5.0 RESULT

A total of 194 isolates of *Salmonella Typhi* streaked on brain heart infusion agar slant were obtained from various laboratories from Chennai including those from Sri Ramachandra University, Porur, Chennai, during June 2003-August 2009.

5.1 Sample Criteria

The cultures were isolated from patients having typhoid fever with a widal titer of 1:80 and above.

Sample details

Patients from whom these isolates were obtained included both adults and children. The breakup was given in Table 7.

Out of the total number of isolates 30% were from male and 51.5% were from female and 19.5% from children. The majority of isolates were from females. Break up of age of patients from whom the isolates were obtained was given in Table 8.

It was found that the most number of isolates were from the group 11-20 years. There was a decrease in the number beyond 30 years. The isolates obtained were isolated from various samples of patients like blood, urine and faeces. The details of the sample source were given in Table 9. The titre of *Salmonella Typhi* O and H antigen were shown in Table 10. Most of the isolates (110) had a titre of 1:160, for both O and H antigens.

5.2 Growth of *Salmonella Typhi* on Different Media

The samples obtained were streaked on different media. The confirmation of *Salmonella Typhi* was done by using a series of
morphological and biochemical tests including sugar fermentation studies. The presumptive identification of *Salmonella Typhi* was based on formation of non-lactose fermenting colonies on MacConkey agar and jet black colonies on Wilson Blair bismuth sulphite agar and colorless luxurious growth on XLD agar. (Figure. 8)

Identification of isolate was further confirmed by H₂S production and K/A on TSI slant. (Figure. 10). Motility was observed in MMA (Figure. 9);

**5.3 Serological Identification of *S. Typhi***

To further confirm, serological identification was performed on the isolates using antisera (King’s institute) against *S. Typhi*. Strong positive agglutination with the antisera was observed within 30 seconds.

**5.4 Conventional typing**

The collected samples were grouped based on conventional and molecular methods. The two conventional methods routinely used were (i) sugar fermentation (arabinose and xylose) method and (ii) Antibiotic susceptible pattern

**5.4.1 Biotyping Based on Sugar Fermentation**

The isolates were classified into 4 biotypes based on their sugar fermentation (Xylose and Arabinose) as shown (Table. 11; Figure. 11).

Out of 194 isolated samples, 182 isolates (93%) were Biotype I; 11 isolates (6%) were Biotype II and only 1 sample (0.5%) was found to be Biotype III. No organism belonging to Biotype IV was observed.
5.4.2 Antibiotic Susceptible Pattern of *Salmonella Typhi*

All the 194 isolates were tested for the 14 antibiotics that were routinely used to treat typhoid fever. These antibiotics included Norfloxacin (10µg), Aztreonam (30 µg), Cefotaxime (30µg), Ceftriaxone (30µg), Nalidixic acid (30 µg), Nitrofurantoin (300µg), Cefuroxime (30µg), Gentamycin (10µg), Amikacin (30µg), Ciprofloxacin (5µg), Oxfloxacin (5µg), Cefixime (5µg), Cefdinir (5µg) and Ceftriaxone (5µg) (Table. 12).

Among the 194 isolates, 17 isolates (9%) were found to be susceptible to all the 14 antibiotics used including nalidixic acid. 127 isolates (65%) were resistant to nalidixic acid alone. 32 isolates (16%) were found to be resistant to both nalidixic acid and nitrofurantoin. 4 isolates (2%) were resistant to nalidixic acid, nitrofurantoin, cefuroxim sodium and ceftazidim antibiotics. 14 isolates (7%) were resistant to nalidixic acid, cefuroxim sodium and nitrofurantoin. Based on this result the isolates were grouped into 5 numbers as group A, B, C, D and E (Table. 13; Figure. 12, and 13).

