2.0 MATERIALS AND METHODS

2.1 Bacterial Strains

Fifteen species of *Salmonella*, *S. blockley*, *S. bovismorbificans*, *S. chingola*, *S. enteritidis*, *S. houten*, *S. hvingfoss*, *S. matopeni*, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. raus*, *S. typhimurium*, *S. waycross*, *S. wandsworth*, *S. typhi* (1106), *S. typhi* (111) and *S. typhi* (495) were obtained from sporadic cases of salmonellosis from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya and the *Salmonella* Reference Center, Institute for Medical Research, Kuala Lumpur.

*S. typhi* (819), *S. typhi* (A102), *S. typhi* (A105), *S. typhi* (MM814), *S. typhi* (VC3112), *S. typhi* (11211), *S. typhi* (109894), *S. typhi* (109896), *S. typhi* (109950), *S. typhi* (110098), *S. typhi* (110723), *S. typhi* (PNG1) and *S. typhi* (PNG4) were identified and serotyped at the institutions from which they were obtained. The strains, from either stool or blood samples, were isolated, maintained and identified by using standard methods. All the strains were streaked on bismuth sulphite agar and blood agar plates to check for purity.

Four other gram negative bacteria, *Burkholderia pseudomallei*, *Escherichia coli*, *Shigella sonnei*, *Vibrio cholerae*, were also from the Department of Medical
Microbiology, Faculty of Medicine, University of Malaya. Bacteria was maintained on Luria-Bertani (LB) agar plates and subcultured every 4 weeks. For long term maintenance, all the isolates were kept in LB broth with 20% glycerol at -70°C.

2.2 Materials

All chemicals and solvents used were of Analar Grade or of the highest grade available commercially, obtained from BDH Chemicals Ltd., England; Boehringer Manheim, Germany; Fluka Biochemika, Switzerland; GIBCO BRL, U.S.A; Sigma Chemical Co., U.S.A.; and United States Biochemical Corp. (USB), U.S.A.

The common chemicals included acetone, boric acid, chloroform, diethyl pyrocarbonate, ethanol, disodium ethylene diaminetetraacetic acid, formamide, glacial acetic acid, glucose, glycerol, hydrochloric acid, isoamyl alcohol, magnesium chloride (6-hydrate), methylated spirit, phenol, potassium chloride, sodium acetate, sodium chloride, disodium hydrogen phosphate (anhydrous), sodium dihydrogen phosphate (monohydrate), sodium dodecyl sulphate, sodium hydroxide and Trizma base (Tris [hydroxymethyl] aminomethane).

Agarose powder and bovine serum albumin were purchased from Sigma. Bromophenol blue and xylene cyanol (FF) were obtained from Bio-Rad Laboratories, U.S.A. Ethidium bromide was obtained from BDH.
For sequencing and PCR, Taq DNA polymerase was from Promega Corp., Madison, Wisconsin, U.S.A; deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP) and dideoxyribonucleoside triphosphates (ddATP, ddCTP, ddGTP, ddTTP) were from Pharmacia Fine Chemicals, Sweden. PCR products were purified using the Geneclean II kit, BIO 101, Inc., U.S.A. Sequencing of PCR products was carried out using the Sequenase kit, Version 2.0 from USB. Deoxyadenosine 5'-α-32P triphosphate[(α-32P)dATP], with a specific activity of 3,000 Ci/mmol and at a concentration of 10 mCi/ml, was obtained from Amersham International plc, England.

For the preparation of sequencing gels, acrylamide and bis-acrylamide were obtained from Clontech Laboratories, Inc., U.S.A. Ammonium persulphate was from Sigma, N,N,N',N'-tetramethylenediamine (TEMED) from Bio-Rad, and urea (Ultrapure, Electrophoresis grade) from BRL.

For autoradiography, Cronex X-ray film and X-ray Hyperfilm-MP were purchased from DuPont, U.S.A and Amersham International plc., U.K. ECL Direct Nucleic Acid Labeling Kit was purchased from Amersham International plc., U.K.. Nitrocellulose membrane filters (Type GS, 0.22 μm, 25 mm) were from Millipore Corp., U.S.A. Nylon membranes (Hybond N') used for Southern blotting were from Amersham International plc., U.K. Quick seal centrifuge tubes were from Beckman Instruments, Inc., U.S.A.
2.3 Media, Buffers and Other Solutions

2.3.1 Luria- Bertani (LB) medium (Miller, 1972)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g/l</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0 g/l</td>
</tr>
</tbody>
</table>

All ingredients were dissolved and the resulting solution was autoclaved.

For solid medium, 1.2 g of Grade A agar per 100 ml was added. For soft agar, agar was added to a final concentration of 0.65%(w/v).

