3.0 RESULTS

3.1 Isolation of Genomic DNA from *Salmonella* Species

Genomic DNA from fifteen species of *Salmonella* and four other gram negative bacteria was extracted by a modified method of Saito and Miura (Section 2.5.1). Figure 6 illustrates the results obtained. Generally, all samples were intact high molecular weight genomic DNA.

3.2 Determination of DNA Concentration

Quantification of DNA samples was performed according to the method described in section 2.5.3. Table 2 shows the results obtained. The concentrations of the DNA samples ranged from 1150 to 3940 μg/ml.

3.3 Amplification of rRNA Gene Regions

Genomic DNAs extracted from the various *Salmonella* species and the other gram negative bacteria were analyzed to determine if the two primer sets, TP1-TP2 targeting the V2 region and TP3-TP4 targeting the V6 region, could work in a universal manner.

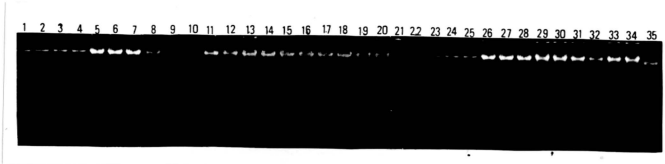

Figure 6 : Ethidium-bromide stained agarose gel (0.5% w/v) of genomic DNA from *Salmonella* species and four other gram negative bacteria (5 µl/well with concentration of 100 ng/µl).

Lane 1 : *S. blockley*,
2 : *S. bovismorbificans*,
3 : *S. chingola*,
4 : *S. enteritidis*,
5 : *S. houten*,
6 : *S. hvittingfoss*,
7 : *S. matopeni*,
8 : *S. paratyphi* A,
9 : *S. paratyphi* B,
10 : *S. paratyphi* C,
11 : *S. raus*,
12 : *S. typhimurium*,

Lane 13 : *S. wandsworth*,
14 : *S. waycross*,
15 : *S. typhi* (1106),
16 : *S. typhi* (819),
17 : *S. typhi* (111),
18 : *S. typhi* (495),
19 : *S. typhi* (PNG1),
20 : *S. typhi* (PNG4),
21 : *S. typhi* (VC3112),
22 : *S. typhi* (MM814),
23 : *S. typhi* (A102),
24 : *S. typhi* (A105),

Lane 25 : *S. typhi* (11211),
26 : *S. typhi* (109894),
27 : *S. typhi* (109896),
28 : *S. typhi* (109950),
29 : *S. typhi* (110098),
30 : *S. typhi* (110723),
31 : *B. pseudomallei*,
32 : *E. coli*,
33 : *S. sonnei*,
34 : *V. cholerae*,
35 : λ *Hin*dIII marker.

Table 2: Determination of concentration and purity of *Salmonella* spp and other gram negative bacteria genomic DNA by spectrophotometry at OD_{260} and OD_{280}.

Sample	OD_{260}	OD_{280}	OD_{260}/OD_{280}	DNA conc. (μg/ml)[a]
S. blockley	0.123	0.064	1.922	1230
S. bovismorbificans	0.219	0.117	1.872	2190
S. chingola	0.124	0.067	1.851	1240
S. enteritidis	0.225	0.120	1.875	2250
S. houten	0.335	0.173	1.936	3350
S. hvittingfoss	0.332	0.187	1.775	3320
S. matopeni	0.339	0.171	1.982	3390
S. paratyphi A	0.238	0.127	1.874	2380
S. paratyphi B	0.115	0.064	1.797	1150
S. paratyphi C	0.225	0.124	1.815	2250
S. raus	0.212	0.120	1.767	2120
S. typhimurium	0.228	0.125	1.824	2280
S. wandsworth	0.394	0.206	1.913	3940
S. waycross	0.253	0.140	1.807	2530
S. typhi (1106)	0.295	0.154	1.916	2950
S. typhi (819)	0.210	0.115	1.826	2100
S. typhi (111)	0.223	0.118	1.949	2230
S. typhi (495)	0.327	1.703	1.920	3270
S. typhi (PNG1)	0.223	0.124	1.798	2230
S. typhi (PNG4)	0.331	0.174	1.902	3310
S. typhi (VC3112)	0.133	0.071	1.873	1330
S. ytphi (MM814)	0.129	0.073	1.767	1290
S. typhi (A102)	0.130	0.072	1.805	1300
S. typhi (A105)	0.134	0.073	1.836	1340
S. typhi (11211)	0.116	0.065	1.785	1160
S. typhi (109894)	0.208	0.115	1.809	2080
S. typhi(109896)	0.125	0.069	1.812	1250
S. typhi (109950)	0.219	0.119	1.840	2190
S. typhi (110098)	0.123	0.066	1.864	1230
S. typhi (110723)	0.218	0.121	1.802	2180
Escherichia coli	0.126	0.070	1.800	1260
Burkholderia pseudomallei	0.158	0.087	1.816	1580
Shigella sonnei	0.209	0.115	1.817	2090
Vibrio cholerae	0.133	0.078	1.705	1330

[a] 5μl of each DNA sample was diluted in 1000μl of sterile distilled water before reading.

