## Chapter 3 Genomic diversity assessed by standard typing methods

### 3.1 Introduction

Typhoid fever is caused by the human-adapted serovar, *Salmonella typhi* and is still a serious health problem in many geographic areas. Large outbreaks frequently involve developing regions especially tropical parts of the world, but epidemics have also been reported in recent years from developed countries. Annually, there are close to 17 million cases of typhoid fever, with nearly 600,000 deaths mostly occurring in Asia and Africa. Papua New Guinea and Indonesia have the highest annual incidences with more than 1,000 cases per 100,000 population being reported yearly (Pang et al., 1995; Pang et al., 1998a).

Some factors that make this disease a continuous health hazard includes rapidly increasing population and urbanization, lack of proper sanitation and water supply, emergence of antibiotic resistant strains and the limited use of vaccines (Edelman and Levine, 1986; Pang, 1992b).

The continuing presence of the disease in endemic areas and its potential for spread means that improved and effective epidemiological surveillance is needed. The development of rational control strategies for this important human disease as well as the availability of detailed and accurate data related to the molecular epidemiology of *S. typhi* is crucial. However, early epidemiological investigations of *S. typhi* relied on classical typing methods such as phage typing (which still remains as the primary typing method of this organism), serotyping, biotyping and antibiogram typing which detected unstable phenotypic traits. These methods are thus not discriminatory enough in differentiating individual strains beyond the species level (Hickman and Farmer III, 1978; Hickman-Brenner and Farmer III, 1983).

The limitations of phenotypically based typing methods have led to the development of typing methods based on the microbial genotype or DNA sequence. These molecular methods minimize the problems with typeability and reproducibility and, in some instances, allow the establishment of large databases of characterized organisms (Olive and Bean, 1999). In recent years, the value of molecular methods for discriminating between strains of pathogenic bacteria in defined epidemiological settings has been well proven in numerous studies of *S. typhi* and other *Salmonella* spp. For example, PFGE, IS200 typing, RAPD-PCR and plasmid profiling was able to discriminate multidrug resistant *S. typhi* isolates from sensitive strains (Threlfall et al., 1993; Hermans et al., 1996; Shanahan et al., 1998; Hampton et al., 1998). Ribotyping and PFGE analysis have shown diversity among *S. typhi* isolates associated with sporadic and outbreak cases (Altwegg et al., 1989; Thong et al., 1994; Echeita and Usera, 1998). Analysis of *S. typhi* strains from sequential blood samples by a barrage of methods such as PFGE, plasmid profiling and ribotyping was able to distinguish relapse from reinfection cases in individuals suffering from typhoid fever (Wain et al., 1999). PFGE, ribotyping and RAPD-PCR used in concordance were able to trace the source of a typhoid outbreak in Zürich to a worker in a restaurant (Gruner et al., 1997). Shangkuan and Lin (1998) used RAPD-PCR to differentiate between the vaccine strain *S. typhi* Ty21a from the rest of the *S. typhi* strains studied. Environmental *S. typhi* isolates also have been characterized by PFGE (Thong et al., 1996c). Clonal lineages and phylogeny studies on *S. typhimurium* and *S. enteritidis* was explored by ribotyping, IS200 typing, PFGE and PCR-RFLP analysis (Olsen et al., 1994; Olsen et al., 1997). A combination of plasmid analysis and PFGE proved to be suitable in differentiating live vaccine strains of *S. choleraesuis* from field strains of this serovar (Weide-botjes et al., 1996). As mentioned previously, the ideal genotyping method should

produce results that are reproducible between laboratories in order to allow unambiguous comparative analysis and the establishment of reliable databases. One of the newest and most promising methods is amplified fragment length polymorphism (AFLP). The technique is relatively new and has not yet been widely used in the study of Salmonellae. Aarts et al. (1998) analysed 62 different *Salmonella* serotypes (excluding *S. typhi*) and showed that the patterns were specific for serotypes. In another study it was shown that AFLP and PFGE have about the same discriminatory power in typing *S. dublin* isolates (Duim et al., 1997).

The clinical manifestations and geographic variations in disease diversity have been well documented to vary markedly in different parts of the world where typhoid fever is endemic. For instance, typhoid fever manifests as a relatively mild illness with low fatality rates in South America and parts of Southeast Asia (Thailand and Malaysia). In contrast there are other regions reporting severe forms of the disease with higher mortality rate as seen in sub-Saharan Africa and Indonesia (Hoffman et al., 1991; Pang, 1998a). The reason for these differences in disease severity are not known but may be related to differences in health care facilities, genetic factors of the host as well as immune response and also it seems reasonable to hypothesize that they could result from *S. typhi* strain variations. Few studies have been performed to determine if variations in clinical presentations are related to strain differences and little correlation between strain characteristics and disease severity has been documented (Heneine et al., 1991; Franco et al., 1992).

The extent of genetic diversity among strains of *S. typhi* is actually greater than previously thought (Altwegg et al., 1989; Pang et al., 1992a; Thong et al., 1996) and more importantly, that the genome of *S. typhi* has undergone major rearrangements (Liu and Sanderson, 1995). It would thus seem appropriate to apply these more discriminative

molecular typing methods in reinvestigating a possible correlation between disease severity and strain differences.

### 3.1.1 Objectives

The first objective of the work in this chapter was to assess the usefulness of pulsed-field gel electrophoresis (PFGE), ribotpying, IS200 typing, PCR-RFLP and PCR-ribotyping as molecular tools for the purpose of delineating epidemiological and evolutionary relationships of sporadic *S. typhi* isolated from Papua New Guinea over a period of 3 years, as well as *S. typhi* isolated from various other geographic locales.

In the present chapter the potential utility of a recently developed PCR-mediated typing method (AFLP) as compared to more established typing schemes such as PFGE and ribotyping was also assessed to study the genetic diversity and molecular characteristic of *S. typhi* isolated from Papua New Guinea from patients with fatal and non-fatal typhoid fever and from different body sites in the same patient.

## 3.2 Materials and methods

### 3.2.1 Bacterial strains

Twenty-five isolates of *S. typhi* obtained from 19 patients from among sporadic cases of typhoid fever in the Eastern Highlands province of Papua New Guinea between February 1992 and April 1994 were studied. The isolates were recovered from blood marrow and feces (rectal swab). In 6 patients, isolates were recovered from both blood and feces of a single patient. These isolates were provided by Dr. A. Clegg and Dr. M. Passey from the Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea.

Nineteen other strains of *S. typhi* used in this study were clinical isolates obtained from patients in Malaysia (3 sporadic strains isolates in 1998, 1990, 1994), Indonesia (3 sporadic strains isolated in 1995), India (2 sporadic strains isolated in 1995), Chile (3 sporadic strains isolated in 1994), Zurich (2 outbreak strains isolated in 1994) and Pakistan (6 sporadic strains isolated in 1997). The *S. typhi* strains were isolated from blood and stool. Table 3.1 shows the list of all the strains that were analysed in this study.

The strains had been identified, serotyped and tested for their antibiotic susceptibility at the institutions from which they were obtained. Phage typing of the isolates was performed according to standard procedures by the *Salmonella* Reference Centre at the Institute for Medical Research Kuala Lumpur. Bacteria were maintained on Luria-Bertani (LB) agar plates. Repeated subculturing of isolates was avoided and for long term maintenance, all the isolates were kept in LB broth with 20% glycerol at –70°C.

**Table 3.1 :** Geographic distribution of *S. typhi* isolates.

| Strain | Isolate Designation | Date of Isolation | Source | Age | Country |
|---|---|---|---|---|---|
| st1 | PNG 4 | 16.2.94 | Stool | 26M | PNG (F) |
| st8 | PNG 45 | 16.2.94 | Blood | 26M | PNG (F) |
| s4 | PNG 8 | 23.2.94 | Blood | 50F | PNG (F) |
| st10 | PNG 57 | 23.2.94 | Stool | 50F | PNG (F) |
| st5 | PNG 17 | 16.3.92 | Stool | 26F | PNG (F) |
| st9 | PNG 55 | 16.3.92 | Blood | 26F | PNG (F) |
| st2 | PNG 5 | 16.11.93 | Stool | 50F | PNG (F) |
| st3 | PNG 7 | 13.2.94 | Stool | 12M | PNG (F) |
| st6 | PNG 18 | 24.5.93 | Marrow | 22M | PNG (F) |
| st7 | PNG 27 | 18.10.93 | Blood | 48F | PNG (F) |
| st11 | PNG 1 | 21.2.94 | Stool | 30M | PNG (NF) |
| st22 | PNG 49 | 21.2.94 | Blood | 30M | PNG (NF) |
| st12 | PNG 2 | 25.3.94 | Stool | 14F | PNG (NF) |
| st13 | PNG 3 | 25.3.94 | Blood | 14F | PNG (NF) |
| st17 | PNG 40 | 6.3.94 | Stool | 32F | PNG (NF) |
| st18 | PNG 42 | 6.3.94 | Blood | 32F | PNG (NF) |
| st14 | PNG 6 | 14.2.94 | Blood | 9M | PNG (NF) |
| st15 | PNG 10 | 13.4.94 | Blood | 14F | PNG (NF) |
| st16 | PNG 37 | 15.11.93 | Blood | 3M | PNG (NF) |
| st19 | PNG 43 | 8.3.93 | Blood | 21M | PNG (NF) |
| st20 | PNG 47 | 12.5.93 | Blood | 14M | PNG (NF) |
| st21 | PNG 48 | 10.3.94 | Stool | 17F | PNG (NF) |
| st23 | PNG 50 | 17.1.94 | Blood | 1M | PNG (NF) |
| st24 | PNG 54 | 22.3.94 | Blood | 13M | PNG (NF) |
| st25 | PNG 51 | 29.3.94 | Stool | 5F | PNG (NF) |
| st27 | 111 | 1990 | Blood | ND | Malaysia |
| st31 | 142 | 1994 | Blood | ND | Malaysia |
| st32 | 495 | 1987 | Blood | 22M | Malaysia |
| st30 | 110959 | 21.3.95 | Blood | 14M | Indonesia |
| st33 | 110961 | 23.5.95 | Blood | 6F | Indonesia |
| st34 | 111217 | 26.4.95 | Blood | 11F | Indonesia |
| st35 | A102 | 1995 | Blood | ND | India |
| st26 | A106 | 1995 | Blood | ND | India |
| st28 | MM814 | 1994 | Stool | ND | Zurich |
| st36 | MM817 | 1994 | Stool | ND | Zurich |
| st37 | 3102 | 1994 | Blood | ND | Chile |
| st38 | 3106 | 1994 | Blood | ND | Chile |
| st29 | 3112 | 1994 | Blood | ND | Chile |
| st39 | PR13 | 1997 | Stool | ND | Pakistan |
| st40 | PR14 | 1997 | Stool | ND | Pakistan |
| st41 | PR15 | 1997 | Stool | ND | Pakistan |
| st42 | PS52 | 1997 | Stool | ND | Pakistan |
| st43 | PS54 | 1997 | Stool | ND | Pakistan |
| st44 | PS55 | 1997 | Stool | ND | Pakistan |

PNG (F) - Strains isolated from Papua New Guinea fatal cases.

PNG (NF) - Strains isolated from Papua New Guinea non-fatal cases.

ND - Not Determined

### 3.2.2 Pulsed-field gel electrophoresis (PFGE)

#### 3.2.2.1 Preparation of genomic DNA

Preparation of *S. typhi* genomic DNA was carried out as described in section 2.2.2.1 This method was chosen to ensure there is homogeneity in the preparation of DNA for the various methods of analysis.

#### 3.2.2.2 Digestion with restriction enzyme

One DNA plug prepared as described above was equilibrated in 150 µl of restriction endonuclease buffer for 30 mins on ice as recommended by the manufacturer. The buffer was then aspirated, the tube filled with fresh buffer and digestion of the DNA was carried out with 10 U of the appropriate endonuclease. In this case *Xba*I (Promega, Madison, Wisconsin, U.S.A.) was used. Incubation in ice was carried out for 15 mins to allow the enzyme to penetrate into the plug. The reaction was incubated overnight at 37°C. After digestion, the plugs could be loaded into a gel or stored in 1×TE buffer overnight at 4°C.

#### 3.2.2.3 Gel electrophoresis

The plugs containing the digested genomic DNA were loaded into slots of a gel consisting of 1% low endosmosis agarose (Sigma Chemical Co., St Louis Mo.) in 0.5×TBE.