2.3.2 Common Solutions for DNA Extraction

The following common solutions, used in DNA extraction, were prepared according to methods described by Sambrook et al.(1989): 0.5M EDTA, pH 8.0; 1M Tris-HCl, pH 8.0 at 25°C; TE buffer (10mM Tris-HCL, 1mM EDTA, pH 8.0); buffered phenol-chloroform (1:1, v/v); (0.15 M NaCl, 0.1M EDTA, pH 8.0) (1% SDS, 0.1M NaCl, 0.1M Tris-HCl, pH 8.0) and 5M NaCl, sodium perchlorate used in the preparation of genomic DNA by a modified method of Saito and Miura (1963); DNase-free RNase A solution (10 mg RNase A per ml of 10 mM Tris-HCl, pH 7.5, 15 mM NaCl); 3M sodium acetate, pH 5.2; and 50% (w/v) polyethylene glycol (PEG)-8000.
2.3.3 Solutions for Agarose Gel Electrophoresis

2.3.3.1 10X Tris-borate EDTA (TBE) buffer, pH 8.3

Tris-base 10.80 g
Boric acid 5.50 g
Na₂EDTA. 2H₂O 0.93 g
Distilled water to 100 ml

The stock solution was diluted to 1 or 0.5X. The 0.5X solution was used in routine electrophoresis and the 1X TBE buffer was used during sequencing of amplified product.

2.3.3.2 6X Bromphenol blue (BPB) loading dye

BPB 0.15% (w/v)
Ficoll 400 9% (w/v)
Glycerol 40% (w/v)
2.3.4 Common Reagents for Slot-blot and Southern Hybridization

2.3.4.1 0.2M NaOH

NaOH 0.8 g
sddH₂O 100.0 ml

(sddH₂O: Sterile distilled deionized water)

2.3.4.2 50X Tris EDTA (TE) buffer

(10 mM Tris- HCl, pH 7.5, 1mM EDTA, pH 8.0)

Tris Base 60.55 g
EDTA 14.61 g
sddH₂O 800.0 ml

Adjust pH to 8.2 with HCl and top up to 1 liter with sddH₂O. Autoclave and store at room temperature. Dilute to 1X with sddH₂O before use.

2.3.4.3 20X SSC

NaCl 175.32 g
Trisodium citrate.2H₂O 88.32 g
sddH₂O to 1.0 liter

Autoclaved to sterilize.
2.3.4.4 2X SSC

Sterile 20X SSC 10.0 ml
sddH$_2$O 90.0 ml

2.3.4.5 Depurination solution

Concentrated HCl 10.8 ml
sddH$_2$O 490.0 ml

2.3.4.6 1M Tris-HCl, pH 7.5

Tris-HCl 121.4 g
sddH$_2$O to 1 liter

pH adjusted with concentrated HCl and stored at room temperature.

2.3.4.7 Denaturation solution

NaOH 20.0 g
NaCl 87.66 g
sddH$_2$O to 1 liter

Autoclaved and stored at room temperature
2.3.4.8 Neutralization solution for Slot Blots (3M Sodium Acetate)

Sodium acetate 24.61 g
sddH₂O to 100.0 ml

The pH was adjusted to 5.5 using glacial acetic acid, the solution autoclaved and stored at room temperature.

2.3.4.9 Neutralization solution for Southern Blots

1M Tris-HCl, pH 7.5 500.0 ml
NaCl 87.66 g
sddH₂O to 1 liter

Autoclaved and stored at room temperature

2.3.4.10 15X SSPE

NaCl 15.8 g
NaH₂PO₄.2H₂O 2.34 g
Na₂EDTA.2H₂O 0.558 g
sddH₂O to 90.0 ml
pH was adjusted to 7.7 with 5M NaOH and the volume adjusted to 100.0 ml with sddH₂O. The solution was sterilized by autoclaving and stored at room temperature.

2.3.4.11 10 % SDS

SDS 10.0 g
sddH₂O 100.0 ml

Solution was heated to 68°C to assist dissolution.

2.3.4.12 50% PEG 8000

PEG 8000 50.0 g
sddH₂O 100.0 ml

Solution was heated to 70°C to dissolve completely and filter sterilised.

2.3.4.13 Prehybridization buffer

Skim milk 0.5 g
sddH₂O to 68.0 ml

The skim milk was thoroughly dissolved in the sterile water and then following solutions added:
15X SSPE 10.0 ml
10% SDS 10.0 ml
50% PEG 8000 12.0 ml

2.3.4.14 2X SSC + 0.1% SDS

Sterile 20X SSC 10.0 ml
10% SDS 1.0 ml
sddH₂O to 100.0 ml

2.3.4.15 0.2X SSC + 0.1% SDS

Sterile 20X SSC 1.0 ml
10% SDS 1.0 ml
sddH₂O to 100.0 ml

2.3.4.16 0.5M EDTA, pH 8.0

EDTA 14.61 g
sddH₂O 100.0 ml

pH was adjusted with 5M NaOH
2.3.4.17 Deprobing solution

- 0.5 M EDTA, pH 8.0 10.0 ml
- 10% SDS 10.0 ml
- sddH₂O to 1.0 liter

The solution was autoclaved and stored at room temperature.