Figures 7 and 8 show the gel electrophoresis patterns obtained when primers TP1/TP2 and TP3/TP4 were used in PCR amplification at 60°C annealing temperature. Amplification of the V2 region of the 16S rRNA genes gave rise to a product of approximately 120 base pairs. The amplified product of the V6 region was an approximately 100 base pairs in size. Results show that the number of PCR amplified products generated for the V2 region to be less than the V6 region, thus suggesting that the V2 region to be more specific than the V6 region. Importantly, no amplification was detected for the V2 region of all other species of gram negative bacteria such as *Escherichia coli*, *Burkholderia pseudomallei*, *Shigella sonnei* and *Vibrio cholerae* (Fig.7, lanes 27-30). For the V6 region, however, there was amplification for *E. coli* although the other gram negative bacteria were not amplified (Fig. 8, lane23).

PCR was also done at 65°C annealing temperature for the V2 and V6 region. Figure 9 shows amplification for the V6 region (lanes 7-11) but no amplification for the V2 region (lanes 3-6). Based on these early experiments, it seemed more likely that the V2 region would contain *S. typhi*-specific sequence compared to V6.

Figure 7 : Ethidium bromide-stained agarose gel (1.5% w/v) of PCR-amplified products of the V2 region at 60°C annealing temperature.

Lane 1 : 123 bp marker,
2 : negative control,
3 : *S. blockley*,
4 : *S. paratyphi* A,
5 : *S. paratyphi* B,
6 : *S. paratyphi* C,
7 : *S. typhimurium*,
8 : *S. wandsworth*,
9 : *S. typhi* (1106),
10 : *S. typhi* (819),
11 : *S. typhi* (111),
12 : *S. hvittingfoss*,
13 : *S. raus*,
14 : *S. waycross*,
15 : *S. typhi* (PNG1),
16 : *S. typhi* (PNG4),
17 : *S. typhi* (MM814),
18 : *S. typhi* (VC3112),
19 : *S. typhi* (109894),
20 : *S. typhi* (109950),
21 : *S. typhi* (495),
22 : *S. typhi* (A102),
23 : *S. typhi* (A105),
24 : *S. typhi* (110098),
25 : *S. typhi* (110723),
26 : *S. typhi* (109896),
27 : *E. coli*,
28 : *Burkholderia pseudomallei*,
29 : *Shigella sonnei*,
30 : *Vibrio cholerae*

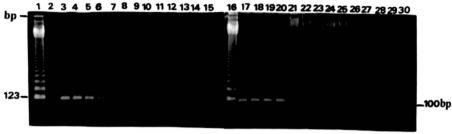

Figure 8 : Ethidium bromide-stained agarose gel (1.5% w/v) of PCR amplfied products of the V6 region at 60°C annealing temperature.

Lane
1 : 123 bp marker,
2 : negative control,
3 : *S. blockley*,
4 : *S. bovismorbificans*,
5 : *S. chingola*,
6 : *S. enteritidis*,
7 : *S. houten*,
8 : *S. hvittingfoss*,
9 : *S. matopeni*,
10 : *S. paratyphi* A,
11 : *S. paratyphi* B,
12 : *S. paratyphi* C,
13 : *S. typhi* (MM814),
14 : *S. typhi* (111),
15 : *S. typhi* (495),
16 : 123 bp marker,
17 : *S. typhimurium*,
18 : *S. wandsworth*,
19 : *S. typhi* (1106),
20 : *S. typhi* (819),
21 : *S. raus*,
22 : *S. waycross*,
23 : *E. coli*,
24 : *V. cholerae*,
25 : *B. pseudomallei*,
26 : *Shigella sonnei*,
27 : *S. typhi* (PNG1),
28 : *S. typhi* (A105),
29 : *S. typhi* (109894),
30 : *S. typhi*(110723)

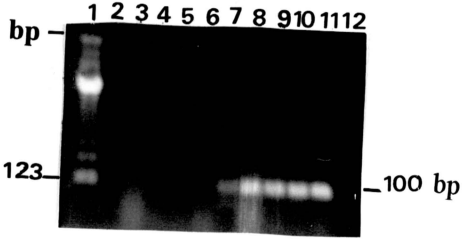

Figure 9 : Ethidium bromide-stained agarose gel (1.5%,w/v) showing PCR amplification for the V2 and V6 region at 65°C annealing temperature as stated below:

Lanes 3-6 [V2 region]
7-11 [V6 region]

Lane 1 : 123 bp marker,
2 : negative control (V2 region),
3 : *S. blockley*,
4 : *S. paratyphi* C,
5 : *S. typhi* (1106),
6 : *S. typhi* (819),
7 : *S. blockley*,
8 : *S. paratyphi* C,
9 : *S. typhi* (1106),
10 : *S. typhi* (819),
11 : *S. typhi* (111),
12 : negative control (V6 region).

3.4 Optimization of PCR Conditions

In establishing important parameters for PCR, *Taq* polymerase, dNTP and primer concentrations were optimized and determined prior to PCR optimization based on annealing temperature, $MgCl_2$ concentration and input DNA concentration (Table 3). The conditions used subsequently for all PCR optimization studies concentrated mainly on the V2 region as shown in Table 3 .

Table 3 : Stock and working concentrations of PCR reagents used.