Electrophoresis of digested DNA fragments was carried out by PFGE using a Contour-clamped homogeneous electric field apparatus with a hexagonal electrode array (CHEF-DR III system; Biorad laboratories, Richmond, California).

PFGE was performed at 12°C for 26 h at 6 V/cm with a pulse time ranging 1-40 s and pulsing angles were set at 120°. The run condition was chosen such that the distribution of the fragments was even, facilitating the comparison of the restriction profiles.

The DNA size standard used was a bacteriological lambda ladder consisting of concatemers starting at 48.5 kb and increasing to approximately 1,000 kb (Biorad Laboratories, Richmond, California).

After a PFGE run, the gel was stained in deionized water containing 1 μg/ml ethidium bromide for 10-15 mins. The DNA restriction patterns were photographed using a Polaroid MP4 camera.

### 3.2.2.4 Data analysis

Restriction enzyme analysis (REA) patterns generated by PFGE were subjected to comparative analysis, either visually or using RFLP (restriction fragment length polymorphism) patterns analysis software (GelCompar; Applied Maths, Kortrijk, Belgium), in order to assess diversity. Cluster analysis (dendogram) involves 2 steps:

i) Calculation of similarity index between all possible strains analysed

ii) Cluster analysis based on the matrix of similarity by using the unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

Visually assessed PFGE patterns were assigned arbitrary patterns and compared by similarity coefficients (F, proportion of shared fragments between 2 isolates) (El-Adhami et al., 1991). The F value was calculated by using the following formula:

$$F = 2n_{xy} / (n_x + n_y)$$

Where $n_x$ is the total number of DNA fragments from isolate x, $n_y$ is the total number of DNA fragments from isolate y, and $n_{xy}$ is the total number of DNA fragments that were identical in the 2 isolates.

Isolates were considered to be genetically similar or identical if there was complete concordance of the DNA fragment profiles and considered different if there was a difference of one or more DNA bands with this method, F value of 1.0 indicates identical patterns and a value of 0 indicates complete dissimilarity.

### 3.2.3 Ribotyping

#### 3.2.3.1 Preparation of genomic DNA

Preparation of *S. typhi* genomic DNA was carried out as described in section 2.2.2.1. This is to ensure homogeneity in DNA preparation for the various methods of analysis.

#### 3.2.3.2 Preparation of rRNA gene probe

The preparation of the 16S rRNA gene probe has been described in secton 2.2.2.2.

#### 3.2.3.3 Gel electrophoresis of digested *S. typhi* genomic DNA

Digestion of *S. typhi* genomic DNA with restriction enzyme *Pst*I (Promega, Madison, Wisconsin, U.S.A.) and conventional gel electrophoresis has been described in section 2.2.2.3.

### 3.2.3.4 Southern blotting and hybridization

Both the Southern blotting and hybridization process has been described from section 2.2.2.4 to 2.2.2.9.

### 3.2.3.5 Data Analysis

Ribotype patterns were subjected to the same data analysis as in section 3.2.2.4.

## 3.2.4 IS200 typing

### 3.2.4.1 Preparation of genomic DNA

Preparation of genomic DNA was carried out in accordance to section 2.2.2.1 to standardize the DNA preparation for various typing methods.

### 3.2.4.2 Preparation of the IS200 probe

#### 3.2.4.2.1 Extraction of plasmid DNA

*E. coli* K-12 harbouring the recombinant plasmid DH5/plZ45 which contains a 300 bp IS200 sequence was a kind gift from Dr. Ken Sanderson, Department of Biological Sciences, University of Calgary, Canada.

Plasmid extraction was carried out according to the alkaline lysis method of Wang et al. (1994). A single colony of *E. coli* K-12 carrying the plasmid DH5/plZ45 was grown overnight at 37°C in 1.5 ml of LB broth + ampicilin (50 μg/ml). The cells were then harvested by centrifugation at 5,000 rpm at room temperature for 10 mins.

The cell pellet was resuspended in 100 μl ice cold SET buffer (appendix 2) containing lysozyme (20 mg/ml) and incubated at room temperature for 10 mins. Two hundred microlitres of freshly prepared lysis solution (appendix 2) was added. The mixture

was mixed thoroughly by inverting the eppendorf tube and then kept on ice for 10 mins. The lysed cells were mixed with 150 µl of ice cold 3M KAc (potassium acetate) pH4 and the tube was inverted gently until a white precipitate was formed, after which it was incubated on ice for 30 mins.

The suspension was centrifuge at 10,000 rpm for 15 mins at 4°C. Supernatant was transferred to a clean eppendorf tube and 5 µl RNase A (10 mg/ml) was added. The mixture was incubated for 20 mins at 37°C. Two hundred microlitres of TE saturated phenol chloroform was added. Once again the tube was vortexed followed by a spin at 10,000 rpm for 10 mins at room temperature.

The supernatant was transferred to a clean tube and 600 µl of ice cold 100% ethanol was added. The tube was vortexed and left at –20°C for at least 30 mins (preferably 1 to 2 hrs).

Pellet was obtained by centrifugation for 5 mins at 10,000 rpm. The pellet was washed with 1 ml cold 70% ethanol. The tube was centrifuged, supernatant poured off and the pellet was dried under vacuum for 5 mins. The pellet was resuspended in 25 µl of 1×TE and the plasmid DNA preparation was stored at 4°C.

#### 3.2.4.2.2 Gel electrophoresis of plasmid

Ten microlitres of the plasmid DNA was subjected to electrophoresis in a 1% low melting agarose (Gibco-BRL, Gaitherburg, Maryland, U.S.A.) gel in a TAE buffer system with lambda DNA digested with *Hind*III (New England Biolabs, U.S.A.) as a molecular weight marker. This was followed by ethidium bromide (1 µg/ml) staining and photographed under UV light.

#### 3.2.4.2.3 Purification of IS200 probe

The 2.3 kb fragment (2 kb plasmid vector DH5/plZ45 + 300 bp IS200 sequence) was cut out from the gel and purified using a Glassmilk DNA Isolation Matrix System (Gibco-BRL, U.S.A.) according to the manufacturer's instruction to give a probe concentration of 15-30 ng. Finally the IS200 probe (entire 2.3 kb fragment) was stored at 4°C for further use.

### 3.2.4.3 Gel electrophoresis of digested *S. typhi* genomic DNA

*S. typhi* genomic DNA was digested with restriction enzyme *Hinc*II (Promega, Madison, Wisconsin, U.S.A.) in accordance to the method in section 2.2.2.3. Digested genomic DNA was electrophoresed at 35 V for 24 hrs in a horizontal 0.8% agarose gel (type II medium electroendosmosis grade, Sigma, U.S.A.) in a 0.5×TBE buffer system. The gel was stained with EtBr (1 μg/ml) after electrophoresis, examined by UV transilluminator and photographed.

### 3.2.4.4 Southern blotting and hybridization

Both the Southern blotting and hybridization process for the IS200 typing was similar to that of ribotyping (section 2.2.2.4 to 2.2.2.9), except that the probe used was the 2.3 kb IS200 probe instead of the 16S rRNA gene probe for ribotyping.

### 3.2.4.5 Data analysis

IS200 profiles were subjected to the same data analysis as PFGE, which was been described in section 3.2.2.4.

### 3.2.5 Polymerase chain reaction (PCR) analysis

#### 3.2.5.1 Genomic DNA isolation

Genomic DNA of a Malaysian *S. typhi* isolate (strain St 111) was prepared by a modified method of Saito and Miura (1963). A single colony of *S. typhi* was grown overnight at 37°C in 5 ml of LB broth. The cells were harvested by centrifugation at 7,000 rpm for 10 mins at room temperature. The cell pellet was resuspended in 50 µl of solution I (appendix 2) by repeated pipetting and 10 mg lysozyme (20 mg/ml) was added. The cell suspension was then incubated at 37°C with gentle shaking for 30 mins in a shaking waterbath. Forty microlitres of solution II (appendix 2) was then added, followed by 10 mins incubation at 60°C. The solution was cooled down to room temperature and 100 µl of 5M Sodium perchlorate was added to help in the dissociation of proteins from nucleic acids. The suspension was mixed thoroughly but gently. Four hundred and fifty microlitres of TE saturated phenol chloroform was mixed with the suspension and shaken gently for 20 mins. The aqueous phase was recovered by centrifugation at 8,000 rpm for 10-15 mins at room temperature and transferred to a new tube. Two volumes of ice cold 100% ethanol were added. The tube was vortexed and left at –20°C for at least 2 hrs.

Pellet was obtained by centrifugation for 5 mins at 9,000 rpm. The pellet was washed with 1 ml cold 70% ethanol. The ethanol was discarded and the pellet was dried under vacuum for 5 mins. The pellet was resuspended in 200 µl 1×TE. The DNA solution was treated with 5 µl of RNase (20 µg/ml) at 37°C for 30 mins. DNA concentration was determined spectrophotometrically at wavelength of 260 and 280 nm (Novaspec® II, Pharmacia LKB).

### 3.2.5.2 PCR-ribotyping

#### 3.2.5.2.1 Primer selection

Two oligonucleotide PCR primers were chosen to amplify the 16S-23S intergenic spacer region of the bacterial rRNA operon as previously reported by Dasen et al. (1994). The sequence of the primers used were: 16S 5'-TTGTACACACCGCCCGTCA-3' and 23S 5'-GGTACCTTAGATGT-3'.

#### 3.2.5.2.2 PCR reaction

PCR reactions were performed in a volume of 25 µl containing 50mM Tris-HCl (pH 9.0), 50mM KCl, 2.5mM $MgCl_2$, 200mM (each) of dCTP, dGTP, dATP and dTTP (Promega, Madison, Wisconsin, U.S.A.), 0.2µM of each primer and 1 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, U.S.A.). The template was 100 ng of purified genomic DNA of *S. typhi.*

#### 3.2.5.2.3 PCR amplification

Amplification was performed in a DNA thermal cycler 480 (Perkin Elmer, Norwalk, Connecticut). An initial denaturing step of 95°C for 3 mins was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and an extension step of 4 mins at 72°C during the last cycle.

#### 3.2.5.2.4 Detection of PCR product

After PCR, 10 µl of the amplified product was subjected to electrophoresis at 100 V on a 1.5% agarose gel (type II medium electrophoresis grade, Sigma, U.S.A.) in a 0.5×TBE

buffer system. Following electrophoresis, the gel was stained in ethidium bromide (1 μg/ml) and photographed under UV light.

### 3.2.5.3 PCR-RFLP

#### 3.2.5.3.1 Primer selection

##### 3.2.5.3.1.1 *via*B gene

Two oligonucleotide PCR primers which amplify a 599 bp region of the *via*B sequence encoding the Vi-antigen of *S. typhi* were chosen (Hashimoto et al., 1995). The sequences of the primers were R1 forward, 5'-GTTATTTCAGCATAAGGAG-3' and R1 reverse, 5'-ACTTGTCCGTGTTTTACTC-3'.

##### 3.2.5.3.1.2 *gro*EL gene

Two oligonucleotide PCR primers which amplify the 1.6 kb *gro*EL gene encoding heatshock protein (HSP60) of *S. typhi* were used (Lindler and Hayes, 1994). The sequence of the primers were primer 5', 5'-GAT CCA TAT GGC AGC TAA AGA CGT AAA ATT CGG-3' and primer 3', 5'-CTA GGT CGA CTT ACA TCA TGC GGC CCA TGC CAC-3'.

##### 3.2.5.3.1.3 *omp*C gene

Two oligonucleotide PCR primers which amplify a 500 bp region of the *omp*C gene encoding the outer membrane protein of *S. typhi* were chosen (Dr. S. Geetha, personal communication). The sequence of the primers were Omp3, 5'-CCG TAA CAG CGA CTT CTT-3' and Omp4, 5'-CCG TTG CTG ATG TCC TTA CC-3'.

#### 3.2.5.3.1.4 *rfb*S gene

Two oligonucleotide PCR primers that amplify a 700 bp region of the *rfb*S gene encoding ADP-paratose which is part of the lipopolysaccharide (LPS) component of Salmonellae serogroup A and D were used (Luk et al., 1997). The sequence of the primers were rfbS1, 5'-TCA CGA CTTA CAT CCT AC-3' and rfbS2, 5'-TCG CTA TAT CAG CAC AAC-3'.

#### 3.2.5.3.1.5 16S rRNA gene

Primer sequences to amplify the 1.5 kb 16S rRNA gene can be obtained from section 2.2.2.2.

#### 3.2.5.3.1.6 16S-23S rRNA spacer region

Primers used to amplify 16S-23S rRNA intergenic spacer region have been discussed in section 3.2.5.2.1.