2.3.5 Solutions for Southern Blotting

2.3.5.1 Solutions for labeling DNA probes

Solutions for producing labeled DNA probes were supplied in the ECL Direct Nucleic Acid Labeling and Detection System. The system contained a DNA labeling reagent with positively-charged complexes of peroxidase and a glutaraldehyde solution for the preparation of labeled probes.

2.3.5.2 Solutions for hybridization and blot washing

The solutions for hybridization and blot washes were prepared according to the protocols suggested by Amersham International plc.
2.3.5.3 Hybridization buffer

The hybridization buffer was supplied with the ECL Direct Nucleic Acid Labeling and Detection System. Before use, 0.5M NaCl and 5% (w/v) blocking agent were added to the hybridization buffer.

2.3.5.4 Primary wash buffer (without urea)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>4.0 g</td>
</tr>
<tr>
<td>20X SSC</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 liter</td>
</tr>
</tbody>
</table>

The ingredient were dissolved and the solution was autoclaved.

2.3.5.5 Secondary wash buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X SSC</td>
<td>100 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 liter</td>
</tr>
</tbody>
</table>

The buffer was autoclaved.
2.3.5.6 Signal generation and detection

The ECL Direct Nucleic Acid Labeling and Detection System was supplied with detection reagents 1 and 2. Detection reagent 1 is a substrate for peroxidase. Detection reagent 2 contains luminol, which produces blue light on oxidation and at the same time, couples the reduction of hydrogen peroxide by peroxidase. Detection reagent 2 also contains an enhancer which increases and prolongs the light output. The light was detected on an X-ray film.

2.3.6 Solutions for DNA Sequencing

2.3.6.1 10mM deoxyribonucleoside 5'-triphosphate (dATP, dCTP, dGTP, dTTP)

Each deoxyribonucleoside triphosphate (100 mM) was diluted with sterile ddH₂O to 10mM. Further dilutions of 1 or 2.5 mM were prepared by diluting the respective stock solutions as required. All solutions were stored at -20°C.

2.3.6.2 10mM dideoxyribonucleoside 5'-triphosphate (ddATP, ddCTP, ddGTP, ddTTP)

The dideoxyribonucleoside triphosphate stock solutions were prepared and stored as described for the deoxyribonucleoside triphosphate stock solutions.
2.3.6.3 Solutions for 8% sequencing gels

10% (v/v) acetic acid

Glacial acetic acid 300 ml
ddH₂O to 3 litres

The solution was kept at room temperature.

40% (w/v) acrylamide-bisacrylamide (19:1)

Acrylamide 38.0 g
Bisacrylamide 2.0 g
ddH₂O to 100 ml

This solution was filtered through a Whatman 3 MM filter paper and stored in a brown bottle at 4°C.

10% (w/v) ammonium persulphate (APS)

APS crystals 0.1 g
ddH₂O 1 ml

This solution was prepared and used fresh.
2.3.6.4 8% sequencing gel solutions for a gel of 30.0 X 40.0 X 0.04 cm³

Sequencing gels were prepared according to the Instruction Manual of Model S2 Sequencing Apparatus (Catalogue No. 1105), BRL. The following components were used:

**40% (w/v) acrylamide**

bisacrylamide (19:1) 30.0 ml
Urea 63.0 g
10X TBE 15.0 ml
ddH₂O to 150 ml

The 8% gel solution was filtered through a Whatman 3 mM filter paper and stored at 4°C in the dark.

**8% sequencing gel**

8% gel solution 80 ml
10% APS 480 µl
TEMED 48 µl

Once the TEMED was added, the solution was gently swirled and poured immediately.
2.4 Sterilization Techniques

2.4.1 Moist Heat

Distilled water, micropipette tips, microcentrifuge tubes, chemicals, centrifuge tubes (polycarbonate, polypropylene and Corex) and Schott bottles were sterilized for 20 minutes at 15 psi at a temperature of 121°C.

2.4.2 Dry Heat

Pasteur pipettes, glass pipettes and measuring cylinders were sterilized at 180°C for 1 hour in an oven.

2.5 Methods

2.5.1 Preparation of Bacterial Genomic DNA

of *Salmonella* and the four gram negative bacteria were grown overnight at 37°C in 100 ml of LB broth (Section 2.3.1) with shaking at 150 rpm (Orbital Shaker Incubator, Model 706, Hotech Instruments Corp., Taiwan).