Group A- Isolates sensitive to all 14 antibiotics
Group B- Isolates resistant only to Nalidixic acid
Group C- Isolates resistant only to NA and Nitrofurantoin
Group D- Isolates resistant only to NA, FU, CR, CZ
Group E- Isolates resistant only to NA, CR, FU

The dendrogram analysis based on the antibiogram data showed one major cluster (177 isolates) and a minor cluster consisting of 17 isolates (Squared Euclidean distance method, SPSS software). The minor cluster
had a cluster of 14 isolates and three out clusters comprising only one isolates. The major cluster had two sub-clusters (Table. 13; Figure. 14).

5.5 Molecular Typing

RAPD (Random Amplified Polymorphic DNA) method was used for molecular typing of *Salmonella Typhi*. This method is highly reliable and reproducible and its applicability in typing *Salmonella Typhi* cultures was tested. Genomic DNA was extracted from the samples that were prominent and pure. RAPD was done using three primers (A, B and C) and at least 13-15 bands were obtained for each of the primers (Figure. 16, 17 and 18). The sizes of the fragment (molecular weight in base pairs) were calculated by using λDNA/HindIII marker, which was run along with the amplified products.

Figure 16 (16 a,b,c,d,e,f,g,h,i,j,k,l and 16m) for primer 784
Figure 17 (17 a,b,c,d,e,f,g,h,i,j,k,l,m,n,o and 17p) for primer 787
Figure 18(18 a,b,c,d,e,f,g,h,i,j,k,l and 18m) for primer 797

Bands were scored 1 for the presence and 0 for the absence; the binary data was used for analysis using the software “SPSS”. A genetic dissimilarity matrix was calculated according to Squared Euclidean Distance which estimated all pair-wise differences in the amplification product and Cluster analysis was done by Wards method using a minimum variance algorithm (Ward, 1963).

From the RAPD data, a dendrogram was obtained using Squared Euclidean distance method using SPSS software. Two clusters, one major cluster comprising of 190 isolates and a minor cluster comprising of 4 isolates were observed. The major cluster had 2 sub clusters- an out cluster
consisting of 1 isolate (193) and the other sub cluster had remaining 189 isolates. The sub cluster having 189 isolates was further divided into many clusters. The dendrogram was given in Figure 19.

5.6 Simple Method for S. Typhi Antigen Detection

For rapid detection of S. Typhi, two methods – dot blot and FTIR were employed in this study. For dot blot and slot blot analysis, the antibody was raised against antigens (O, H and Vi) in chicken. The antigens of S. Typhi (O, H and Vi) were immunized in white leghorn layer chickens to raise IgY antibodies. The polyclonal antibodies were purified and characterized using various immunological techniques.

5.6.1 Polyclonal Antibody (IgY) Production Against S. Typhi Antigens and its Characterization:
5.6.1.1 Immunization of Chickens

The commercial antigens (O, H and Vi) of S. Typhi were used to immunize the White leghorn chicken (35-40 weeks old). Approximately, 75 eggs were collected from the chickens immunized with each of the antigens and stored at 4ºC or processed immediately.

5.6.1.2 Purification of IgY

The IgY was purified according to the procedure of Akita and Nakai (1992). The purified antibodies were quantified by Lowry method and it was found to be in the range of ~27-40 µg per egg. The purified IgY and the
commercial antigens were then separated on SDS-PAGE; intact IgY and antigens were found (Figure 20).

5.6.1.3 Characterization of IgY

The purified polyclonal antibodies (IgY) were first characterized using agglutination techniques like slide agglutination and tube agglutination. The slide agglutination assay was done using the specific commercially available antigens (O, H and Vi) of S. Typhi along with culture S. Typhi as internal control. Agglutination was observed with the purified IgY and their corresponding antigens; agglutination was also observed with S. Typhi organism (Figure 21). The tube agglutination was done using different concentrations of purified IgY with fixed concentration of corresponding antigens (Figure 22). Agglutination was observed even at dilution of 1:320 and complete agglutination was observed at 1:40 dilution.