PCR REAGENTS	STOCK CONC.	PER REACTION (20µl)	WORKING CONC.
10X Buffer IV	-	2.0 µl	-
$MgCl_2$	25 mM	2.8 µl	3.5 mM
100mM dNTP	10 mM	2.0 µl	1.0 mM
5U/µl *Taq* pol.	0.5 Unit/µl	1.0 µl	0.025 U/µl
TP1	7.6 µM	1.0 µl	0.38 µM
TP2	6.57 µM	1.0 µl	0.33 µM
DNA	50 ng/µl	1.5 µl	3.75 ng/µl

* Thermal cycles : 35

3.5 PCR Optimization and Specificity of Primers

PCR was optimized by testing the primers under various conditions such as a range of $MgCl_2$ concentrations, varying *Taq* polymerase and primer concentrations and annealing temperature. The best conditions were defined as the ones yielding the sharpest, most distinct band with a minimum of non-specific bands or primer-dimers. Eigure 10 shows the effect of different primer concentrations on the V6 region. At diluted concentration of primers (0.3 μM) the primer-dimers disappeared. These conditions, as shown in Table 3 above, once established were used for all subsequent PCR experiments.

3.5.1 Effect of Different Annealing Temperatures on PCR

PCR amplification results obtained for the V2 region using primers TP1 and TP 2 by varying the annealing temperature are shown in Table 4. At 55°C annealing temperature, amplification was seen for 27 out of 30 samples tested, of which 16 were *S. typhi*. At 60°C annealing temperature (Figure 7), amplification was seen for 10 out of 15 samples and 4 isolates, *S. hvittingfoss, S. paratyphi* B, *S.waycross* and *S. raus*, gave unpredictable results with poor reproduciblity.

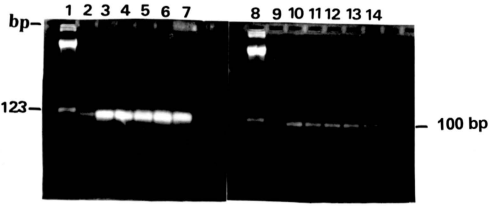

Figure 10 : Ethidium bromide-stained agarose gel (1.5%,w/v) showing PCR amplification of the V6 region at different primer concentrations.

Lanes 2-7 [undiluted primer concentration, 1 μM.]
9-14 [diluted primer concentration, 0.3 μM]

Lane 1 : 123 bp marker,
2 : negative control,
3 : *S. blockley*,
4 : *S. typhimurium*,
5 : *S. paratyphi* C,
6 : *S. typhi* (1106),
7 : *S. typhi* (819),
8 : 123 bp marker,
9 : negative control,
10 : *S. blockley*,
11 : *S. typhimurium*,
12 : *S. paratyphi* C,
13 : *S. typhi* (1106),
14 : *S. typhi* (819).

Table 4 : Effect of different annealing temperarures on PCR

PCR CONDITIONS		SAMPLES																								
		Salmonella blockley	*S. bovismorbificans*	*S. chingola*	*S. enteritidis*	*S. houten*	*S. hvittingfoss*	*S. matopeni*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*	*S. raus*	*S. typhimurium*	*S. wandsworth*	*S. weycross*	*S. typhi(1106)*	*S. typhi(819)*	*S. typhi(111)*	*S. typhi(495)*	*S. typhi(PNG1)*	*S. typhi(PNG4)*	*S. typhi(VC3112)*	*S. typhi(MM814)*	*S. typhi(A102)*	*S. typhi(A105)*	*S. typhi(109894)*	*S. typhi(109896)*	*S. typhi(109950)*	*S. typhi(110098)*	*S. typhi(110723)*	*S. typhi(112111)*
Buffer IV dNTP TP1/TP2 *Taq* Polymerase Input DNA: 3 ,0 ng/µl $MgCl_2$ conc., 2.5mM Thermal Cycles : 35																				-										
Annealing Temperature (°C)	55	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-
	60	+	-	-	-	-	±	-	+	±	+	+	+	+	±	+	±	+	+	-	±	-	±	±	+	+	+	+	+	-
	61	+	-	-	-	-	±	-	+	±	+	-	+	+	-	+	+	+	+	-	±	+	+	+	+	+	+	+	+	-
	63	+	-	-	-	-	-	-	±	±	+	-	±	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
	65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key + : Positive amplification
- : Negative amplification
± : Inconsistent amplification

Figure 11 shows that at 61°C annealing temperature, no amplification was seen for *S. raus* (Lane 3) while the remaining samples were identical to results obtained at 60°C. Inconsistent results were also noticed for *S. hvittingfoss*, *S. paratyphi* B (Lanes 5 and 6) and *S. waycross* (not shown). At 63°C annealing temperature, as seen in Figure 12, the amplification was greatly reduced and only *S. blockley*, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhimurium*, *S. wandsworth* (Lanes 3, 8, 9, 10, 11 and 12) and most of the *S. typhi* (Lanes 13 to 18) isolates were amplified. However, *S .paratyphi* A, *S. paratyphi* B, *S. typhimurium* and a number of *S. typhi* amplified inconsistently. Repeated PCR cycles at 63°C annealing temperature gave the same results. No amplification was seen for any sample at 65°C annealing temperature.