The cells were harvested by centrifugation and the cell pellet was resuspended in 1.0 ml of 0.15 M NaCl, 0.1 M EDTA, pH 8.0, Lysozyme (10mg), was added to lyse the cells and the mixture was then incubated in a 37°C water bath with gentle shaking for 45 minutes. Lysis buffer [1% (w/w) SDS, 0.1 M NaCl 0.1 M Tris-HCl, pH 8.0], 8 ml, was added to achieve complete lysis. After 10 minutes at 60°C, the mixture was cooled to room temperature and 2.2 ml of 5M Na-perchlorate was added to help in dissociating proteins from nucleic acid. The mixture was then gently extracted with buffered phenol-chloroform for 20 minutes.

The aqueous phase was recovered by centrifugation and nucleic acid was precipitated with 0.6 volume of isopropanol/2X volume of cold absolute or 95% ethanol. After 20 minutes at room temperature, the nucleic acid was pelleted by centrifugation, washed with 70% (v/v) ethanol, and dried under vacuum.

The pellet was resuspended in 4 ml of TE buffer or sterile distilled water and was treated with DNase-free RNase (50 µl/ml) at 37°C for 30 minutes. The DNA was stored at 4°C.
2.5.2 DNA Extraction from Serum Samples

The vials containing either the clinical samples or serum suspensions were extensively vortexed and briefly centrifuged (1-2 seconds). A total of 50.0 µl of serum suspension was then added to a tube containing the following components:

- 1X TE buffer: 50.0 µl
- 10% SDS: 50.0 µl

Proteinase K was added to a final concentration of 50 µg/ml and the components thoroughly mixed. The mixture was then incubated at 55°C for three hours in a water-bath. The Proteinase K was inactivated by heating at 95°C for 15 minutes after which the tubes were immediately placed on ice. A total of 150.0 µl Tris-buffered phenol-chloroform was added and the mixture mixed well. The tubes were then centrifuged at 10,000-11,000 rpm for 10 minutes. After centrifugation, the tubes were carefully placed on ice and the clear, aqueous layer collected and placed into another clean microcentrifuge tube. Steps 6 to 8 were repeated and the aqueous pooled. Approximately 2 volumes of ice-cold absolute alcohol and 1/10 volume 3M sodium acetate was added and the mixture gently but thoroughly mixed by repeatedly inverting the tube. The tubes were then left overnight at -20°C. The next day the tubes were centrifuged in a refrigerated centrifuge (Braun, Sigma, Germany) at 13,000 rpm for 40 minutes. The supernatant was discarded and the pellet carefully rinsed with 70% ethanol by gently pipetting. The pellet was then
dried in a vacuum drier (Savant, NY, USA) for 30 minutes. The resulting DNA pellet was redissolved in 50-70 µl of sterile, distilled, deionized water (sddH₂O) depending on the size of the pellet.

2.5.3 Determination of DNA Concentrations

The extracted DNA was suitably diluted and the concentration estimated by measuring the OD_{260nm} using a UV spectrophotometer (Pharmacia, Novaspec, LKB Biochrome, England). The DNA samples were then diluted to 100 ng/µl concentrations, aliquoted and stored at -20°C.

2.5.4 Oligonucleotide Primers for PCR Amplification

Four, 20-mer oligonucleotide primers (TP1 to TP4) were used. They were based on sequence data of the *E.coli* 16S rRNA genes (Brosius et al., 1981). Based on the assumption that all eubacteria contain 16S rRNA, it was postulated that these primers may be used to amplify the V2 and V6 regions of *Salmonella* species. The sequences of the primers are as follows:

TP1 : 5’-AATTGAAGAGTTTGATCATG-3’
TP2 : 5’-ACATTACTCACCGTCGGGC-3’
TP3 : 5’-GCAACGCGAAGAACCCTTACC-3’
TP4 : 5’-AGCCATGCAGCACCCTCTCTC-3’
TP1 and TP2 flank the V2 region, and TP3 and TP4 the V6 region (Figure 3).

2.5.5 PCR Amplification of rRNA Gene Regions and Analysis of PCR Products

The following components were added to a 0.6 ml microfuge tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer IV</td>
<td>2 µl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.8 µl</td>
</tr>
<tr>
<td>dNTP</td>
<td>2 µl</td>
</tr>
<tr>
<td>Primer TP1/2</td>
<td>1 µl</td>
</tr>
<tr>
<td>TP3/4</td>
<td>1 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>8.7 µl</td>
</tr>
</tbody>
</table>