5.6.1.4 Double Immuno Diffusion

The double immuno diffusion was done with the purified IgY and antigens (commercial and urine sample) and pre-immune IgY was used as control. The precipitation was observed only with the IgY raised against the different antigens whereas no precipitation was observed in control IgY (pre-immune) (Figure. 23). The different IgY isolated (anti-O, anti-H and anti-Vi) gave precipitation for their corresponding antigens. These IgY also detected S. Typhi organism present in the patient urine sample as precipitation was observed (Figure. 23).
5.6.1.6 Immuno Electrophoresis

Counter immuno electrophoresis (CIE) was done with the purified IgY (10 µg) and their corresponding commercial antigens. For anti-H antibody, (15 µg) of H antigens were used. Similarly for anti-O and anti-Vi the concentrations of corresponding antigen were 25 and 20 µg respectively. Healthy urine sample was also used as control in these experiments. The antibody IgY detected the corresponding antigens (Figure. 24).

5.7 Dot-Blot Analysis

Dot blot analysis was done with commercial O antigen and its corresponding anti-O (IgY) with a typhoid patient’s urine for comparison. The anti-O IgY was able to detect both the commercial antigen as well as S. Typhi from patient’s urine (Figure. 25). Different concentrations of antigens (0.5 – 5 µg; O or H or Vi) were used for dot blot analysis. Healthy urine sample served as control and the different volumes ranging from 0.5 – 5 µl were used. The antibody IgY (1:100 dilution) was able to detect the presence of antigen even as low as 1 µg and 1 µl of patient urine sample (Figure. 25).

One OD culture of S. Typhi was serially diluted using healthy urine sample and used both for plating on MacConkey agar medium and dot blot analysis(fig 26). The different dilutions ($10^{-1}$ to $10^{10}$) of urine sample along with a neat sample were blotted on to C' Nylon membrane and probed using purified polyclonal antibody. The commercial antigen (O, H and Vi) was used as positive control and a healthy urine sample served as negative control. The antibody (1:100 dilution) was able to detect most of the
dilutions of the urine sample (Figure. 27). The patient serum, which was used as positive control, was able to detect the commercial antigens (O, Vi and H) (Figure. 27 and 28). The antibodies raised against the antigens detected specifically the corresponding antigens alone; cross-reactivity was not observed (Figure. 29).

The slot blot analysis was done using these polyclonal antibodies with the three antigens and different cultures. Prominent bands were observed in typhoid patient’s urine, *S. Typhi* suspended in saline and commercial *S. Typhi* antigens. Whereas bands were not observed in lanes that carried *E. coli* and *Pseudomonas aeruginosa* (Figure. 30).

### 5.8 FTIR

The entire IR range will usually be taken for analysis of different samples. The region spans from wave numbers 400 cm$^{-1}$ to 4000 cm$^{-1}$ (Figure. 31 and 32). The spectra collected for the samples were consolidated into overlay plots depicting the patient samples and an average control spectrum in absorbance units against the wave number, typical of the Fourier transforms. The spectrum showed the peaks of the average control and the test sample with the difference in intensity due to the difference in the concentration ranges of the test sample viz 1:80, 1:160, 1:320. The samples B863, B431 and B313 alone showed some differences statistically than control samples around the wave number 700-736 cm$^{-1}$ corresponding to nucleic acids. The samples B237, B304, B346, B652 and B863 when compared to average control had difference in protein components (wave number 1025 cm$^{-1}$).
The samples B237, B304, B346, B652 and B863 had some statistical differences at 1076 cm\(^{-1}\) that represents carbohydrate moieties. The sample B278 had a split peak at the wave number 1120 cm\(^{-1}\), that represents methylated mannose vibrations. The samples A237, A304, A313, A357, A652, A905 and A346 showed differences in the carbohydrate regions 1200-1460 cm\(^{-1}\) and 3435-3442 cm\(^{-1}\) when compared with control samples. The samples A431 and A863 had statistically significant difference only in the region 1200 to 1400 cm\(^{-1}\) when compared with control samples.