### 3.2.5.3.2 PCR reaction

#### 3.2.5.3.2.1 *gro*EL and 16S rRNA genes

PCR reaction for *gro*EL gene is similar to the 16S rRNA gene and this has been shown in section 2.2.2.2.

#### 3.2.5.3.2.2 *via*B gene, *omp*C gene, *rfb*S gene and 16S-23S rRNA spacer region

The PCR reactions for *via*B, *omp*C and *rfb*S genes are similar to that of the 16S-23S rRNA intergenic spacer region which has been described in section 3.2.5.2.2.

#### 3.2.5.3.3 PCR amplification

Conditions for each amplifications are as follows:

##### 3.2.5.3.3.1 *via*B gene

Initial denaturing step: 96°C for 30 s, annealing at 56°C for 15 s and extension at 74°C for 30 s. Final extension step at 74°C for 10 mins.

##### 3.2.5.3.3.2 *gro*EL gene

Initial denaturing step: 95°C for 3 mins. Followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 55°C for 2 mins and extension at 72°C for 1.5 mins.

##### 3.2.5.3.3.3 *omp*C gene

Initial denaturing step: 95°C for 3 mins. Followed by 28 cycles of denaturation at 93°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 mins. Final extension step at 72°C for 10 mins.

##### 3.2.5.3.3.4 *rfb*S gene

Thirty cycles of denaturing at 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 2 mins.

##### 3.2.5.3.3.5 16S rRNA gene

PCR amplification of the 16S rRNA gene has been described in section 2.2.2.2.

##### 3.2.5.3.3.6 16S-23S rRNA spacer region

The amplification of the spacer region has been shown in section 3.2.5.2.3.

same tube. The reaction was incubated at 37°C for 2 hours (or 'at room temperature overnight). A 20-fold volume of TE (10mM Tris; 0.1mM EDTA pH8.0) was added to stop the reaction.

#### 3.2.5.4.3 Preselective amplification

The AFLP protocols calls for a preselective amplification step to enrich the bulk of genomic DNA fragments. A 4 µl aliquot of the diluted restriction/ligation material was combined with 15 µl AFLP Amplification Core Mix containing PCR reagents (PE Applied Biosystems, Foster City, CA, U.S.A.) and 0.5 µl each of *Eco*RI and *Mse*I preselective primer. These primers match the respective adapter sequence.

PCR parameters for preselective amplification on a PCR system 9700 (PE Applied Biosystems, Foster City, CA, U.S.A.) were: 94°C 20 sec, 56°C 30 sec, 72°C 2 mins for 25 cycles. The finished reaction was diluted with 10-fold volume of TE (10mM Tris; 0.1mM EDTA pH8.0) and kept frozen at -20°C until used for selective AFLP amplification.

#### 3.2.5.4.4 Selective amplification

For selective AFLP amplifications, 1.5 µl of diluted preselective amplification product was combined with 0.5 µl each of fluorescent dye-labeled selective *Eco*RI primer and unlabeled selective *Mse*I primer, and 7.5 µl AFLP Amplification Core Mix (PE Applied Biosystems, Foster City, CA, U.S.A.). The AFLP microbial Fingerprinting Kit (PE Applied Biosystems, Foster City, CA, U.S.A.) provides 9 unlabeled selective *Mse*I primers and 9 fluorescent dye-labeled *Eco*R1 primers, both having 0, +1, +2 nucleotide extensions which specifies selective amplification.

Thermal cycling was done on a PE Applied Biosystems 9700 thermal cycler using the following parameter:

94°C 2 mins initial template denaturation followed by a 10-step "touchdown" cycling which is programmed as follows: 2 cycles 94°C 20sec, 65°C 30 sec, 72°C 60 sec; 2 cycles 94°C 20 sec, 64°C 30 sec, 72°C 60sec; 2 cycles 94°C 20sec, 63°C 30 sec, 72°C 60 sec; 2 cycles 94°C 20sec, 62°C 30 sec, 72°C 60 sec; 2 cycles 94°C 20sec, 61°C 30 sec, 72°C 60 sec; 2 cycles 94°C 20sec, 60°C 30 sec, 72°C 60 sec; 2 cycles 94°C 20sec, 59°C 30 sec, 72°C 60 sec; 2 cycles 94°C 20sec, 58°C 30 sec, 72°C 60 sec; 2 cycles 94°C 20sec, 57°C 30 sec, 72°C 60 sec. This is followed by 20 cycles at 94°C 20sec, 56°C 30 sec, 72°C 60 sec and a final incubation for 1 cycle at 60°C 30 mins.

#### 3.2.5.4.5 Electrophoresis

1.5 µl of selective PCR product was mixed with 16 µl formamide containing 1 µl GeneScan ROX-500 Size Standard (PE Applied Biosystems, Foster City, CA, U.S.A.) and electrophoresed on a PE Applied Biosystems ABI Prism® 310 DNA Genetic Analyzer using POP-4 polymer in a 36 cm capillary for 30 mins at 15 kV.

The DNA fragments generated were automatically analyzed and sized by the ABI Prism GeneScan® software (PE Biosystems, U.S.A.) according to size curve generated for each sample from migration of the internal size standard fragments. An example of an output from the GeneScan Software displaying a typical AFLP electropherogram is shown in appendix 5.

#### 3.2.5.4.6 Data analysis

GeneScan data files were analyzed by comparing electropherograms of 5 isolates with the electropherogram of the first isolate which was chosen arbitrary as a reference. Differences in fragments were documented in a binary table (appendix 4) generated with Excel® Microsoft software by manually transferring fragment sizes ranging from 50-500bp derived from GeneScan software, into the table. A "0" indicates absence of a fragment and a "1" indicates presence of a given fragment. (A new software application termed Autograph Fragment Typer software (PE Biosystems, U.S.A.) has been developed to automate this process). The binary table was then imported into the phologenetic analysis software application PAUP* 4.0 (Sinauer Associates, Cambridge, MA, U.S.A.) and computed using parsimony or distance analysis (UPGMA) algorithms.

### 3.2.6 Discriminatory index analysis

The discriminatory index (DI) for typing methods was used based on the probability that two unrelated strains sampled from a test population will be placed into different typing group (Hunter and Gason, 1988). This discriminatory index is an application of Simpson's Index of Diversity and is given by the following equation.

$$D = 1 - 1/[N(N-1)] \sum_{j=1}^{S} n_j(n_j-1)$$

Where N is the total number of strains in the sample population, S is the total number of types described, and $n_j$ is the number of strains belonging to the $j^{th}$ type.

This equation can be applied both to directly compare the discriminatory power of each of the typing methods investigated and to analyze the discriminatory power of combined typing schemes (Hunter and Gason, 1988).

## 3.3 Results

### 3.3.1 Phage typing

The 25 Papua New Guinea clinical isolates were represented by a single phage type (PT) D2. Three Pakistani strains (st39, st49 and st41), both the Indian strains (A102 and A106) and one Chilean strain (st38) exhibited PT E1. Two Chilean strains (st37 and st32) were Vi-untypeable, while 3 other Pakistani strains (st42, st43 and st 44) were comprised of PT J1, PT J3 and PT 46 respectively. All the Malaysian isolates (st27, st 31 and st32) were classified as PT D1. The phage types of the three Indonesian strains were not determined.

### 3.3.2 Antibiograms

Forty of the 44 isolates were sensitive to the antibiotic tested except for three Pakistani strains (st39, st40, st41) and one Indian strain (A106) that was resistant to the following antibiotics: ampicillin, chloramphenicol and tetracycline.

### 3.3.3 Pulsed-field gel electrophoresis

Molecular characterization of the 44 human isolates of *S. typhi* was performed by using PFGE after digestion of chromosomal DNA with restriction endonuclease *Xba*I. The restriction endonuclease *Xba*I, which recognize genomic sequences containing the rare tetranucleotide CTAG, was chosen based on previous work (Nair et al., 1994 and Thong et

al., 1994). The *Xba*I digestion patterns produced clearly resolvable fragments which were easily interpreted. Digestion with *Xba*I produced fingerprints consisting of 14-20 fragments ranging in size from 23 kb to 540 kb (Fig. 3.1).

Of the 44 strains tested, 10 Papua New Guinea (PNG) strains were obtained from patients with fatal typhoid fever, 15 PNG strains were obtained from patients with non-fatal disease and the other 19 strains were of diverse geographic origin (Table 3.1).

The relatedness of PFGE profiles between 2 isolates was scored by the coefficient of similarity (El-Adhami et al., 1991). The value (F) reflected the proportion of shared fragments in the 2 genomes. On the basis of F values generated, the 25 PNG strains showed limited genetic diversity as evidenced by only 2 major PFGE patterns and a few minor clonal variants (with 1-3 band differences from the major PFGE patterns) detected following digestion with *Xba*I (F=0.81-1.0) (Table 3.2). The 10 PNG strains isolated from fatal cases were closely related (F=0.92-1.0) with the PFGE X1 pattern being dominant (8 out of 10 strains) while pattern X1a and X1b were the other 2 clonal variants. This data corresponds to work previously done (Thong et al., 1996) and suggests that there could be an association among strains of *S. typhi* between genotype patterns and the capability to cause fatal illness. In contrast, the 15 non-fatal PNG strains had various PFGE patterns (X1, X1a, X1d, X2, X2a, and X2b) and they were grouped in 2 clusters at 85% similarity (Fig. 3.4). The geographic strains were highly diverse with F values ranging from 0.62-0.94. A total of 13 major PFGE types were established by *Xba*I digestion of genomic DNA from the 19 geographic isolates (Table 3.2). Within some of the major PFGE types, there exist strains which were classified as clonal variants and these were indicated by numerical suffixes (e.g.: X3 and X3a, X4 and X4a, X13 and X13a). Clonal variants were found to differ from the major PFGE profiles by up to three DNA fragments according to the

standardization of Tenover et al. (1995) and the generally expressed F values above 0.9. The PFGE profiles of the geographic isolates were different to that of the Papua New Guinea strains.

The stability of the PFGE restriction profiles were determined by comparing the profiles obtained after 3 isolates had undergone four serial passages. The genomic DNA isolated from each passage was digested with *Xba*I and run on a PFGE gel. The restriction profiles were found to be identical throughout (Fig. 3.3) and this demonstrated that the restriction profiles were stable within the period of study

Reproducibility of the *Xba*I generated PFGE profiles were confirmed by testing 13 geographic isolates on a separate occasion (Fig. 3.2).

The discrimination index for a particular typing method was calculated according to the studies carried out by Hunter and Gason (1988). The discrimination index of PFGE for the 44 isolates in this study was 0.88, meaning that when 2 isolates were sampled individually from the population studied, then on 88% of occasions they would be classified as 2 different types.