The PCR was performed in 20 µl reaction mix which included buffer, deoxyribonucleoside triphosphates (1 mM), 0.2 µM of each of two relevant primers specific for the target sequence, and 0.5 unit of thermostable Taq DNA polymerase in addition to the DNA to be amplified. The MgCl₂ free 10X buffer for PCR contained 50 mM Tris-HCl (pH 8.0 at 25°C), 100 mM NaCl, 0.1mM EDTA, 1 mM DTT, 50% glycerol and 1% Triton X-100. The separate tube of 25 mM MgCl₂ was used in varying concentrations to optimize PCR conditions to obtain best results. The solution was centrifuged briefly to ensure complete mixing of the components at the bottom of the tube. The PCR mix was then heat-denatured at 95°C for 10 minutes followed by rapid chilling on ice for 5 minutes. The tube was
briefly centrifuged to collect the solution at the bottom of the tube. Taq DNA polymerase, 0.5 units, was added to the mixture which was then overlaid with 20 μl of sterile liquid paraffin to prevent evaporation. Thirty five PCR cycles were then performed in a Perkin-Elmer Cetus Thermocycler (Model 480 DNA Thermal Cycler). After the last cycle, the tubes were maintained at 72°C for 12 minutes to complete the synthesis on all strands.

The PCR thermal profiles used were based on those of Barry et al. (1990):

- Denaturation step: 94°C for 1 minute,
- Primer annealing step: 60°C for 1 minute,
- Primer extension step: 72°C for 1 minute.

A small quantity of the PCR product was evaluated by eletrophoresis in a submerged horizontal agarose slab gel (1.5% w/v) in 0.5X TBE buffer (see stock solution, 2.3.3.1) alongside the 123 bp markers. After thermocycling was complete, the samples were stored at 4°C until visualization by conventional agarose gel electrophoresis. This was done within 18 hours of PCR.

2.5.6 Electrophoresis of DNA

Electrophoresis of PCR products after addition of 6X BPB loading dye (see 2.3.3.2) was performed on agarose (1.5% or 2%) gels prepared in 0.5X TBE.
Agarose powder, 1.5 or 2.0 g, was dissolved in 100 ml of 0.5X TBE (pH 8.3) by heating. The gel was incorporated with EtBr (0.5 μg/ml) when it was cooled to 45°C to 50°C. The resultant mixture was then poured into a horizontal gel tray, fitted with an 8- or 12- well comb. The gel was then placed in a buffer tank and submerged in 0.5X TBE buffer containing EtBr (0.5 μg/ml). Samples, 10 μl, were each loaded into a well and electrophoresis was carried out at room temperature at a constant voltage of 100 V until the BPB was at the anode end of the gel.

The gel was then viewed on a 302 nm UV transilluminator. The gel was photographed with a Polaroid MP-4 Land camera fitted with a yellow filter and Polaroid Land 665 black-and-white films.

2.5.7 Purification of PCR Products

The PCR products were extracted once with an equal volume of chloroform to remove all traces of paraffin and centrifuged at 10,000 rpm for 10 minutes. The aqueous phase was then purified with Geneclean (GlassMax DNA isolation Matrix System, Gibco BRL).

Initially, 2 to 4 volumes of NaI (6 M) and 10 μl of glass milk suspension were added to the aqueous phase. The mixture was incubated at room temperature for 10 minutes with occasional shaking. The mixture was then centrifuged for 30 seconds at 10,000 rpm after which the supernatant was removed. The pellet was
washed thrice with ice-cold New Wash solution for 30 seconds at 10,000 rpm. After the final wash, the pellet was resuspended in 25 μl sterile ddH₂O and eluted for 10 minutes at 50°C. The mixture was centrifuged for 2 minutes at 10,000 rpm and the supernatant was transferred to a fresh tube. The pellet was resuspended in 15 μl sterile ddH₂O and eluted for 5 minutes at 50°C. The mixture was centrifuged for 2 minutes at 10,000 rpm. The supernatant was removed and pooled with the first eluate and stored at -20°C.

2.5.8 Slot-Blot Hybridization

2.5.8.1 Preparation of Hybridization membrane

The membranes for slot-blot hybridizations were prepared using the Slot-Blot apparatus (Hoefer Scientific Instruments, San Francisco, USA).

The Slot-Blot apparatus was thoroughly cleaned by soaking the entire assembly, including the screws, in 0.2 M NaOH for 30 minutes. This was to ensure that residual nucleic acids from previous uses will be denatured. The apparatus was then rinsed three times in distilled water and wiped dry with clean tissue. A piece of nylon membrane (Hybond-N⁺ Amersham International, Buckinghamshire, England) was cut to the dimensions of 11.7 cm X 4.0 cm. It is imperative that a clean pair of scissors, forceps and gloves be used to reduce risk of contamination. One corner of the membrane was nicked to determine the orientation. Using a clean pair of forceps
dipped in 70% EtOH, the membrane was picked up and briefly soaked in TE buffer for a couple of minutes.