3.5.2 Effect of Different $MgCl_2$ Concentrations on PCR

PCR optimized for varying concentrations of $MgCl_2$ gave results as shown in Table 5. At 63°C annealing temperature no amplification was seen for any of the samples at 0.75 mM and 1 mM $MgCl_2$. At 2 mM $MgCl_2$ concentration, amplification was seen for *S. blockley*, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhimurium*, *S. wandsworth* and most of the *S. typhi* isolates but inconsistency in results was still seen for *S. paratyphi* A, *S. paratyphi* B, *S. typhimurium* and a number of *S. typhi* isolates. There was no difference in results at 3 mM concentration of $MgCl_2$ except that some inconsistency in results was eliminated.

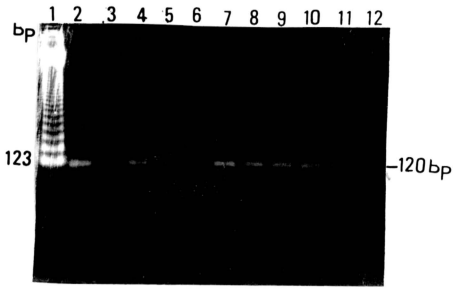

Figure 11 : Ethidium bromide-stained agarose gel (1.5%,w/v) showing PCR amplification of the V2 region at 61°C annealing temperature.

Lane 1 : 123 bp marker,
2 : *S. typhimurium*,
3 : *S. raus*,
4 : *S. wandsworth*,
5 : *S. hvittingfoss*,
6 : *S. paratyphi* B,
7 : *S. paratyphi* A,
8 : *S. typhi* (1106),
9 : *S. typhi* (PNG1),
10 : *S. typhi* (109894),
11 : *S. typhi* (A102).
12 : negative control

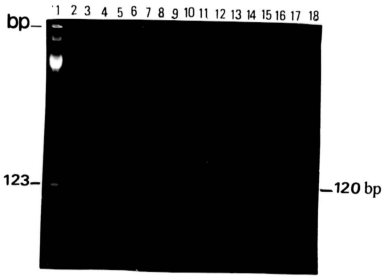

Figure 12 : Ethidium bromide-stained agarose gel (1.5%,w/v) showing PCR amplification of the V2 region at 63°C annealing temperature.

Lane 1 : 123 bp marker,
2 : negative control,
3 : *S. blockley*,
4 : *S. bovismorbificans*,
5 : *S. chingola*,
6 : *S. enteritidis*,
7 : *S. hvittingfoss*,
8 : *S. paratyphi* A,
9 : *S. paratyphi* B,
10 : *S. paratyphi* C,
11 : *S. typhimurium*,
12 : *S. wandsworth*,
13 : *S. typhi* (1106),
14 : *S. typhi* (819),
15 : *S. typhi* (495),
16 : *S. typhi* (A102),
17 : *S. typhi* (PNG4),
18 : *S. typhi* (109894).

Table 5 : Effect of different $MgCl_2$ concentrations on PCR

PCR CONDITIONS		SAMPLES																								
		Salmonella blockley	*S. bovismorbificans*	*S. chingola*	*S. enteritidis*	*S. houten*	*S. Hvittingfoss*	*S. mateperi*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*	*S. taus*	*S. typhimurium*	*S. wandsworth*	*S. weycross*	*S. typhi/(1106)*	*S. typhi/(819)*	*S. typhi/(111)*	*S. typhi/(495)*	*S. typhi/(PNG1)*	*S. typhi/(PNG4)*	*S. typhi/(VC3112)*	*S. typhi/(MM814)*	*S. typhi/(A102)*	*S. typhi/(A105)*	*S. typhi/(109894)*	*S. typhi/(109896)*	*S. typhi/(109950)*	*S. typhi/(110098)*	*S. typhi/(110723)*	*S. typhi/(112111)*
Buffer IV dNTP TP1/TP2 *Taq* Polymerase Input DNA: 3 ng/µl AnnealingTem., 63°C Thermal Cycles : 35																														
$MgCl_2$ Concentration (mM)	0.75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.00	+	-	-	-	-	-	-	±	±	+	-	±	+	-	+	±	±	+	±	+	±	+	±	±	+	+	+	-	-	+
	3.00	+	+	-	-	-	-	-	+	±	+	-	+	+	-	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+
	3.50	+	-	-	-	-	-	-	+	±	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	4.00	+	-	-	-	-	-	-	+	±	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Key + : Positive amplification
- : Negative amplification
± : Inconsistent amplification

When 3.5 mM $MgCl_2$ was used, all the inconsistency in results disappeared and repeated PCR showed positive amplification for all the *S. typhi* isolates which previously showed inconsistent results. *S. paratyphi B* remained inconsistent throughout. Non-specific results and smearing effects were seen when concentrations of 4 mM and above was used.

PCR was also performed at different concentrations of $MgCl_2$ and annealing temperatures. Two samples, *S. wandsworth* and *S. typhi* (1106) were selected to be tested and Figure 13 shows the results of PCR amplification at 60°C annealing temperature for different $MgCl_2$ concentrations. No amplification was seen at 0.75 and 1 mM for both samples but at 2 and 3 mM there was amplification for *S. wandsworth* but *S. typhi* was amplified only at 2 mM. Figure 14 shows results of PCR amplification at two different annealing temperatures, 63°C and 65°C at two different $MgCl_2$ concentrations, 2 and 3 mM for *S. wandsworth* and *S. typhi* (1106). Amplification was seen for both samples at 2 and 3 mM $MgCl_2$ concentration at 63°C annealing temperature while no amplification was seen at 65°C annealing temperature except for *S. typhi* at 2 mM. After repeated amplification and optimization, the results obtained at 63°C annealing temperature and 3.5 mM $MgCl_2$ concentration were consistent with good reproduciblity. All the inconsistency in amplification results for *S. paratyphi* A, *S. paratyphi* B, *S. typhimurium* and the *S. typhi* isolates which were previously inconsistent disappeared.