**Table 3.2 :** Genotypic characteristics of *S. typhi* strains.

| No. | Strain | Date of Isolation | Source | Age | Country | PFGE (*Xba* I) | Ribotyping (*Pst* I) | AFLP (*Eco* RI / *Mse* I) | IS200 (*Hinc* II) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | st1 | 16.2.94 | Stool | 26M | PNG (F) | X1 | P1 | A1 | H1 |
| 2 | st8 | 16.2.94 | Blood | 26M | PNG (F) | X1 | P1 | A4 | H1 |
| 3 | s4 | 23.2.94 | Blood | 50F | PNG (F) | X1 | P2 | A5 | H1 |
| 4 | st10 | 23.2.94 | Stool | 50F | PNG (F) | X1 | P1 | A7 | H1 |
| 5 | st5 | 16.3.92 | Stool | 26F | PNG (F) | X1 | P1 | A4 | H1 |
| 6 | st9 | 16.3.92 | Blood | 26F | PNG (F) | X1 | P1 | A2 | H1 |
| 7 | st2 | 16.11.93 | Stool | 50F | PNG (F) | X1 | P1 | A3 | H1 |
| 8 | st3 | 13.2.94 | Stool | 12M | PNG (F) | X1 | P1 | A2 | H1 |
| 9 | st6 | 24.5.93 | Marrow | 22M | PNG (F) | X1 (a) | P1 | A6 | H1 |
| 10 | st7 | 18.10.93 | Blood | 48F | PNG (F) | X1 (b) | P2 | A13 | H1 |
| 11 | st11 | 21.2.94 | Stool | 30M | PNG (NF) | X2 | P3 | A14 | H1 |
| 12 | st22 | 21.2.94 | Blood | 30M | PNG (NF) | X1 | P1 | A4 | H1 |
| 13 | st12 | 25.3.94 | Stool | 14F | PNG (NF) | X2(a) | P3 | A4 | H1 |
| 14 | st13 | 25.3.94 | Blood | 14F | PNG (NF) | X2(b) | P3 | | H1 |
| 15 | st17 | 6.3.94 | Stool | 32F | PNG (NF) | X1(c) | P1 | A2 | H1 |
| 16 | st18 | 6.3.94 | Blood | 32F | PNG (NF) | X1 | P1 | A4 | H1 |
| 17 | st14 | 14.2.94 | Blood | 9M | PNG (NF) | X1 | P1 | | H1 |
| 18 | st15 | 13.4.94 | Blood | 14F | PNG (NF) | X1 | P1 | A4 | H1 |
| 19 | st16 | 15.11.93 | Blood | 3M | PNG (NF) | X1(d) | P6 | A6 | H1 |
| 20 | st19 | 8.3.93 | Blood | 21M | PNG (NF) | X1 | P1 | A2 | H1 |
| 21 | st20 | 12.5.93 | Blood | 14M | PNG (NF) | X1 | P1 | A4 | H1 |
| 22 | st21 | 10.3.94 | Stool | 17F | PNG (NF) | X2(a) | P3 | A4 | H1 |
| 23 | st23 | 17.1.94 | Blood | 1M | PNG (NF) | X1 | P1 | A2 | H1 |
| 24 | st24 | 22.3.94 | Blood | 13M | PNG (NF) | X2(a) | P3 | A2 | H1 |
| 25 | st25 | 29.3.94 | Stool | 5F | PNG (NF) | X2(a) | P3 | A2 | H1 |
| 26 | st27 | 1990 | Blood | ND | Malaysia | X4 | P1 | A11 | H2 |
| 27 | st31 | 1994 | Blood | ND | Malaysia | X12 | P7 | ND | H2 |
| 28 | st32 | 1987 | Blood | 22M | Malaysia | X4(a) | P8 | ND | H3 |
| 29 | st30 | 21.3.95 | Blood | 14M | Indonesia | X7 | P4 | A9 | H2 |
| 30 | st33 | 23.5.95 | Blood | 6F | Indonesia | X8 | P4 | ND | H2 |
| 31 | st34 | 26.4.95 | Blood | 11F | Indonesia | X9 | P4 | ND | H4 |

| No. | Strain | Date of Isolation | Source | Age | Country | PFGE (*Xba* I) | Ribotyping (*Pst* I) | AFLP (*Eco* RI / *Mse* I) | IS200 (*Hinc* II) |
|---|---|---|---|---|---|---|---|---|---|
| 32 | st35 | 1995 | Blood | ND | India | X3(a) | P3 | ND | H2 |
| 33 | st26 | 1995 | Blood | ND | India | X3 | P3 | A10 | H2 |
| 34 | st28 | 1994 | Stool | ND | Zurich | X5 | P5 | A12 | H2 |
| 35 | st36 | 1994 | Stool | ND | Zurich | X5 | P5 | ND | H2 |
| 36 | st37 | 1994 | Blood | ND | Chile | X10 | P6 | ND | H2 |
| 37 | st38 | 1994 | Blood | ND | Chile | X11 | P6 | ND | H3 |
| 38 | st29 | 1994 | Blood | ND | Chile | X6 | P4 | A16 | H2 |
| 39 | st39 | 1997 | Stool | ND | Pakistan | X3 | P3 | ND | H2 |
| 40 | st40 | 1997 | Stool | ND | Pakistan | X3 | P3 | ND | H5 |
| 41 | st41 | 1997 | Stool | ND | Pakistan | X3 | P3 | ND | H2 |
| 42 | st42 | 1997 | Stool | ND | Pakistan | X13 | P3 | ND | H6 |
| 43 | st43 | 1997 | Stool | ND | Pakistan | X14 | P9 | ND | H2 |
| 44 | st44 | 1997 | Stool | ND | Pakistan | X13(a) | P3 | ND | H2 |

PNG (F) - Strains isolated from Papua New Guinea fatal cases.

PNG (NF) - Strains isolated from Papua New Guinea non-fatal cases.

ND - Not Determined

Different colours symbolizes different profiles for the particular typing method (to simplify comparision between strains).

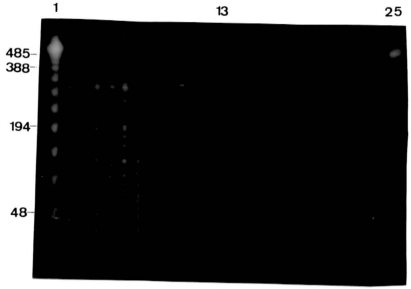



**Fig. 3.1**: Pulsed-field gel electrophoresis of *XbaI* digested *S. typhi* isolates representing all the PFGE profiles.
Lanes 2, 3 and 4 – PNG fatal isolates (st2, st6, st7) representing profiles X1, X1(a), X1(b)
Lanes 5, 6, 7, 8, 9 – PNG non-fatal isolates (st15, 16, 11, 12, 13) representing profiles X1(c), X1(d), X2, X2(a), X2(b)
Lanes 10 and 19 – Indian isolates (st26, st35) representing profiles X3 and X3(a)
Lanes 11, 15, 16 – Malaysian isolates (st27, st32, st31) representing profiles X4, X4 (c), X12
Lane 12 – Zürich isolate (st28) representing profile X5
Lanes 13, 20, 21 – Chilean isolates (st29, st37, st38) representing profiles X6, X10, X11
Lanes 14, 17 and 18 – Indonesian isolates (st30, st33, st34) representing profiles X7, X8, X9
Lanes 22, 23 and 24 – Pakistan isolates (st42, st43, st44) representing profiles X13, X14 and X13(a)
Lanes 1 and 25 – Lambda PFG marker (in kilobases)

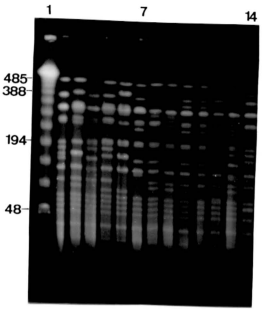



**Fig 3.2**: Reproducibility of *Xba*I restriction patterns of *S. typhi* isolates run on a separate occasion.
Lanes 2, 3 and 4 – Malaysian isolates st27, st32 and st31
Lanes 5, 6 and 7 – Indonesian isolates st30, st33, and st34
Lanes 8 and 9 – Indian isolates st35 and st26
Lanes 10 and 11 – Zürich isolates st28, st36
Lanes 12, 13 and 14 – Chilean isolates st37, st38 and st29
Lane 1 – Lambda PFG marker (in kilobases)

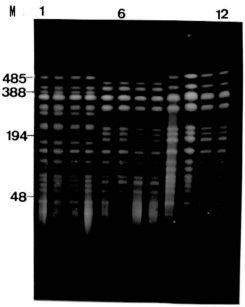



**Fig 3.3**: *XbaI* digested genomic DNA of serially passaged *S. typhi* isolates st28 (lanes 1, 2, 3 and 4), st29 (lanes 5, 6, 7 and 8) and st10 (lanes 9, 10, 11 and 12).
M- molecular weight marker in kilobases.

**A**

Clustering: UPGMA

X4a 100
X4 88.2 100
X5 82.4 88.2 100
X9 80.0 80.0 80.0 100
X3a 70.6 70.6 76.5 68.6 100
X3 72.2 72.2 77.8 70.3 94.4 100
X2 77.8 77.8 83.3 75.7 86.9 94.7 100
X2b 75.7 75.7 81.1 73.7 91.9 92.3 97.4 100
X2a 80.0 80.0 85.7 77.8 91.4 91.9 97.3 94.7 100
X1b 77.8 83.3 77.8 70.3 83.3 84.2 88.5 87.2 86.5 100
X7 81.1 81.1 81.1 73.7 81.1 82.1 92.3 90.0 84.2 97.4 100
X12 77.8 83.3 77.8 75.7 83.3 84.2 88.5 92.3 86.5 94.7 87.2 100
X1 83.3 88.9 83.3 75.7 83.3 84.2 89.5 87.2 86.5 94.7 92.3 89.5 100
X1d 86.5 86.5 86.5 78.9 81.1 87.2 92.3 90.0 89.5 92.3 90.0 87.2 97.4 100
X1a 80.0 85.7 80.0 72.2 80.0 81.1 86.5 84.2 83.3 91.9 89.5 86.5 97.3 94.7 100
X1c 77.8 83.3 77.8 70.3 83.3 84.2 84.2 82.1 81.1 89.5 87.2 84.2 94.7 92.3 91.9 100
X11 77.8 77.8 77.8 75.7 86.9 84.2 89.5 87.2 86.5 89.5 92.3 84.2 89.5 92.3 86.5 84.2 100
X8 70.6 70.6 76.5 74.3 76.5 77.8 88.9 91.9 85.7 83.3 86.5 88.9 83.3 86.5 85.7 77.8 83.3 100
X10 77.8 83.3 77.8 70.3 77.8 73.7 78.9 82.1 81.1 84.2 82.1 84.2 89.5 97.2 86.5 84.2 84.2 77.8 100
X13a 76.5 76.5 82.4 74.3 88.2 83.3 88.9 86.5 91.4 77.8 81.1 77.8 83.3 86.5 80.0 77.8 83.3 76.5 83.3 100
X13 82.4 76.5 82.4 80.0 88.2 83.3 88.9 86.5 91.4 77.8 75.7 77.8 83.3 86.5 80.0 77.8 83.3 76.5 83.3 94.1 100
X14 76.5 76.5 82.4 80.0 82.4 77.8 83.3 81.1 85.7 72.2 75.7 72.2 77.8 81.1 74.3 72.2 77.8 70.6 77.8 88.2 88.2 100
X6 68.8 81.3 68.8 66.7 62.5 64.7 64.7 62.9 60.6 76.5 74.3 76.5 76.5 74.3 78.8 76.5 64.7 68.8 76.5 62.5 62.5 62.5 100

**B**

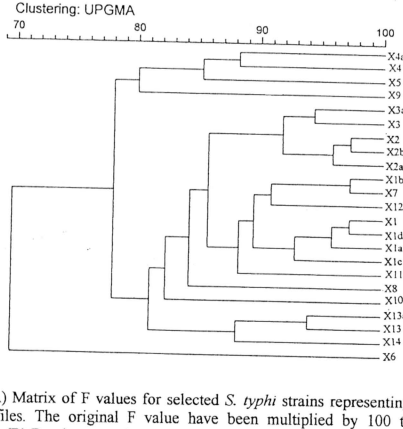



**Fig 3.4**: (A) Matrix of F values for selected *S. typhi* strains representing all the different PFGE profiles. The original F value have been multiplied by 100 to give similarity percentages.(B) Dendrogram shows the cluster analysis of selected strains representing all the different PFGE profiles generated by GelCompar program on the basis of F values and by using the unweighted pair group arithmetic means method (UPGMA).

### 3.3.4 Ribotyping

Genomic DNA's of the 25 Papua New Guinea and 19 geographic isolates were digested with restriction enzyme *Pst*I. The sensitivity of the method is dependent on the restriction enzymes used to digest chromosomal DNA and in this case *Pst*I was chosen based on previous results (Altwegg et al., 1989; Pang et al., 1992a). Electrophoresis of the digested DNA on agarose gels produced too many fragments and the resolution of these fragments was generally poor. By probing the DNA fragments with a 1.5 kb PCR generated fragment of the 16S rRNA gene (Nair et al., 1999) (Fig. 3.5), the number of bands in the fingerprints were greatly reduced thus making it easier to compare restriction patterns (ribotypes) between strains. *Pst*I generated 9 ribotypes which contained 5-6 bands ranging in size from 6 kb to 21.5 kb (Fig 3.6A). Eighty percent of the PNG fatal strains had ribotype pattern P1 and the other 20% had pattern P2. F values between 0.6 and 1.0 showed diversity among the PNG fatal isolates. The PNG non-fatal strains were diverse as well with 3 ribotype patterns (P1 [8 strains], P3 [6 strains] and P6 [1 strain]) and F values between 0.4-1.0. The 3 ribotypes fell into different dendrogram clusters (Fig 3.7B)

Eight ribotypes were generated from the 19 geographic strains (Table 3.2). Limited numbers of ribotype patterns were also observed, as ribotyping is a technique to detect polymorphisms at the ribosomal gene level, while PFGE analyses the entire genome. However, these geographic strains also had ribotype patterns (P1 belongs to a Malaysian strain, P6 to a Chilean strain and P3 to majority of the strains from the Indian subcontinent) similar to that of Papua New Guinea strains. The geographic strains also showed considerable diversity with F values between 0.36-1.0 (Fig. 3.7A). The rather high genetic diversity between strains of various ribotype patterns could be due to the unstable nature of the ribosomal genes in *S. typhi* (Liu and Sanderson, 1996).