After this, the membrane was carefully placed on the Slot-Blot apparatus (again with forceps dipped in 70% EtOH) ensuring there were no trapped air bubbles beneath it. The top block of the apparatus was placed onto the membrane and the alignment pins tightened. The apparatus was connected to a vacuum pump (GAST, MI, USA) and the pump switched on for 5 minutes at 5-10 mm Hg. After this, the pump was turned off before the samples were loaded. The suitably diluted samples (diluted in sddH₂O) were applied to the individual slots. A minimum volume of 100 µl is recommended at a target DNA concentration of 100 ng/µl. One ml of TE buffer was added to each slot and the vacuum pump was turned on at 10-15 mm Hg to draw the solution down. As the level of TE decreased, it was topped up with another 1.0 ml. This was repeated three times to wash any remaining sample DNA down the slot to the membrane surface. When this was complete and WITHOUT turning the vacuum off, the screws were removed and the top block of the apparatus removed. Using forceps dipped in 70% EtOH, the membrane was carefully lifted off the apparatus and placed on a piece of Whatman 3 mM (Whatman International Ltd, Maidstone, England) filter paper to air dry.

When dry, the membrane was placed into denaturing solution for 5 minutes and then air dried again. Then, it was placed in neutralizing solution also for 5 minutes after which it was air dried for 30 minutes. The membrane was then
wrapped in cling film (Saran Wrap) and exposed, face down, to UV irradiation at 312 nm (on a Spectroline transilluminator) for 5 minutes to fix the DNA onto the membrane. The membrane was then dried in an oven at 50°C overnight and stored in a dry, air-tight container until needed.

2.5.8.2 Labeling of Hybridization Probes

Labeling of double stranded DNA hybridization probes was done using the Rediprime DNA labeling system (Amersham Life Science, Buckinghamshire, England) according to the manufacturer's instructions detailed below. This system is based on the random priming labeling technique.

The DNA to be labeled, in this case the purified PCR product, was diluted to a concentration of 25 ng in 45 μl of ddH2O. The DNA sample was then denatured by boiling at 95-100°C for 5 minutes in a boiling water-bath. The contents of the tube were briefly centrifuged and the mixture added to the labeling mix supplied in the kit. This mixture was reconstituted by gently flicking the tube until the blue color was evenly distributed. The tube was briefly centrifuged again and 2 μl of Redivue [α-32P] CTP (Amersham Life Science, Buckinghamshire, England) added and mixed by gentle pipetting. The mixture turns a light shade of purple. All subsequent steps were carried out behind a protective perspex shield. Another brief centrifugation was followed by a 10 minute incubation at 37°C. The reaction was stopped by adding 5 μl 0.2M EDTA, pH 8.0. The labeled probe was
denatured by heating to 95-100°C for 5 minutes immediately prior to use in hybridization.

2.5.8.3 Hybridization experiments

Prehybridization was carried out by first washing the hybridization flasks with 70% EtOH, rinsing them out with distilled water and drying them overnight in a 50°C oven. The hybridization nylon meshes were also washed by rinsing in distilled water. A clean nylon mesh was soaked in 2X SSC and placed on a clean piece of cling film. The membrane, also briefly soaked in 2X SSC was carefully placed on top of the mesh ensuring there were no air bubbles trapped underneath.

The mesh was then rolled and placed into a hybridization bottle (Hybaid, Hybaid Ltd., Middlesex, UK) and 7.0 ml prehybridization buffer dispensed into the flask. The bottle was then placed into a hybridization oven (Hybaid, Hybaid Ltd., Middlesex, UK)(pre-warmed to 42°C) to rotate overnight, also at 42°C and at a setting of 11. It is important to ensure that the membrane rolls against the inside of the bottle not in onto itself. It is also important to ensure that the hybridization bottles are balanced on the rotating wheel. The next day, the labeled probe was added in to the prehybridization buffer and hybridization allowed to proceed for another 18-24 hours.
2.5.8.4 Washing the Hybridization Membranes

The meshes were removed from the hybridization flasks and the blots placed inside plastic containers. Care was taken to ensure that blots with different probes were placed in separate containers. Enough 2X SSC was added to cover the membranes and washing done at 50°C for 40 minutes. The wash solution was discarded and replaced with 2X SSC + 0.1% SDS and the wash repeated at 53°C for 30 minutes. The procedure was repeated again with 0.2X SSC + 0.1% SDS at 55°C for another 30 minutes. This set of stringency conditions was found applicable in most cases but had to be altered in some cases. Generally, the higher the temperature and the lower the salt concentration the higher the stringency of the washes. After this the membrane was wrapped in cling-film and exposed face down on X-ray film (Hyperfilm-MP, Amersham Int., Amersham, UK) in a cassette (Kodak X-omatic Regular Screen, Kodak, Rochester, NY, USA) for 1-3 days at room temperature.