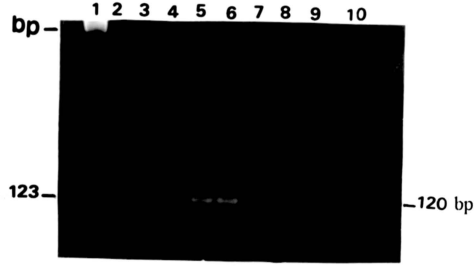

Figure 13 : Ethidium bromide-stained agarose gel (1.5%, w/v) showing PCR amplification of the V2 region at 60°C annealing temperature at different concentrations of $MgCl_2$ given in brackets.

Lane 1 : 123 bp marker,
2 : negative control,
3 : *S. wandsworth* (0.75 mM),
4 : *S. wandsworth* (1 mM),
5 : *S.wandsworth* (2 mM),
6 : *S.wandsworth* (3 mM),
7 : *S. typhi* 1106 (0.75 mM),
8 : *S. typhi* 1106 (1 mM),
9 : *S. typhi* 1106 (2 mM),
10 : *S. typhi* 1106 (3 mM).

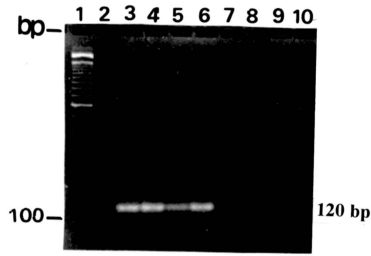

Figure 14 : Ethidium bromide-stained agarose gel (1.5%,w/v) showing PCR amplification of the V2 region at two different annealing temperatures lanes 3-6 (63°C) and lanes 7-10 (65°C) and different $MgCl_2$ concentrations (given in brackets).

Lane 1 : 100 bp marker,
2 : negative control,
3 : *S. wandsworth* (2 mM),
4 : *S. wandsworth* (3 mM),
5 : *S. typhi* 1106 (2 mM),
6 : *S. typhi* 1106 (3 mM),
7 : *S. wandsworth* (2 mM),
8 : *S. wandsworth* (3 mM),
9 : *S. typhi* 1106 (2 mM),
10 : *S. typhi* 1106 (3 mM).

3.5.3 Effect of Different DNA Concentrations on PCR

Having fixed the annealing temperature at 63°C and $MgCl_2$ concentration at 3.5 mM conditions, which invariably gave the best results, the effect of different DNA concentrations on PCR was studied.

PCR was performed on a range of different DNA concentrations as shown on Table 6. At 1.25 ng/µl DNA concentration, no amplification was seen for any of the samples. This clearly indicated that the amount of template used was too low for amplification. In contrast, 3.75 ng/µl of DNA gave positive PCR amplification for 7 out of 15 different *Salmonella* species which were *S. blockley, S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhimurium, S. wandsworth* and all isolates of *S. typhi* (Table 6). Increasing the amount of input DNA to 6.25 ng/µl gave the best results where amplification for *S. paratyphi* B was consistent with high reproducibility and amplification for all the other positive samples were of higher intensity (Table 6).

A total of 16 different *S. typhi* isolates were also tested under the optimized PCR conditions and all isolates generated a positive result. Sterile distilled water which was used as negative control, was negative throughout, showing that the PCR product was free from contaminants. To further obtain specific PCR conditions for *S. typhi,* other gram negative bacteria such as *Escherichia coli, Vibrio cholerae,*

Table 6 : Effect of different DNA concentrations on PCR

PCR CONDITIONS		SAMPLES																							
		Salmonella blockley	*S. bovismorbificans*	*S. chingola*	*S. enteritidis*	*S. houten*	*S. Hvittingfoss*	*S. matopeni*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*	*S. raus*	*S. typhimurium*	*S. wandsworth*	*S. waycross*	*S. typhi(1106)*	*S. typhi(819)*	*S. typhi(111)*	*S. typhi(495)*	*S. typhi(PNG1)*	*S. typhi(PNG4)*	*S. typhi(VC3112)*	*S. typhi(MM814)*	*S. typhi(A102)*	*S. typhi(A105)*
Buffer IV dNTP TP1/TP2 *Taq* Polymerase $MgCl_2$ con. : 3.5 mM AnnealingTem., 63°C Thermal Cycles : 35		-	-	-	-	-	-	-	+	±	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
Input DNA Concentration (ng/µl)	1.25	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+
	3.75	+	-	-	-	-	-	-	+	±	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
	6.25	+	-	-	-	-	-	-	+	±	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+

PCR CONDITIONS		SAMPLES																							
		S. typhi(109894)	*S. typhi(109896)*	*S. typhi(109950)*	*S. typhi(110098)*	*S. typhi(110723)*	*S. typhi(112111)*																		
Buffer IV dNTP TP1/TP2 *Taq* Polymerase $MgCl_2$ con. : 3.5 mM AnnealingTem., 63°C Thermal Cycles : 35		+	+	+	+	+	+																		
Input DNA Concentration (ng/µl)	1.25	+	+	+	+	+	+																		
	3.75	+	+	+	+	+	+																		
	6.25	+	+	+	+	+	+																		

Key + : Positive amplification
- : Negative amplification
± : Inconsistent amplification

Burkholderia pseudomallei and *Shigella sonnei* were also used as negative controls. Figure 15 shows result of PCR amplification for eight different *Salmonella* species, four different strains of *S. typhi* and two other gram negative bacteria at the optimized conditions as stated above.