Reproducibility of ribotype profiles was also established. Three isolates that generated *PstI* ribotypes P1, P2 and P3 were repeatedly tested on a separate occasion (Fig. 3.6B) and the profiles obtained were identical to that from the first run (Fig. 3.6A).

The discriminatory index of ribotyping for the 44 isolates studied was 0.76, lower than that observed with PFGE.

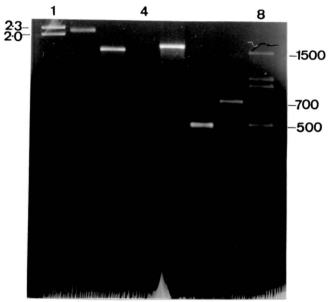



**Fig. 3.5**: Various gene probes used in the study
Lane 2 – DH5/plZ45 plasmid vector containing a 300 bp IS200 sequence
Lane 3 (16S rRNA), Lane 4(the 599 bp *via*B band is very faint), Lane 5 (*gro*EL), Lane 6 (ompC) and Lane 7 (*rfb*S) were all PCR generated
Lane 1 – λ *Hind* III marker (in kilobases)
Lane 8 – 100 bp marker (in base pair)

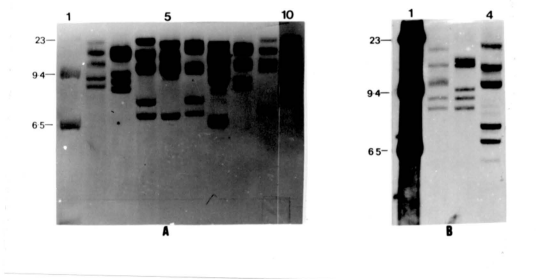
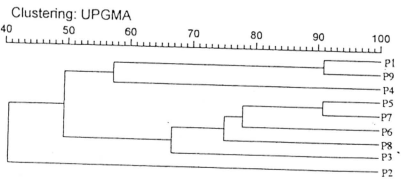



**Fig 3.6**: (A) Ribosomal gene polymorphisms in the 44 *S. typhi* isolates. Genomic Southern blot made with *Pst*I and probed with a 1.5 kb 16S rRNA gene. Lanes 2-10 represent ribotypes P1, P2, P3, P6, P5, P4, P7, P8 and P9 respectively. Lane 1 – λ *Hind* III marker indicated in kilobases. (B) Reproducibility of genomic Southern blot made with *Pst*I and probed with a 1.5 kb 16S rRNA gene. Lanes 2, 3 and 4 represent ribotypes P1, P2 and P3. Lane 1 – λ *Hind* III marker indicated in kilobases.

Clustering: UPGMA

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P1 | 100 | | | | | | | |
| P9 | 90.9 | 100 | | | | | | |
| P4 | 60.0 | 54.5 | 100 | | | | | |
| P5 | 36.4 | 50.0 | 54.5 | 100 | | | | |
| P7 | 40.0 | 54.5 | 40.0 | 90.9 | 100 | | | |
| P6 | 54.5 | 50.0 | 72.7 | 83.3 | 72.7 | 100 | | |
| P8 | 60.0 | 54.5 | 40.0 | 72.7 | 80.0 | 72.7 | 100 | |
| P3 | 40.0 | 54.5 | 40.0 | 72.7 | 80.0 | 54.5 | 60.0 | 100 |
| P2 | 60.0 | 54.5 | 20.0 | 36.4 | 40.0 | 54.5 | 40.0 | 20.0 | 100 |

A




B

**Fig 3.7** (A)Matrix of F values of selected *S. typhi* strains representing all the different ribotypes. F values have been multiplied by 100 to give similarity percentages.
(B)Dendrogram showing the cluster analysis of selected *S. typhi* strains representing all the different ribotypes generated using UPGMA method based on the F values.

### 3.3.5 IS200 typing

The genomic DNAs of the 25 PNG and 19 geographic *S. typhi* clinical isolates were digested with *Hinc*II, and probed with a 2.3 kb plasmid vector DH5/plZ45 containing a 300 bp internal fragment of IS200 (Fig. 3.5). *Hinc*II was used for digestion as it lacked the restriction site within the IS200 sequence (Stanley et al., 1993).

Strains digested with *Hinc*II were found to possess 22-25 bands carried on fragments that ranged in size from 23 kb to below 2 kb. Six fingerprints were identified and they were designated as types H1 to H6 (Fig. 3.8). Type H1 pattern was uniquely observed in all the 25 PNG strains and this pattern was not found in any of the geographic isolates (Table 3.2). The H2 pattern predominated among the geographic isolates, being represented by 14 out of the 19 isolates examined (74%). Two isolates exhibited H3 pattern while H4, H5 and H6 patterns were comprised of a single strain each (Table 3.2). Close genetic diversity (F=0.82-1.0) was observed among all the isolates studied by this typing method (Fig 3.10). The lack of diversity could be due to the slow transposition rate of the IS200 element in nature among bacterial strains.

The stability of the IS200 fingerprints were determined by comparing the fingerprints obtained after a single isolate had undergone four serial passages. Isolated genomic DNA from each passage was digested with *Hinc*II and probed with the IS200 element. The IS200 fingerprints (H1 patterns in this case) were found to be identical throughout (Fig. 3.9) and this demonstrated the stable nature of the IS200 element within the period of study. Reproducibility was also confirmed as the H1 pattern (Fig. 3.9) was found to be identical to that of the first run (Fig. 3.8).

The discriminatory index of IS200 typing for the 44 isolates studied was 0.56.

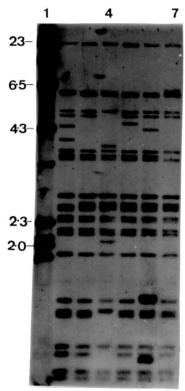



**Fig. 3.8**: IS200 profiles in the 44 *S. typhi* isolates studied. A genomic Southern blot was made with *Hinc*II. Hybridization was carried out with a 2.3 kb DH5/plZ45 plasmid vector containing a 300 bp IS200 internal probe. Lanes 2 to 7 represent IS200 types H1, H2, H3, H4, H5 and H6 respectively. Lane 1 – λ *Hind* III marker (in kilobases). Extra bands were seen in the λ *Hind*III marker due to partial degradation of the marker.

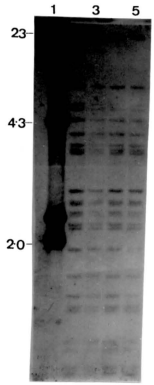
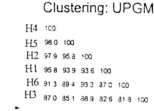
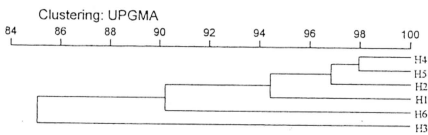



**Fig. 3.9**: Reproducibility and stability of IS200 profiles in *S. typhi*. *Hinc*II digested genomic DNA of serially passaged *S. typhi* isolate st4 representing type H1 was probed with the IS200 fragment (lanes 2,3, 4 and 5).
Lane 1 – λ *Hind*III marker (in kilobases)




A




B

**Fig. 3.10 :** (A)Matrix of F values of selected *S. typhi* strains representing all the different IS200 types. F values have been multiplied by 100 to give similarity percentages. (B)Dendrogram showing the cluster analysis of selected *S. typhi* representing all the different IS200 types generated using the UPGMA method based on the F value.

### 3.3.6 PCR-ribotyping

PCR amplification of the spacer region between the 16S and 23S rRNA genetic loci were carried out for the 44 *S. typhi* isolates. PCR-ribotypes consisted of patterns comprising of 3 major bands with the sizes of bands varying from approximately 700 to 1100 bp (Fig. 3.11).

Amplifications of the spacer region only produced a single profile for all the 44 isolates (Table 3.3). Therefore, PCR-ribotyping was not discriminative even though the typeability and reproducibility (data not shown) was 100%.

### 3.3.7 PCR-RFLP

The 1.5 kb 16S rRNA gene (Weisburg et al., 1991), 16S-23S rRNA intergenic spacer region (Dasen et al., 1994), 599 bp fragment of the *via*B gene (Hashimoto et al., 1995), the 1.6 kb *gro*EL gene (Linder and Hayers, 1994), a 500 bp region of the *omp*C gene (S. Geetha, personal communications), and 700 bp region of the *rfb*S gene (Luk et al., 1997) were amplified as previously described. PCR amplification of all the genes were obtained for the 44 isolates studied, confirming the well conserved nature of the primers.

The published 16S rRNA, 16S-23S rRNA, *via*B, *gro*EL, *omp*C and *rfb*s gene sequences were analyzed using the DNASIS software programme (Hitachi, Brisbane, CA, U.S.A.) to search for useful restriction enzyme cleavage sites. Based on this programme, *Xma*I was chosen to restrict the 16S rRNA gene, *Hae*III was used to restrict the 16S-23S rRNA spacer region and *gro*EL gene independently, *via*B and *rfb*S amplicons were digested independently with *Sau*3A and the *omp*C amplicon digested with *Kpn*I.

There was no enzyme found capable of differentiating any of the genes studied as only a single restriction profile was generated for each gene among all the 44 *S. typhi*

isolates tested (Table 3.3). Thus, this typing approach was not useful for discriminating *S. typhi* isolates.

Fig. 3.12(A) shows the single restriction profile of the 16S rRNA gene after digestion with *Xma*I. Three DNA bands were produced, ranging in size from approximately 200 bp to 800 bp. Faint fragments seen on the gel (perhaps due to incomplete digestion) are related to overloading and are not considered in differentiating profiles.

Fig. 3.12(B) shows the single restriction profile of the 16S-23S rRNA intergenic spacer region after digestion with *Hae*III. Four fragments ranging from 250 bp to 620 bp were produced.

Fig. 3.12(C) shows the single restriction profile of the *via*B gene. Two bands were produced in the size range of 240 bp to 400 bp. The extra fragments on the gel could be due to incomplete digestion or gel overloading.

Fig. 3.12(D) shows the single restriction profile of the *gro*EL gene after digestion with *Hae*III. Five bands were produced ranging from 150 bp to 650 bp.

Fig. 3.12(E) shows the single *Kpn*I restriction profile of the *omp*C gene. Two bands ranging from 150 bp to 350 bp were generated.

Fig. 3.12(F) shows the single restriction profile of the *rfb*S gene after digestion with *Sau*3A. Three bands were produced ranging from 100 bp to 400 bp. The extra bands on the gel could be due to incomplete digestion or gel overloading.