2.5.8.5 Developing the X-ray film

When the film was ready to be developed it was removed from its surface and the film immersed in developing solution until the required intensity is seen. It was then placed in stop solution for 5 minutes to stop the developing reaction and immediately placed in a fixing solution for at least 10 minutes. The film was then
washed in ordinary tap water and allowed to drip dry. All work was done in a light-proof photography dark room.

2.5.8.6 Deprobing the Hybridization Membranes

It was often time saving and economical to deprobe used hybridization membranes and use them again, preferably with the same kind of probe by re-labeling the probe and repeating the hybridization. The used membranes were immersed in deprobing solution at 75°C for 45-60 minutes in a shaking water-bath. This wash was repeated three times and the membrane wrapped in cling-film while still moist and stored at 4°C. All radioactive waste was collected in dark bottles, neatly labeled and stored behind perspex shields to be collected for proper disposal.

2.5.9 Southern Hybridization

2.5.9.1 Southern transfer of DNA from agarose gel to nylon membrane by capillary transfer

After agarose (1.5% or 2%, w/v) gel electrophoresis, a photograph of the DNA samples in the gel was taken. After photography, the size of the gel was measured. A piece of nylon membrane with dimensions slightly larger than the gel was cut and soaked in distilled water.
2.5.9.2 Processing the gel

The gel was placed in a suitable plastic tray, sufficient depurination solution was added to cover the gel. The gel was shaken gently for 20 minutes at room temperature and the depurination solution was discarded. The gel was then rinsed three times with deionized water. A similar volume of denaturation solution was added to the gel which was further shaken at room temperature for 30 minutes. The solution was discarded and the gel rinsed with deionized water 3 times. The procedure was repeated with a similar volume of neutralization solution.

2.5.9.3 Capillary blotting

A plastic tray was filled with 20X SSC solution and a supporting platform made using a perspex gel casting tray and a glass plate as the base. The platform was then covered with a wick made from three sheets of filter paper (Whatman 3 MM) saturated with 20X SSC. The gel was placed on the 3 MM paper. Care was taken to avoid trapping air bubbles. The gel was surrounded with saranwrap to prevent the SSC from being absorbed directly by the paper towels.

The Hybond-N+ nylon membrane (Amersham, U.K.) used for blotting was cut to the size of the gel, soaked in 20X SSC, and placed on top of the gel without trapping any air bubbles beneath it. Three sheets of 3 MM paper which was cut to size, soaked in 20X SSC, was in turn placed on top of the nylon membrane. Again,
care was taken to ensure that no air bubbles were trapped. A stack of absorbent paper towels (newspaper were found to do just as well) was placed on top of the 3 MM paper. Lastly, a glass plate was then placed on top of the paper towels and a weight of about 750 g was applied. The capillary blotting stack was left overnight.

2.5.9.4 Processing the blot

The nylon membrane containing the DNA samples was wrapped in cling film. The DNA samples on the membrane were fixed to the membrane by ultraviolet cross-linking with a 302-nm transilluminator for 5 minutes.

ECL system

The methods of DNA labeling, prehybridization, hybridization, membrane washing, and signal generation/detection of the ECL Direct Nucleic Acid Labeling and Detection System were performed according to the protocols suggested by the manufacturer, Amersham International. Figure 4 illustrates the principles of the ECL Direct Nucleic Acid Labeling and Detection System.

2.5.9.5 DNA labeling

20-30 microlitres (10 ng/μl) of double stranded PCR product to be labeled as the probe was denatured for 5 minutes at 100°C and cooled immediately on ice for 5 minutes. The tube containing the cooled denatured PCR product was spun
Figure 4: Principles of the ECL direct nucleic acid labeling and detection system. (From the Manual of the ECL labeling and detection system)
equivalent volume (20-30 µl) of DNA labeling reagent was added to the cooled briefly in a microcentrifuge to settle the liquid in the bottom of the tube. An single-stranded PCR product. The contents in the tube were mixed before addition of (20-30 µl) of glutaraldehyde solution. The final content was then mixed thoroughly using a vortex mixer. The tube was then spun briefly in a microcentrifuge and incubated for 10 minutes at 37°C.

2.5.9.6 Prehybridization

The Southern blot was incubated for 1 hour in hybridization buffer (0.25 ml per cm² of membrane) in a sealed plastic container at 42°C. All air bubbles were removed from the plastic container before it was sealed.

2.5.9.7 Hybridization

Some of the buffer that had been used during prehybridization was withdrawn for mixing with the labeled DNA probe (Section 2.5.9.5). The mixture was then added to the bulk of the buffer in the plastic container containing the Southern blot. All air bubbles were removed from the plastic container before it was sealed. Hybridization was done by incubation with gentle agitation at 42°C overnight.
2.5.9.8 Post-hybridization membrane washing

After hybridization at 42°C overnight, the blot was removed from the hybridization medium and incubated in primary wash buffer (2 ml buffer per cm² membrane) at 42°C for 25 minutes with constant agitation. After two times of primary washing, the blot was incubated in secondary wash buffer (2 ml buffer per cm² membrane) at room temperature for 5 minutes with constant agitation. Secondary washing was done twice. Both primary and secondary washes were to remove any unbound probes.