3.6 Amplification of DNA from Clinical (Serum) Samples

To assess the possible clinical applications of the study, a collection of serum samples were also included. These serum samples could be classified as follows:

1. Widal test positive; culture positive,
2. Widal test positive; culture negative,
3. Widal test negative; culture negative.

DNA was extracted from these samples according to the method described in Section 2.5.2 and the optimized PCR conditions were used to amplify the samples using primers TP1 and TP2. Figure 16 shows the results of PCR amplified serum samples, where all the Widal and culture positive samples were amplified while none of the Widal and culture negative samples were amplified. Of the 5 samples which were Widal positive and culture negative, 2 were amplified.

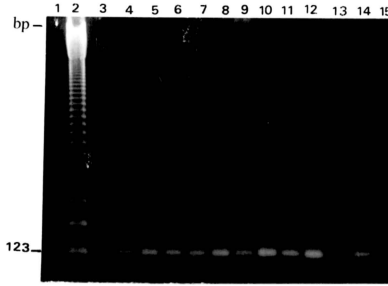

Figure 15 : Ethidium bromide-stained agarose gel (1.5%, w/v) showing PCR amplification of the V2 region at optimized conditions as stated below:

Lane 1 : negative control,
2 : 123 bp marker,
3 : *S. enteritidis*,
4 : *S. blockley*,
5 : *S. paratyphi* A,
6 : *S. paratyphi* B,
7 : *S. paratyphi* C,
8 : *S. wandsworth*,
9 : *S. typhimurium*,
10 : *S. typhi* (1106),
11 : *S. typhi* (A102),
12 : *S. typhi* (819),
13 : *B. pseudomallei*,
14 : *S. typhi* (MM814),
15 : *V. cholerae*.

PCR conditions:

1. Concentration of dNTP	1 mM
2. Concentration of primers	0.2 μM
3. *Taq* DNA polymerase	0.5 Unit
4. $MgCl_2$ concentration	3.5 mM
5. Target DNA concentration	6. 25 ng/μl
6. Denaturation step	94°C, 1 minute
7. Annealing step	63°C, 1 minute
8. Extension step	72°C, 1 minute

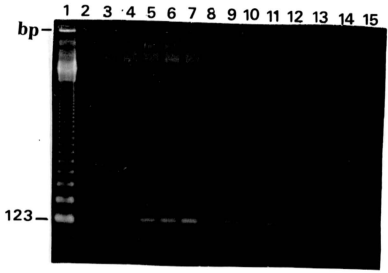

Figure 16 : Ethidium bromide-stained agarose gel (1.5%,w/v) showing PCR amplification of DNA from clinical (serum) samples at optimized conditions.

Lanes 3-7 [Widal+, culture+]
8-12 [Widal+, culture-]
13-15 [Widal-, culture-]

Lane 1 : 123 bp marker,
2 : negative control,
3 : W^+C^+ 1,
3 : W^+C^+ 2,
5 : W^+C^+ 3,
6 : W^+C^+ 4,
7 : W^+C^+ 5,
8 : W^+C^- 1,
9 : W^+C^- 2,
10 : W^+C^-3,
11 : W^+C^-4,
12 : W^+C^-5,
13 : W^-C^-1,
14 : W^-C^-2,
15 : W^-C^-3.

3.7 Southern Hybridization with the PCR-amplified V2 Region of *S. typhi* as the Probe

The specificity of the amplified products was established by a series of hybridizations experiments. Firstly, a direct Southern transfer was done of the PCR products of *S. blockley, S. enteritidis, S. typhimurium, S. hvittingfoss, S. paratyphi* A, B and C, *S. wandsworth, S. typhi* (1106), *S. typhi* (MM 814) and probed with *S. typhi* (1106) genomic DNA PCR product. The optimal conditions for washing the membrane after probing were determined by the instructions of the manufacturer (Section 2.5.9.8) and by trial and error. The hybridization results are shown in Figure 17 where strong binding was observed between the labeled probe, PCR amplified V2 region of *S. typhi* (1106) and PCR products of *S. blockley, S. paratyphi* A, B, C, *S. typhimurium, S. wandsworth* and both the *S. typhi* strains. The result was consistent with the PCR result thus confirming the high degree of homology between these strains.

Another Southern transfer was performed using genomic DNA (100 ng/μl) from a series of *Salmonella* species, *S. paratyphi* A, B and C, *S. typhimurium, S. wandsworth, S. typhi* (1106), and two gram negative bacteria, *Burkholderia pseudomallei* and *Vibrio cholerae.* was probed with *S. typhi* (1106) genomic DNA PCR product (Figure 18). The result obtained showed strong binding for genomic DNA of *S. typhi* (1106) and genomic DNA of *S. paratyphi* A, B and C and also faint binding for *S. typhimurium* and *S. wandsworth.*

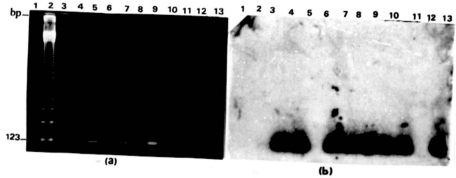

Figure 17 : Southern hybridization of the V2 PCR-amplified product of *S.typhi* (1106) probe to PCR amplified V2 regions of *Salmonella* species and other gram negative bacteria.