**Table 3.3 :** PCR-RFLP and PCR-ribotyping characterization of *S. typhi* strains.

| No. | Strain | Date of Isolation | Source | Age | Country | PCR-RFLP | | | | | | PCR-Ribotyping (16S-23S rRNA) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 16S rRNA (*Xma* 1) | 16S-23S rRNA (*Hae* 111) | *groEL* (*Hae* 111) | *ompC* (*Kpn* 1) | *rfbS* (*Sau* 3A) | *viaB* (*Sau* 3A) | |
| 1 | st1 | 16.2.94 | Stool | 26M | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | st8 | 16.2.94 | Blood | 26M | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | s4 | 23.2.94 | Blood | 50F | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 4 | st10 | 23.2.94 | Stool | 50F | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | st5 | 16.3.92 | Stool | 26F | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | st9 | 16.3.92 | Blood | 26F | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7 | st2 | 16.11.93 | Stool | 50F | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 8 | st3 | 13.2.94 | Stool | 12M | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | st6 | 24.5.93 | Marrow | 22M | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 10 | st7 | 18.10.93 | Blood | 48F | PNG (F) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 11 | st11 | 21.2.94 | Stool | 30M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 12 | st22 | 21.2.94 | Blood | 30M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 13 | st12 | 25.3.94 | Stool | 14F | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 14 | st13 | 25.3.94 | Blood | 14F | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 15 | st17 | 6.3.94 | Stool | 32F | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 16 | st18 | 6.3.94 | Blood | 32F | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 17 | st14 | 14.2.94 | Blood | 9M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 18 | st15 | 13.4.94 | Blood | 14F | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 19 | st16 | 15.11.93 | Blood | 3M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 20 | st19 | 8.3.93 | Blood | 21M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 21 | st20 | 12.5.93 | Blood | 14M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 22 | st21 | 10.3.94 | Stool | 17F | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 23 | st23 | 17.1.94 | Blood | 1M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 24 | st24 | 22.3.94 | Blood | 13M | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 25 | st25 | 29.3.94 | Stool | 5F | PNG (NF) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 26 | st27 | 1990 | Blood | ND | Malaysia | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 27 | st31 | 1994 | Blood | ND | Malaysia | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 28 | st32 | 1987 | Blood | 22M | Malaysia | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 29 | st30 | 21.3.95 | Blood | 14M | Indonesia | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 30 | st33 | 23.5.95 | Blood | 6F | Indonesia | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 31 | st34 | 26.4.95 | Blood | 11F | Indonesia | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

| No. | Strain | Date of Isolation | Source | Age | Country | PCR-RFLP | | | | | | PCR-Ribotyping (16S-23S rRNA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 16S rRNA (*Xma* 1) | 16S-23S rRNA (*Hae* 111) | *groEL* (*Hae* 111) | *ompC* (*Kpn* 1) | *rfbS* (*Sau* 3A) | *viaB* (*Sau* 3A) | |
| 32 | st35 | 1995 | Blood | ND | India | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 33 | st26 | 1995 | Blood | ND | India | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 34 | st28 | 1994 | Stool | ND | Zurich | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 35 | st36 | 1994 | Stool | ND | Zurich | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 36 | st37 | 1994 | Blood | ND | Chile | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 37 | st38 | 1994 | Blood | ND | Chile | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 38 | st29 | 1994 | Blood | ND | Chile | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 39 | st39 | 1997 | Stool | ND | Pakistan | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 40 | st40 | 1997 | Stool | ND | Pakistan | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 41 | st41 | 1997 | Stool | ND | Pakistan | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 42 | st42 | 1997 | Stool | ND | Pakistan | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 43 | st43 | 1997 | Stool | ND | Pakistan | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 44 | st44 | 1997 | Stool | ND | Pakistan | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

PNG (F) - Strains isolated from Papua New Guinea fatal cases.

PNG (NF) - Strains isolated from Papua New Guinea non-fatal cases.

ND - Not Determined

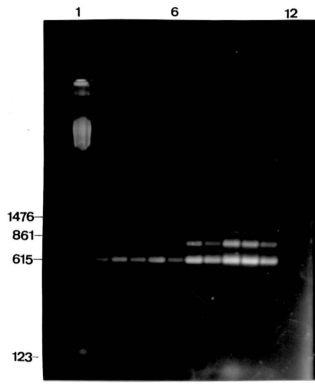



**Fig. 3.11**: PCR-ribotyping analysis of the 44 *S. typhi* isolates. Lanes 2 (st4), 3(st8), 4(st11), 5(st12), 6(st27), 7(st30), 8(st26), 9(st28), 10(st37) and 11(st39) represent the single PCR-ribotype profile produced. Lane 12 – negative control (blank). Lane 1 – 123 bp DNA standard ladder (in base pair)

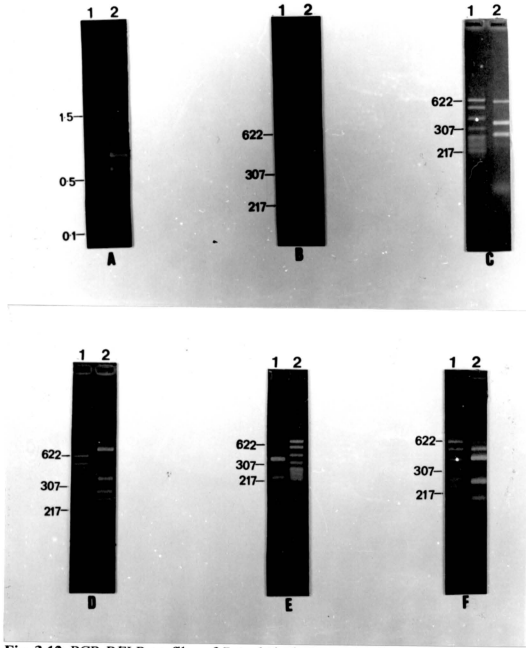



**Fig. 3.12**: PCR-RFLP profiles of *S. typhi* isolates.
(A) The single *Xma*I restriction pattern of the 16S rRNA gene
Lane 1: 100 bp marker indicated in kilobases, Lane 2: RFLP profile
(B) The single *Hae*III restriction pattern of the 16S-23S intergenic spacer region
Lane 1: NEB marker II indicated in base pair, Lane 2: RFLP profile
(C) The single *Sau*3A restriction pattern of the *via*B gene
Lane 1: NEB marker II indicated in base pair, Lane 2: RFLP profile
(D) The single *Hae*III restriction pattern of the *gro*EL gene
Lane 1: NEB marker II indicated in base pair, Lane 2: RFLP profile
(E) The single *Kpn*I restriction pattern of the *omp*C gene
Lane 1: RFLP profile, Lane 2: NEB marker II indicated in base pair
(F) The single *Sau*3A restriction pattern of the *rfb*S gene
Lane 1: NEB marker II indicated in base pair, Lane 2: RFLP profile

### 3.3.8 AFLP

Genomic DNA's from the 25 Papua New Guinea (PNG) *S. typhi* strains and 5 representatives of *S. typhi* geographic strains from Malaysia, India, Switzerland (Zürich), Indonesia and Chile were analysed with 18 AFLP primer combinations (Table 3.4). A total of 780 fragments were scored. This represents about 2.5% of the *S. typhi* genome, assuming an average fragment length of 150bp. Fifty-eight fragments (7.4%) were polymorphic.

The 25 PNG strains isolated from fatal and non-fatal cases exhibited 11 AFLP profiles. Profile A4 predominated with 8 out of the 25 isolates (28%), profile A15 was seen in 2 isolates and profiles A1, A3, A5, A6, A7, A8, A13 and A14 in a single isolate each (Table 3.2). The 5 geographic strains produced 5 different AFLP profiles as seen in Table 3.2.

A dendrogram was generated using the PAUP*4 program to ascertain the genetic relationships among the strains studied. Based on the cluster analysis (Fig. 3.13) it was shown that the geographic strains are genetically diverse. The PNG strains from fatal and non-fatal cases also fell into different but closely related clusters in the dendogram indicating slight genetic diversity among these strains (Fig. 3.13). Like PFGE, AFLP analysis provides a way of examining DNA segments distributed over the entire genome of an organism and thus explained the genetic diversity observed.

AFLP was also used to differentiate PNG *S. typhi* strains recovered from different body sites of the same patient. It was found that strains isolated from blood and feces from the same patient had different AFLP profiles (Table 3.5). The results for this typing method was compared to other established methods such as PFGE and ribotyping (Table 3.5).

Discrimination index analysis of the 30 strains analyzed by AFLP, PFGE and ribotyping showed that AFLP with a DI of 0.88 was more discriminative than PFGE (DI=0.74) and ribotyping (DI=0.63).

**Table 3.4**: AFLP primer combination used in this study and the number of fragments.

| Primer combination | *EcoRI* | *MseI* | # Fragments | Polymorphisms |
|---|---|---|---|---|
| e0mct | | CT | 34 | 3 |
| e0mg | | G | 82 | 10 |
| eagmg | AG | G | 8 | 1 |
| eatm0 | AT | | 51 | 6 |
| ecm0 | C | | 67 | 7 |
| egmo | G | | 126 | 2 |
| egma | G | A | 29 | 0 |
| egmg | G | G | 16 | 0 |
| ecmg | C | G | 40 | 6 |
| eacmg | AC | G | 23 | 2 |
| etm0 | T | | 66 | 4 |
| eama | A | A | 58 | 8 |
| eamcc | A | CC | 29 | 0 |
| eamg | A | G | 31 | 3 |
| ecma | C | A | 41 | 4 |
| ecmcc | C | CC | 28 | 0 |
| egma | G | A | 34 | 2 |
| egmcc | G | CC | 17 | 0 |

**Table 3.5 :** Molecular characteristics of *S. typhi* strains isolated from different body sites.

| Strain | Isolate Designation | Date of Isolation | Source | Age | Country | Classification | PFGE (*Xba* I) | Ribotyping (*Pst* I) | AFLP (*Eco* RI / *Mse* I) |
|---|---|---|---|---|---|---|---|---|---|
| st1 | PNG 4 | 16.2.94 | Stool | 26M | PNG (F) | Patient A | X1 | P1 | A1 |
| st8 | PNG 45 | 16.2.94 | Blood | 26M | PNG (F) | Patient A | X1 | P1 | A4 |
| s4 | PNG 8 | 23.2.94 | Blood | 50F | PNG (F) | Patient B | X1 | P2 | A5 |
| st10 | PNG 57 | 23.2.94 | Stool | 50F | PNG (F) | Patient B | X1 | P1 | A7 |
| st5 | PNG 17 | 16.3.92 | Stool | 26F | PNG (F) | Patient C | X1 | P1 | A4 |
| st9 | PNG 55 | 16.3.92 | Blood | 26F | PNG (F) | Patient C | X1 | P1 | A2 |
| st11 | PNG 1 | 21.2.94 | Stool | 30M | PNG (NF) | Patient D | X2 | P3 | A14 |
| st22 | PNG 49 | 21.2.94 | Blood | 30M | PNG (NF) | Patient D | X1 | P1 | A4 |
| st12 | PNG 2 | 25.3.94 | Stool | 14F | PNG (NF) | Patient E | X2(a) | P3 | A4 |
| st13 | PNG 3 | 25.3.94 | Blood | 14F | PNG (NF) | Patient E | X2(b) | P3 | |
| st17 | PNG 40 | 6.3.94 | Stool | 32F | PNG (NF) | Patient F | X1(c) | P1 | A2 |
| st18 | PNG 42 | 6.3.94 | Blood | 32F | PNG (NF) | Patient F | X1 | P1 | A4 |

PNG (F) - Strains isolated from Papua New Guinea fatal cases.

PNG (NF) - Strains isolated from Papua New Guinea non-fatal cases.

Different colours symbolizes different profiles for the particular typing method (to simplify comparision between strains).

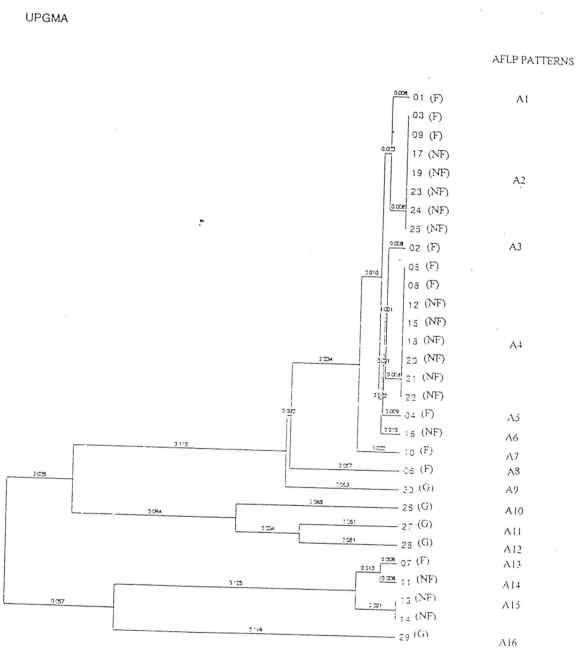



**Fig. 3.13**: Dendrogram representing the genetic relationship among the 25 Papua New Guinea (PNG) *S. typhi* strains and 5 geographic strains based on AFLP patterns using PAUP*4.0. Strain number corresponds to that of Table 3.2. F denotes PNG fatal strains; NF symbolizes PNG non-fatal strains while G stands for geographic strains.

### 3.4 Discussion

Differentiation of strains involved in infections with *S. typhi* depend on accurate epidemiological markers. The discriminatory power of typing methods used in the epidemiological investigations are critical to conclusions drawn regarding infections caused by *S. typhi* strains. It is important to establish whether strains involved in outbreaks are homogeneous, as it will help in the understanding of epidemiological relationships of strains to patients, carriers and possible source of infection. The application of precise typing method(s) to determine the genetic relatedness of *S. typhi* isolates is also critical in identifying outbreaks and designing preventive measures such as vaccination programs.

The most common method used to date for the demonstration of epidemiologic associations among isolates of *S. typhi* has been phage typing. However, this typing method has certain limitations. It is technically more demanding and is subject to considerable experimental as well as biological variability (Hickman-Brenner and Farmer, 1983). It may also be of limited value if a common phage type or a limited number of phage types represent the majority of the strains isolated over a wide geographic locale. Acquisition or loss of lysogenic phages could result in changes in lysis patterns. The need to maintain stocks of biologically active phages and control strains may limit its use to reference laboratories (Edelman and Levine, 1986).

