant strains**

In this present study, when the animals were inoculated with LD$_{50}$ it was found to be $4 \times 10^9$ CFU of SS3 and SS5 wild strains respectively. The animals died within 72 hrs of intraperitoneal inoculation, thereby indicating that the strains used for immunization were virulent. In the animal study also no clinical symptoms were observed when the mice were immunized with the mutants. Hone *et al.* (1991) have also reported similar results that intraperitoneal injection of wild *S.typhi* strains in mice results in death within 72 hrs of inoculation.

6.5.7 **Mice immunization using *OmpR* mutants**

Passive immunization studies by injecting rabbit antiserum into mice 1 hour before a challenge with the live bacteria in mucin was done. OMP immune rabbit serum was able to confer 100% protection against challenge with 100 LD$_{50}$ of *S.typhi* strains and 80% protection against *S.typhimurium*. These results indicate that humoral immunity plays an important role in the protection against infection against infection with *S.typhi* in mice. Moreover, the results suggest that
besides serum antibodies, other mechanisms are implicated in the protection against mice (Isibasi et al., 1988).

The protective role of *Salmonella* virulence (Vi) antigen has been demonstrated using the mouse typhoid model. Immunization with Vi 4072 at $5 \times 10^7$ CFU afforded complete protection against intraperitoneal challenge with virulent Ty2, whereas same dose of Vi negative strain failed to protect (Cao et al., 1992). *Salmonella* OMPs and porins also appear to contribute protection against typhoid infections, as documented in the mouse model (Udhaya Kumar and Muthukkaruppan, 1987; Matsui and Arai, 1991).

The *in-vivo* relevance of these findings to the mechanisms of host defense and pathogenesis of *S. typhi* still remains speculative and nothing is known about the regulation of Vi synthesis *in-vivo*. However, the finding that Vi expression in *S. typhi* is regulated by members of the family of two component system which are known to be important in controlling gene expression *in-vivo* coupled with what is known about its role in virulence suggests that there is a need to regulate Vi antigen in different host microenvironment in order for *S. typhi* to express full virulence. In the present study mice was immunized with *S.typhi* mutant showing protected immune response. The immunized animal’s serum antibody titre was found to be 1:80 in this present investigation.

Previously, inactivated whole cell vaccines given parenterally have been used to provide protection against typhoid fever. However, due to the high incidence of associated adverse systemic and local reactions, they are generally considered to be unsuitable for use as public health vaccines. Thus two vaccine developed is first parenteral capsular polysaccharide vaccine based on the *S.typhi* Vi antigen. Second, a live attenuated oral vaccine containing *S.typhi* strain Ty21a. Both of these vaccines have been used successfully to immunize against typhoid fever. However, as a result of their short falls, such as unknown basis of attenuation in Ty21a, a number of new genetically defined attenuated strains of *S. typhi* have been constructed as candidate live oral vaccine (Garmory et al., 2002).
Vi polysaccharide down regulated expression would therefore be a logical target against which to elicit opsonizing antibody for a candidate of typhoid vaccine. Thus attenuated S. *typhi* strains that express Vi antigen constrictively or whose expression of Vi antigen is under the control of promoters that are activated in host intracellular compartment in order to determine whether the response against this antigen can be improved as a step for improving the efficacy of live oral typhoid vaccine.

6.6 Typhoid prevention and control measures

1. Protect and chlorinate public water supplies. Provide safe water supplies and avoid possible back flow connections between sewers and water supplies.

2. Dispose of human faeces in a sanitary manner and maintain fly-proof latrines.

3. Use scrupulous cleanliness in food preparation and handling.

4. Educate the public regarding the importance of hand washing with disinfectants. This is important for food handlers or attendants involved in the care of patients. Thorough and frequent hand washing is essential, especially after a bowl movement.

5. Mass screening of typhoid carriers among food handlers and treat them with antibiotics.

6. Immunization of typhoid fever is recommended for the international travelers to endemic areas, especially if travel will involve exposure to unsafe food and water or close contact in rural area and with indigenous populations.