2.5.9.9 Signal generation/detection

After the secondary wash, excess wash buffer on the blot was removed by placing the wet membrane on a paper towel. Meanwhile, 500 µl of detection reagent 1 (Section 2.3.5.6) was mixed with an equal volume of detection reagent 2 (Section 2.3.5.6). The resultant mixed detection reagent was added directly to the blot on the surface containing the DNA.

The membrane was then left at room temperature for 5 minutes. Excess detection reagent was drained off and the membrane was immediately wrapped in cling film. Air pockets were smoothed out.

The membrane was then exposed to a piece of X-ray film (Kodak XAR05) for 15 minutes at room temperature in a Kodak X-Omatic cassette. The X-ray film
was developed manually in Kodak GBX Developer and Replenisher for 1 to 2 minutes, rinsed in water, fixed in Kodak GBX Fixer and Replenisher for 1 to 2 minutes and rinsed again with water. The autoradiograph was then air-dried. The result obtained was documented by photography.

2.5.10 DNA Sequencing

Direct sequencing of the PCR products was performed using the Sequenase kit Version 2.0(USB) with the incorporation of a radiolabel (α-32P dATP). The method of Seetharam and Dicker (1991) with modifications by Chen and Chasin (1992) was followed.

Four 1.5 ml microfuge tubes, labeled A,C,G or T, were filled with 2.5 μl each of termination mixes and kept at room temperature. The Sequenase labeling mix (dGTP) was diluted to 5-fold to working concentration.

The Sequenase and Pyrophosphatase (0.33 volume pyrophosphatase to 1 volume polymerase) mix was diluted 1: 6 in ice-cold Sequenase diluent and stored on ice for no more than 1 hour.
The following were combined in a 1.5 μl microfuge tube:

- PCR product (1 μg/μl) 5 μl
- Primer solution (50 pmole) 1 μl
- Reaction buffer 2 μl
- Sterile ddH₂O 2 μl

The mixture was boiled for 5 minutes, quickly cooled on ice and centrifuged briefly to get all the solutions to the bottom of the tube. The ice-cold annealed mixture was added to a 1.5 ml microfuge tube containing the following:

- DTT, 0.1M 1.0 μl
- Diluted labeling mix (dGTP) 2.0 μl
- (α-³²P)dATP 0.5 μl
- Mn buffer 1.0 μl

The mixture was briefly centrifuged in a microcentrifuge to ensure complete mixing of the components at the bottom of the tube. Diluted Sequenase-pyrophosphatase mix, (2 μl) was added to the mixture. The labeling reaction was then incubated at room temperature for 5 minutes, after which 3.5 μl of the mixture was transferred to each of the four termination tubes which were prewarmed at 37°C for at least 1 minute. The mixture was then incubated at 37°C for 5 minutes. The reaction was stopped by adding 4 μl stop-loading dye to each tube. The
samples were heated at 75° C for 2 minutes and immediately loaded onto a preheated sequencing gel (2-3 μl/well). The samples were electrophoresised at a constant current of 50 mA for 2-3 hours.

2.5.10.1 Autoradiography

After the run was complete, the gel was fixed in fixer solution and transferred to a Whatman 3MM filter paper, covered with cling-film and dried at 80°C for approximately 2 hours on a gel drier (Hoefer Scientific Instruments, San Francisco, USA). The dried gel was placed in an exposure cassette (Kodak X-omatic Regular Screen, Kodak, Rochester, NY, USA) for autoradiography. A piece of X-ray film (Hyperfilm-MP, Amersham Int., Amersham, UK) was placed on the gel and exposed for 24-48 hours at room temperature with the aid of intensifying screens. Following exposure, the X-ray film was processed with Kodak GBX Developer and Fixer. The bands in the various lanes of the autoradiographs were read with the aid of a light box.

Figure 5 shows the flow chart of the experiments performed to achieve the objectives of this study.
PCR Optimization of Bacterial Culture and Serum Suspension of *Salmonella* sp

Bacterial culture of *Salmonella* sp

Extraction of genomic DNA from *Salmonella* sp

Quantification of DNA

Agarose gel electrophoresis

PCR amplification of two variable regions V2 and V6

PCR optimization to obtain a *S. typhi* specific band

Agarose gel electrophoresis

Gene clean

Southern and slot-blot hybridizations

Direct sequencing of PCR product

Serum suspension of *Salmonella* sp

DNA extraction of serum samples

Agarose gel electrophoresis

PCR amplification and optimization for serum samples

Agarose gel electrophoresis

Figure 5: Flowchart of methods used in this study