In view of the limitations of phage typing, new molecular typing methods have been developed for characterization of *S. typhi*. Ribotyping based on rRNA gene restriction patterns was shown by several groups (Altwegg et al., 1989; Pang et al., 1992a) to be effective in subtyping *S. typhi* isolates that either belonged to common phage types or were untypeable. However, ribotyping is more costly, technically more complex and the discrimination achieved is largely dependant on the choice and/or the number of restriction

enzymes used. Moreover, several studies have indicated that ribotyping is less discriminatory than PFGE analysis (Poh et al., 1992a ; Prevost et al., 1992).

Reeves et al. (1989) showed that MLEE analysis could not differentiate *S. typhi* strains represented by different phage types and concluded that a single clone was responsible for *S. typhi* infections despite the widespread geographical origins of the strains. Selander et al. (1990) concluded the presence of only 2 major clones and 5 subclones among 334 *S. typhi* isolates investigated. MLEE analysis is useful in providing quantitative data regarding the population genetics of pathogens species but has relatively limited application to epidemiological studies (Maslow et al., 1993).

Although plasmid analysis is valuable in epidemiologic investigations and may be the most discriminatory method of subtyping for some bacteria, they are not universally applicable as seen with *S. typhi*. Many *S. typhi* isolates do not harbour plasmids and even when plasmids are present, there is often little heterogeneity is plasmid profiles generated, thus rendering them useless for subtyping (Murray et al., 1985; Maher et al., 1986). Another drawback is that plasmids are gained or lost during the evolutionary process and thus may not be satisfactory for long-term follow up studies.

Genetic fingerprinting by *Salmonella* specific insertion sequence IS200 has been used of late for the molecular typing of *S. typhi*. This typing method was found to less discriminatory when compared to phage typing as shown by Threlfall et al. (1994). They also concluded that this typing method was not sufficient in the primary differentiation of this organism. On the other hand, IS200 typing was able to differentiate drug-sensitive and drug-resistant *S. typhi* strains of Vi-phage types E1 and M1 isolated from patients infected in India and Pakistan (Threlfall et al., 1993). The drawback of this typing method is similar to that of ribotyping as it is costly, laborious and technically more complex.

PFGE, which involves the separation of large DNA fragments after digestion with infrequently cutting restriction enzymes has provided a new approach in subtyping *S. typhi* (Thong et al., 1994; Nair et al., 1994). This approach has also been used successfully for epidemiological investigation of outbreaks caused by pathogens such as *Shigella* spp. (Brian et al., 1993) and methicillin-resistant *Staphylococcus aureus* (Struelens et al., 1992). Like most typing methods, PFGE has its disadvantages. It requires long and tedious procedures for isolating and restricting the genomic DNA by the agarose plug method. Large quantities of enzyme and reagents for DNA isolation and restriction are needed as well. Technical complexity such as the need for experimentally optimizing the electrophoretic conditions may also be encountered. However, new developments in instrumentation as seen in the newer PFGE systems (i.e. CHEF-DR III apparatus, Biorad, U.S.A.) and simplification of protocols (Thong et al., 1996a) have rendered PFGE useful for subtyping microorganisms.

To date very few published epidemiological studies on *S. typhi* have been carried out using PCR methodologies such as PCR-ribotyping and PCR-RFLP, even though these techniques have been successfully used for the typing of *S. typhimurium* and other *Salmonella* serovars (Kilger and Grimont, 1993; Nastasi and Mammina, 1995).

### 3.4.1 Assessment of genetic diversity by PFGE, ribotyping, IS200 typing, PCR-ribotyping and PCR-RFLP

The main purpose of this study was to assess the genetic diversity at the genome level of 25 Papua New Guinea (PNG) *S. typhi* isolates and 19 other *S. typhi* strains isolated from different geographic locales by using various molecular typing methods.

Recently, several epidemiological studies to characterize *S. typhi* have been carried out using molecular typing methods such as PFGE, ribotyping and IS200 typing. In this

study, the 3 methods have been tested in parallel using the same method of DNA preparation as a standardization precaution.

IS200 is a particularly useful marker of chromosomal genotype in many *Salmonella* serovars as the element seems to have little if any affinity for extrachromosomal sites in this genus (Stanley and Saunders, 1996). For the interpretation of IS200 profiles, strains with a one band difference were considered to be different strains as the number of bands in Southern blots of IS200 reflects the number of copies of this genetic element in the genomes of the strains studied. IS200 elements are very stable genetic entities and rarely undergo transposition or horizontal gene transfer; hence, they serve as good indicators of clonal lineages (Bisercic and Ochman, 1993). *Hinc*II digested genomic DNA of the 25 Papua New Guinea isolates established only one IS200 fingerprint (Table 3.2). The 19 geographic isolates were represented by 5 IS200 fingerprints which were different from the single fingerprint of the PNG isolates. We consider these IS200 fingerprints to represent clonal lineages of *S. typhi* as the fingerprints were highly stable and did not alter on repeated testing. The IS200 fingerprints indicated the clonal nature of the PNG strains and the multiclonal nature of the geographic strains. Though IS200 profiles were useful for establishing clonal lineages in *S. typhi*, our data and previous findings of Threlfall et al. (1994) have shown IS200 profiling to lack the discriminatory power for the *S. typhi* serovar as a majority of the strains were represented by a very few clones. The DI of IS200 typing for the 44 isolates analyzed was 0.56. This index indicates that there is only a 56% probability that 2 unrelated strains from the test population will be characterized as belonging to 2 different groups.

A higher level of discriminatory power than that seen with IS200 profiling was achieved by ribotyping (DI=0.76), which detects the number of copies of the 16S rRNA

genes in the genome. In contrast to the IS200 fingerprints which generated one profile for the 25 PNG isolates, four ribotypes were produced by ribotyping (Table 3.2). Ribotype P1 and P2, which represented the majority of the ribotypes (19 out of the 25 PNG isolates), were solely found in the PNG isolates (except in one instance where a Malaysian strain had a P1 ribotype), while ribotype P3 and P6 were shared with some of the geographic isolates. As rRNA genes are known to be present in lower copy numbers than IS200 elements, the greater variations of profiles observed could be explained by the unstable nature of the ribosomal genes in *S. typhi* and the genetic recombination occuring between the *rrn* genes (Liu and Sanderson., 1996). Extreme caution should be exercised in the analysis of ribotyping results since a single genetic change can lend to changes in 2 or 3 bands. Due of the low number of bands generated in ribotyping, this could affect 50% of the pattern, and closely or distantly related strains would either fall apart or fall closer together in cluster analysis. However, for the geographic isolates, distinctive ribotype profiles within the same IS200 profile and vice versa were observed.

The discrepancies observed between PFGE fingerprints profiles and profiles established by both IS200 and ribotype analysis could possibly be explained by the different chromosomal parts examined by each of the 3 methods which have not evolved at the same rate. Both IS200 and ribotype analysis only reflect variability in certain parts of the genome. IS200 profiles depend on the restriction fragment length polymorphisms at the insertion sites and from the DNA rearrangements following the transposition of the IS element into the genome, while ribotypes are dependent on genetic rearrangements between rrn genes and polymorphisms at the restriction cut site of the enzymes used (Ng et al., 1999). PFGE analysis on the other hand provides accurate overall representation of the genome (Maslow et al., 1993). Changes in the chromosome structure of strains either

through mutations which affect the restriction cleavage sites or genome rearrangements can be readily detected by PFGE. If these genetic events were occurring in chromosomal regions not carrying either IS200 elements or rRNA genes, differences between strains could not be established by methods involving both IS200 and rRNA hybridizations. This could explain the larger number of clonal lineages established by PFGE when compared to both IS200 and ribotype profiling. PFGE analysis with 100% typeability and reproducibility could provide the best means of discriminating the 44 *S. typhi* strains with a DI of 0.88.

In this study two closely related clones with minor clonal variants were observed among the 25 PNG strains indicating a common ancestry. These 2 clones and their variants were not observed in the 19 geographic strains which had diverse PFGE profiles.

The picture emerging from comparative studies of genome variation as measured by IS200 typing, rRNA gene polymorphisms and PFGE analysis indicates that the PNG strains are derived from the same clone or have the same genetic lineage but have undergone some changes (genetic events) which accounts for the observed variations in PFGE profiles and ribotypes. There was a temporal pattern to these changes as strains first isolated in 1992 had only X1 PFGE profiles and P1 ribotypes as compared to 1994 isolates that had a number of X2, X2(a), X2(b), X1, X1(c) PFGE profiles and P3 ribotype. IS200 has been shown not to transpose easily and due to this, analysis by IS200 profiling cannot follow up on temporal changes in the genome. PFGE analysis monitors the changes occurring in the genome readily and allows genetic relationships between strains to be easily determined. This approach contributes to a greater understanding of the evolutionary and epidemiological relationships of *S. typhi* strains.

There has been a lack of epidemiological studies of *S. typhi* using PCR methods. Due to this reason, PCR ribotyping and PCR-RFLP were carried out in this study in order to determine the capability of each of these typing methods in discriminating isolates within this serovar.

PCR-ribotyping utilizes the structure of operons within which the genes coding for rRNA are located. The rRNA operons usually exist in multiple copies throughout the genome. They consist of a promoter region followed by the 16S rRNA gene, an intergenic spacer region, the 23S rRNA gene, another spacer region and then the 5S rRNA gene. As the intergenic spacer region varies in length, primers complementary to the highly conserved region flanking the intergenic spacers may be used for PCR amplification to detect length polymorphisms in these regions. These polymorphisms are shown to be stable and reproducible over a period of time (Kerr, 1993).

PCR-ribotyping has been used successfully for typing organisms such as *Burkholderia cepacia* (Dansen et al., 1994) and recently for *Salmonella typhimurium* (Nastasi et al., 1995). In this study, it was found to be of limited use as only one profile was generated among the 44 *S. typhi* isolates tested when primers that amplified the 16S-23S rRNA intergenic spacer region was used. The low level of discrimination may be due to the well-conserved nature of the region involved, where interspecific variability is low and there is a lack of polymorphisms in the 16S-23S rRNA spacer region. Amplification of different regions of the rRNA operons such as the 16S rRNA gene, 23S rRNA gene, 5S rRNA gene or even the 5S-23S rRNA intergenic spacer region may increase the discriminatory level of this typing method. A clear advantage of PCR-ribotyping is that highly conserved sequences in eubacteria are amplified and thus enables the use of a single primer set to type many different species.

PCR-RFLP was developed based on the dissatisfaction with the limitations of conventional RFLP typing methods, especially when large amounts of purified DNA are needed and difficulties in the interpretation of results are encountered (Kerr, 1993). This typing method involves the amplification of a target sequence which in turn is cut with a restriction enzyme or several enzymes. Resolution of the fragments by agarose gel electrophoresis permits comparison of different isolates.

In this study the 1.5 kb 16S rRNA gene, the 1.6 kb groEL gene, the 599 bp fragment of the *via*B gene, a 500 bp region of the *omp*C gene, 700 bp segment of the *rfb*S gene and the 16S-23S rRNA intergenic spacer region were digested independently with enzymes specific for each gene (Table 3.3). Resolution of the fragments by electrophoresis produced only one PCR-RFLP profile for each gene (Table 3.3). The discriminatory power of this method for typing the 44 *S. typhi* isolates studied was low and one factor that may have contributed to the low discrimination is that the target sequence for amplification (especially for the *omp*C, *via*B and *rfb*S gene) was too short (between 500-700 bp). Ideally, the target sequences to be amplified should be 1-2 kb in length (Swaminathan and Matar, 1993). This was carried out for the 16S rRNA gene and *gro*EL gene, but in this case the lack of discrimination could be due to the insufficient polymorphisms seen within the target sequence to allow identification of subtypes.

The problem of low discriminatory power may be overcome by testing more restriction enzymes or by choosing target sequences that are longer. Kilger and Grimont (1993) amplified and restricted a 1,240 bp sequence of the *fli*C gene which is involved in the phase 1 flagellar antigen of Salmoenlla serovars. Discriminatory profiles were produced but the only drawback is that the *fli*C gene only exists in motile strains of *S. typhi* which limit its general use. Even though PCR-RFLP may have potential as an efficient typing

tool, certain disadvantages such as the need for primer knowledge of the targeted nucleotide sequence to be amplified and the fact that the PCR-RFLP primers are genus/species specific may limit its use.