(a) Ethidium bromide-stained agarose gel (1.5%,w/v).

Lane 1 : negative control,
2 : 123 bp marker,
3 : *S. enteritidis*,
4 : *S. blockley*,
5 : *S. typhimurium*,
6 : *S. hvittingfoss*,
7 : *S. paratyphi* A,
8 : *S. paratyphi* B,
9 : *S. typhi* (1106),
10 : *S. paratyphi* C,
11 : *S. wandsworth*,
12 : *B. pseudomallei*,
13 : *S. typhi* (MM814).

(b) Autoradiograph of Southern blot hybridization of the gel in (a) with the PCR-amplified V2 region of *S. typhi* (1106) as the probe.

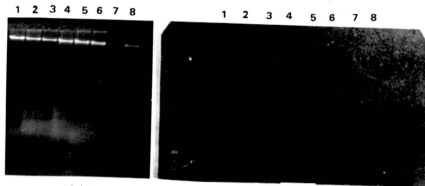

Figure 18 : Southern hybridization of the PCR amplified V2 region of *S. typhi* (1106) probe to genomic DNA (100 ng/ µl) of *Salmonella* species and other gram negative bacteria.

(a) Ethidium bromide- stained agarose gel (0.5%,w/v) of genomic DNA.

Lane 1 : *S. typhi* (1106),
2 : *S. typhimurium*,
3 : *S. wandsworth*,
4 : *S. paratyphi* A,
5 : *S. paratyphi* B,
6 : *S. paratyphi* C,
7 : *Burkholderia pseudomallei*,
8 : *Vibrio cholerae*.

(b) Autoradiograph of Southern blot hybridization of the gel in (a) with the PCR-amplified V2 region of *S. typhi* (1106) as the labeled probe.

There was no hybridization detected between the probe and other samples. This indicated a high degree of homology between the labeled PCR product and the target, genomic DNA of *S. typhi, S. paratyphi* A, B, C and also *S. wandsworth* and *S. typhimurium.*

3.8 Slot-Blot Hybridization with the PCR-amplified V2 Region as the Probe

Two slot-blot hybridizations were conducted at different stringency conditions to demonstrate the specificity of the PCR product and because stringency is measured by high temperature and low salt concentration of the hybridization wash, the temperature of the first slot-blot wash was lower as compared to the temperature of the second slot-blot wash. In the first hybridization experiment, genomic DNA from various *Salmonella* species and three other gram negative bacteria was immobilized on hybridization membrane and probed with PCR amplified V2 region of *S. typhi* (1106). Figure 19 shows the result obtained for the first slot-blot, where strong binding was observed between the labeled probe and PCR amplified V2 region of *S. typhi* (1106) which was used as a positive control and also with genomic DNA of *S. blockley, S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhimurium, S. wandsworth,* and also to *S. typhi* (1106) and *S. typhi* (819). *S. typhi* (PNG1) and *S. typhi* (A102) bound weakly. This could be due to low concentrations of the target DNA (100 ng/μl).

There was no hybridization detected between the probe and other samples. This indicated a high degree of homology between the labeled PCR product and the target, genomic DNA of *S. typhi*, *S. paratyphi* A, B, C and also *S. wandsworth* and *S. typhimurium*.

3.8 Slot-Blot Hybridization with the PCR-amplified V2 Region as the Probe

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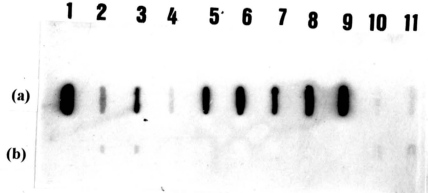

Figure 19 : Autoradiograph of Slot-blot hybridization of the PCR-amplified V2 region of *S. typhi*(1106) probe to genomic DNA (100 ng/µl) of *Salmonella* and other gram negative bacteria.

Lanes	1a : PCR product of *S. typhi* (1106),	1b : *S. bovis*,
	2a : *S. blockley*,	2b : *S. chingola*,
	3a : *S. paratyphi* B,	3b : *S. enteritidis*,
	4a : *S. wandsworth*,	4b : *S. houten*,
	5a : *S. paratyphi* C,	5b : *S. hvittingfoss*,
	6a : *S. typhi* (819),	6b : *S. matopeni*,
	7a : *S. typhimurium*,	7b : *B. pseudomallei*,
	8a : *S. paratyphi* A,	8b : *V. cholerae*,
	9a : *S. typhi* (1106),	9b : *E. coli*,
	10a : *S. typhi* (PNG1),	10b : *S. raus*,
	11a : *S. typhi* (A102),	11b : *S. waycross*.