The samples B237, B304, B346, B652 and B863 had some differences when analysed statistically in the absorbance range of 1658 cm\(^{-1}\) representing Lipid A. The samples A237, A304, A313, A357, A431, A652, A863 and A905 showed differences when compared with control samples in the higher region 2850 – 3125 cm\(^{-1}\).

### 5.8.1 Statistical Analysis of FTIR Spectra

#### 5.8.1.1 Karl Pearson’s Non-Parametric Correlation

The non-parametric Karl Pearson’s correlation coefficient showed highly positively correlated values in the range of r>=0.856 to r<=1.000. Some samples showed very weak correlation with the values in the range of r>=0.200 to r<=0.697. The relatedness of the test samples with the control samples was assessed. The values for the significant regions and non-significant regions were tabulated (Table. 14).

#### 5.8.1.2 Regression Analysis

Multiple regression analysis has been done with backward elimination method. The independent variable was the average control and the
dependent variables being the test. The excluded variables, those which did not fit in the model showed some significance from the spectrum point of view. Hence the excluded variables were considered significant for assessing the samples that were different from the control. Values which were excluded while fitting in the model were tabulated (Table 15) for those specific regions.

5.8.1.3 Mann Whitney U Test

The significant difference between the control and test samples using Mann Whitney U Test values for the several given peaks were identified. The values with the significance and non-significance differences of the control and test samples were tabulated (Table 16).

5.9 Herbal Extract

Methanolic extract of S. mahagoni was analysed for antibacterial activity. The methanolic extract was then analysed using TLC and diffusion methods to test their antibacterial activity. The methanolic extract alone showed antibacterial activity in disc diffusion and well diffusion methods (Figure 33 and 34).

5.9.1 Thin Layer Chromatography

TLC was performed for SM3 extract obtained from S. mahagoni. For TLC hexane: ethyl acetate (6:4) was used as resolving solvent. In the methanolic extract, many bands were observed in the UV – short UV (bands
with Rf - 0.66, 0.60, 0.46, 0.32, 0.20 and 0.14) and long UV (bands with Rf – 0.14, 0.20, 0.32, 0.46, 0.60, 0.72 and 0.78) for long UV were observed (Figure. 35). In case of visible light three bands (Rf – 0.20, 0.32, 0.46) were observed (Figure. 35).

5.9.2 Antibacterial Screening

The methanolic leaf extract of *Swietenia mahagoni* was tested against the human pathogen *Salmonella Typhi* for antibacterial activity. Methanolic extract containing 500 µg/ml discs were used for disc diffusion assay and showed good inhibition zones measuring about 1.2 cm. The thin layer agar diffusion assay with different concentrations of methanolic extract (1000 µg, 750 µg and 500 µg) were tested along with an antibiotic – Ciprofloxacin 90 µg/ml that served as control (Table. 17). Methanol at different concentrations was used as internal control and had no antibacterial effect (Table. 17; Figure. 34).

5.9.3 Bioautography

Bioautography technique was carried out that showed inhibition against *Salmonella Typhi* as clear zone was observed (Figure. 36).

5.9.4 MIC of SM 3

The minimum inhibitory concentration of the methanolic extract of SM 3 on *S. Typhi* was done in triplicates, using 0.6 OD culture on a microtitre plate. Different concentrations of methanolic extract was added to
the culture and observed after overnight incubation. The OD was read at 620 nm and documented. The MIC was observed to be 17.5 mg from this assay (Figure. 37, 37a and 37b; Table. 18).

5.9.5 Brine Shrimp Lethality Test (BSLT)

Brine shrimp lethality test was done in triplicates with different concentrations of SM3 methanolic extract. The organisms were observed at two time intervals 24 hrs and 48 hrs. At 24 hrs (Table. 19) some lethality was observed in brine shrimp, in most of the concentrations whereas at 48 hrs most of the brine shrimp were dead even at 100 µg/ml and above this concentration 100% lethality was observed. It was observed that the LC$_{50}$ for brine shrimp for SM3 methanolic extract was found to be 13.59 µg/ml for 48 hrs incubation was shown in Table. 20 and graph (Figure. 38).