Earlier studies utilizing multilocus enzyme electrophoresis concluded that *S. typhi* represents a single clone that has shown minimal intraspecies divergence in its spread to different parts of the world (Reeves et al., 1989; Selander et al., 1990). Contradictory to these findings, recent PFGE analysis of *S. typhi* isolates from different parts of South East Asia indicated that the extent of genetic diversity among strains of *S. typhi* is much higher than previously thought (Nair et al., 1994; Thong et al., 1995). Ribotyping, which is also, another established typing method showed considerable genetic diversity among *S. typhi* strains (Altwegg et al., 1989; Pang et al., 1992a).

The present study has extended this observation of significant diversity to human isolates of *S. typhi* from various geographic locales not only by PFGE analysis (F=0.62 – 0.94), but as well as ribotyping (F=0.36 – 1.0) and IS200 typing (F=0.82 – 1.0). Papua New Guinea has the highest incidence of typhoid fever in the world with more than 1,200 cases per 100,000 populations being reported annually (Pang et al., 1995). Interestingly, the genetic diversity observed among the PNG *S. typhi* strains in this study by various typing methods was more limited compared to the other geographic isolates. These results correlated with previous findings by Thong et al. (1996b) who used only PFGE to detect diversity of these isolates. The F values observed with the PNG isolates for PFGE (F=0.85 – 1.0), ribotyping (F=0.4 – 1.0) and IS200 (F=1.0) are considerably higher than those observed with the geographic isolates. The limited diversity observed among the PNG isolates could be due to the fact that typhoid fever is a relatively new public health problem, and the disease was rarely seen before 1985 (Passey et al., 1985). Typhoid fever

is reported to have spread only recently in endemic form in the highland regions and in some of the larger costal towns. The population is rather mobile due to improved infrastructure (a highway) and thus making it unlikely that isolation and lack of mobility are the causes of the limited diversity seen among the isolates (Thong, 1996d). Results from this present study and the notion that all *S. typhi* strains belonged to phage type D2 and were sensitive to the same antibiotics, shows that the *S. typhi* strains that were circulating in PNG from 1992-1994 were most likely derived from a single introduced strain (clone), and that these strains have now spread with some limited genetic variation due to the lack of selective pressure to develop such variations. The study also provides further support for the hypothesis that multiple clones of *S. typhi* coexist independently and simultaneously in regions of the world where it is endemic in contrast to the previous notion that all *S. typhi* strains belong to a single clone worldwide (Reeves et al., 1989; Selander et al., 1990).

### 3.4.2 Molecular characterization by AFLP

AFLP allows the differentiation of related bacterial species through comparison of complex banding patterns produced by PCR-amplified restriction fragments. In this study, *S. typhi* genomic DNA was digested using two restriction enzymes (*Mse*I and *Eco*RI) and adaptor DNA sequences were ligated to the fragments prior to PCR. Although AFLP has been used in the genotyping of various species of bacteria (Janssen et al., 1996; Aarts et al., 1998; Boumedine and Rodolakis, 1998; Gibson et al., 1998; Savelkoul et al., 1999), including *Salmonella* serotypes of poultry origin (Aarts et al., 1998), there have been no reported studies with *S. typhi* a strictly human pathogen. In the study of Aarts et al. (1998), the 62 different *Salmonella* serotypes had unique profiles by AFLP fingerprinting and

different strains previously identified as identical could be distinguished. It was thus of great interest to evaluate the usefulness of AFLP in the genotyping of the 25 PNG *S. typhi* strains isolated from fatal and non-fatal typhoid cases which have shown limited genomic diversity by other typing methods. Five geographic isolates form Malaysia, Indonesia, Chile, India and Zürich which showed considerable diversity by PFGE, ribotyping and IS200 typing were also tested to assess the discriminatory power of AFLP.

Like PFGE, AFLP analysis provides a way of examining DNA segments distributed over the entire genome of an organism. AFLP is able to detect mutations at the restriction sites of the frequently cutting enzymes used. These genetic markers change more rapidly than the markers (inversions, deletions, insertions and polymorphisms at restriction cut sites of rare cutting enzymes) assessed by PFGE in evolution. The rapid changes in AFLP genetic markers makes AFLP suitable to monitor changes occurring in the genome readily and thus allows the determination of genetic relationships between strains. This explains why the overall results in the present study have shown that AFLP (DI=0.88) was able to clearly discriminate and differentiate geographically diverse strains of *S. typhi* as well as closely related PNG strains which were identical or very similar by PFGE (DI=0.74) and ribotyping (DI=0.63). Based on the AFLP dendrogram (Fig. 3.14) the PNG fatal and non-fatal strains fell into closely related clusters indicating that these strains were still highly related (clonal).

However it was noted that AFLP patterns A2, A4 and A15 had strains which could be differentiated by PFGE and ribotyping respectively. Discrepancies like these indicates that the strains in questions have no mutations/polymorphisms within the *Mse*I and *Eco*RI restriction sites or no mutations in the sequence adjacent to the restriction sites which are complementary to the selective primer extensions as compared to the polymorphisms

within the *Xba*I sites (detected by PFGE) or rearrangements of the *rrn* genes (detected by ribotyping).

Beyond their direct application in differentiating and discriminating between strains for epidemiological purposes, molecular techniques have also contributed a great deal to phylogenetic and evolutionary studies. In addition, these approaches have also improved our understanding of the biology of pathogens and also their pathogenesis and virulence. For example, the proposal that significant genetic diversity exists among *S. typhi* strains globally (Pang, 1998), which may be related to the fact that their genome is highly plastic in nature (Liu and Sanderson, 1996), was based largely on studies using approaches such as PFGE. Previous studies by Thong et al. (1996b) have shown a correlation between the molecular profile of *S. typhi* as defined by PFGE and its ability to cause fatal disease among typhoid fever cases in Papua New Guinea. The authors concluded from this work that a possible association exists between genotype, as assessed by PFGE, and the capability to cause fatal illness. The advent of AFLP, a potentially more discriminative technique has allowed us to analyze these strains in more detail in the present study. AFLP analysis was able to further subtype these PNG *S. typhi* strains and this finding is in agreement with other studies which have also demonstrated the superior discriminatory power of AFLP towards the differentiation of closely related bacterial strains belonging to the same species or even subspecies/biovar (Janssen et al., 1996; Aarts et al., 1998; Boumedine and Rodolakis, 1998; Gibson et al., 1998).

AFLP data in this study suggested no correlation between genotype and virulence unlike a recent study with *C. psittaci*, where AFLP permitted differentiation among highly related strains in relation to host origin and clinical syndromes (Boumedine and Rodolakis, 1998). Our results show that strains with identical AFLP profiles were present among those

which came from fatal and non-fatal cases and that no particular profile was uniquely associated with disease outcome. Based on these findings, we assume that no particular *S. typhi* genotype assessed by either AFLP or PFGE analysis seems to be particularly more virulent as the ability to cause fatal disease is not associated with the banding patterns produced by either technique. Therefore, other factors (including host factors), which are as yet unknown, may be involved in the causation of fatal disease.

The results of this present study also indicates that *S. typhi* strains isolated from blood and feces from the same patient showed genetic changes that occurred *in vivo* during the course of infection. The two *S. typhi* isolates from blood and feces of the same patient produced different AFLP profiles. This was observed for all the 6 Papua New Guinea patients studied (Table 3.5). PFGE and ribotyping were less sensitive in detecting these genetic changes between strains from the same patient as only 3 and 2 patients showed differences in strains isolated from various regions of the body when analyzed by these methods respectively (Table 3.5). These variations in strains from different bodily sites may arise as a selective pressure owing to host immune response or antibiotic therapy. Such variation in the strains indicates that the strains are not independent clones but are so closely related both epidemiologically and genetically that they are presumed to be the same strain or a clonal variant.

An interesting observation was noticed in one patient (patient D) in this study. There is a possibility that this patient has been infected simultaneously by 2 closely related *S. typhi* strains as the profiles for all 3 typing methods differed between these 2 strains (Table 3.5).

A number of studies have been described previously to show the *in vivo* instability of microorganisms. PFGE was used to detect the *in vivo* emergence of polymorphism in

*Pseudomonas aeruginosa*, in which up to 20% genome divergence occurred over several months (Struelens et al., 1993). Southern blot hybridization with a species-specific probe was used to demonstrate the unstable nature of *Candida albicans* during recurrent vaginitis and that drug treatment can result in the selection of variants of the original strains (Schroppel et al., 1994). A recent study on the genetic relatedness of strains of *Candida* spp. obtained from different parts of the body suggested a possibility of substrain "shuffling" within a patient (Lockhart et al., 1996). It was discovered that out of the 20 simultaneous oral and vulvovaginal samples from 10 patients with recurrent candidiasis, 20% contained isolates that were unrelated and 35% contained isolates that were highly similar. To our knowledge, the present work is probably the first study in which AFLP analysis was used to analyze microbial strains (*S. typhi* in this case) from various parts of the body.

A number of high-resolution molecular typing systems for bacterial pathogens have been developed in recent years. As pointed out recently (Struelens et al., 1998), many of these methods (e.g. ribotyping, PFGE, RAPD-PCR) are largely comparative, allowing differentiation between closely related strains from those markedly different in genetic background (e.g. outbreak investigations). However, for the purpose of epidemiological surveillance, monitoring clonal spread and prevalence in populations over extended periods, as would be important in diseases like typhoid fever, requires what is referred to as "library" typing systems (Struelens et al., 1998). Such methods must be easily standardized, have a high throughput, be quantitative and also adopt a uniform nomenclature (Struelens et al., 1998). The issue of standardization is particularly important, as it would enable results from different laboratories to be compared. Of the many library typing systems, it has been proposed that genotypic profiles generated by AFLP are

particularly suitable, especially if they are more reproducibly and objectively analyzed by using electrophoresis with automated laser detection (Struelens et al., 1998). In addition, AFLP has other advantages including the small amounts of DNA needed, good reproducibility, no requirement for prior knowledge of DNA sequence and the possibility of direct cumulative analysis with appropriate software. The minor drawbacks of this technique previously experienced were its cost and technical complexity, but in light of recent modifications this technique has become relatively rapid and technically simpler (Gibson et al., 1998). AFLP may become an important future method in the epidemiological surveillance of pathogenic bacteria.

### 3.5 Conclusion

The genetic diversity among *S. typhi* isolates from different geographical regions is high as shown by PFGE, ribotyping and IS200 typing, providing further support for the hypothesis that multiple clones of *S. typhi* coexist independently in different regions of the world. The genomic DNA preparation for the 3 methods used were standardized. PFGE was the most discriminatory of the 3 methods followed by ribotyping and IS200 typing. On the other hand, limited amount of diversity was observed among *S. typhi* isolates from Papua New Guinea (PNG) as assessed by the same typing methods. This points to the fact that the *S. typhi* strains circulating in PNG which is endemic for this disease between 1992 – 1994 were most likely to be clonal and were possibly derived from a single clone (ancestor). The various typing methods produced different number of types/profiles (lineages) and so it is reasonable to say that genetic diversity is a function of the typing method used. Each typing method assesses different parts of the chromosome and these markers (mutations, inversions, deletions, insertion, polymorphisms at restriction enzyme

sites) evolve differently through space and time. Thus, genetic diversity is also a function of time.

PCR-ribotyping and PCR-RFLP were shown to have limited value in the epidemiological studies of *S. typhi* as both these techniques failed to differentiate the strains studied.

AFLP, being more discriminatory than PFGE, confirmed the extent of genetic diversity between geographic isolates and was also sensitive enough to discriminate between the closely related PNG fatal and non-fatal strains (but still indicated the clonal nature of these strains).

AFLP analysis of the PNG strains showed there was no association between genotypes and virulence (the ability to cause fatal and non-fatal disease).

AFLP complemented by PFGE and ribotyping has the potential to deduce whether strains isolated from various body parts are of clonal origin (indicating that infecting strains have undergone slight genetic variations) or are independent clones (indicating infection caused by different strains). This might help in the treatment of the disease or patient management and may be important in a clinical setting.

Typing methods like AFLP and PFGE can be useful for epidemiological surveillance of typhoid fever (monitoring clonal spread and prevalence in a population over a period of time). They have the potential to be included in the new "library" typing system concept based on the creation of reference databases by new computerized gel scanning and analysis software, which will enable these typing methods to be standardized easily between laboratories, to be quantitative and to have a uniform nomenclature.