To further assess the specificity of the probe, the second slot-blot was conducted at higher stringency also using the PCR-amplified V2 region of *S. typhi* (1106) as probe against genomic DNA of various *Salmonella* sp and two gram negative bacteria at two different genomic DNA concentrations which was 300 ng/µl Figure 20(a) and 500 ng/µl Figure 20(b). Result of the second slot-blot hybridization Figure 20(b) showed strong binding between the labeled probe and PCR amplified V2 region of *S. typhi*, which was used as positive control and also with genomic DNA of *S. typhi* (1106) of higher target DNA concentration (500 ng/µl). No hybridization was detected between the probe and other samples. This demonstrated the absolute specificity of the probe at higher stringency conditions and confirmed the origin of the probe to be from the genomic DNA of *S. typhi* (1106).

3.9 DNA Sequencing Results of the PCR-amplified V2 Region of *S. typhi* (1106) and *S. typhimurium*

Two samples were selected to be sequenced, *S. typhi* (1106) and *S. typhimurium* and the PCR amplified V2 region of both were sequenced using primers TP1 and TP2. Figures 21 and 22 compares the nucleotide sequences of the PCR-amplified V2 region of *S. typhi* and *S. typhimurium* from various sources. a and a' is the sequence data obtained in this study, by sequencing the PCR-amplified V2 region of *S. typhi* (1106) and *S. typhimurium*. b/b' and c/c' are the sequences of the same region as reported by Chang *et al.* (1997) (Aligned at the

Genestream Ssearch Network server at CRBM Montpellier, France) and Subramaniam, G (personal communication) respectively. There was 100% homology between the sequences obtained in this study with those obtained by Subramaniam, G for *S. typhi* and *S. typhimurium*. A high degree of homology about 98.75% is observed between the results of this study and the sequences reported by Chang *et al.*, 1997 with only 1.25% of mismatch for *S. typhi* and 2.5% for *S. typhimurium*. Figure 23 shows an autoradiograph of the sequence results obtained for *S. typhi* (1106).

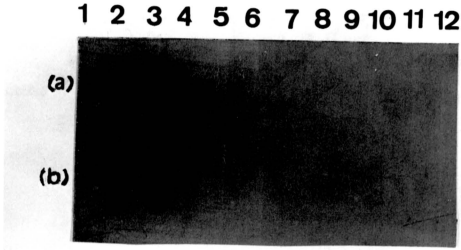

Figure 20 : Slot-blot hybridization of the PCR- amplified V2 region of *S. typhi* (1106) probe to genomic DNA of *Salmonell*a and other gram negative bacteria.

Lanes 1a-12a [genomic DNA, 300ng/μl]
1b-12b [genomic DNA, 500ng/μl]

Lane 1 (a), (b) : negative control,
2 (a), (b) : PCR amplified V2 region of *S. typhi* (1106),
3 (a), (b) : *S. typhi* (1106),
4 (a), (b) : *S. blockley*,
5 (a), (b) : *S. chingola*,
6 (a), (b) : *S. enteritidis*,
7 (a), (b) : *S. paratyphi* B,
8 (a), (b) : *S. paratyphi* C,
9 (a), (b) : *S. typhimurium*,
10 (a), (b) : *S. wandsworth*,
11 (a), (b) : *B. pseudomallei*,
12 (a), (b) : *Vibrio cholerae*.

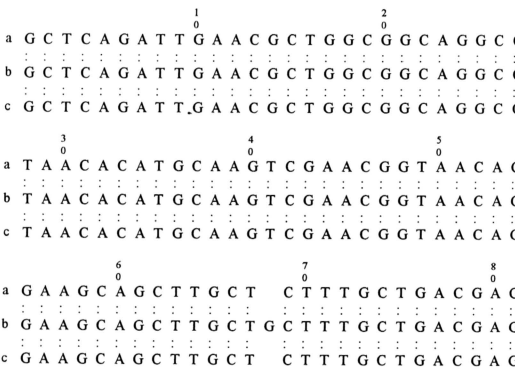

Figure 21 : Alignment of the nucleotide sequences of the PCR-amplified V2 region of the 16S rRNA gene of *S. typhi* from various sources as stated below:

a Sequences of the PCR-amplified V2 region of *S. typhi* (1106) obtained in this study.

b Chang *et al.*, 1997 (Aligned at the Genestream Ssearch Network Server at CRBM Montpellier, France).

c Sequences of the PCR-amplified V2 region of *S. typhi* (Subramaniam, G personal communication)

Figure 22 : Alignment of the nucleotide sequences of the PCR-amplified V2 region of the 16S rRNA gene of *S. typhimurium* from various sources as stated below:

a' Sequences of the PCR-amplified V2 region of *S. typhimurium* (1106) in this study.

b' Chang *et al.*, 1997 (Aligned at the Genestream Ssearch Network Server at CRBM Montpellier, France).

c' Sequences of the PCR-amplified V2 region of *S. typhimurium* (Subramaniam, G personal communication)

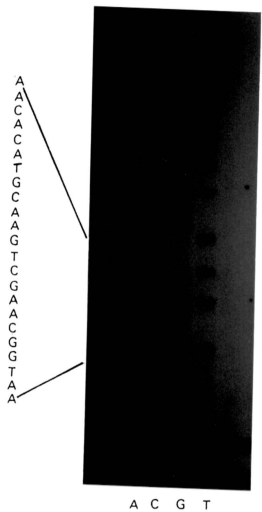

Figure 23 : An autoradiograph showing sequence results of the PCR amplified V2 region of *S. typhi* (1106) using primers TP1 and TP2.