4.0 DISCUSSION

In many developing countries in the tropical parts of the world, typhoid fever remains an important cause of morbidity and mortality with an estimated annual global incidence of 16.6 million cases and 600,000 deaths (Pang et al., 1995). Foodborne salmonellosis is also one of the more prevalent diseases in many parts of the world. The approaches of molecular biology and recombinant DNA technology has had an important impact on the diagnostic options available in diagnosing the various salmonellosis. Because rapid identification is important for clinical management of patients, and also in public health practice with regard to the containment of possible outbreaks, the polymerase chain reaction (PCR) would be a rapid and sensitive method for the detection of the causative organism in preventing the spread of foodborne outbreaks as well as in clinical diagnosis.

In this study, the PCR approach was used to amplify two variable regions in the 16S rRNA gene of *Salmonella* species, to optimize PCR conditions in the attempt to obtain a *S. typhi*-specific product, to characterize the PCR product by sequencing to confirm the origin of the product, and to determine if the optimized PCR conditions could be applied for serum samples for diagnostic purposes.

4.1 Amplification of rRNA Gene Regions

Because some segments of rRNA are conserved while others are variable to different degrees (Gutell *et al.*, 1987), it is possible to generate oligonucleotides which are complementary to segments that are specific for any level of the phylogenetic tree from kingdom to species (Gobel *et al.*, 1987; Giovanni *et al.*, 1988). PCR using oligonucleotide primers directed at sequences conserved throughout the eubacterial kingdom has been used to to amplify the 16S ribosomal RNA gene of a range of genera e.g. *Staphylococcus, Clostridium, Neisseria, Mycobacterium, Rickettsia* and several others (Wilson *et al.*, 1990). Barry *et al.* (1990) have used selective primers to amplify species specific sequences identified in the V2 and V6 regions of *Aeromonas salmonicida*. The primers used were based on the sequence available from the *E. coli* 16S rRNA gene (Brosius *et al.*, 1981) and have been used to amplify unknown sequences from a wide range of eubacteria. This method has been found useful for increasing the amounts of bacterial 16S ribosomal RNA gene sequences for the purposes of sequencing and probing. In addition, this method also has a broad range of application in the detection and identification of known pathogens that are difficult to culture.

In an earlier study by David *et al.*, 1990, which was concentrated on the V2 region of the 16S rRNA gene, they reported that PCR can detect as little as 1 pg of *S. typhi* DNA. Using only a single set of PCR parameters, they concluded that at an early stage, PCR was unable to detect DNA from sera and leukocytes of typhoid

patients. Further study was conducted on the two variable regions, V2 and V6 in the 16S rRNA gene of *Salmonella* sp to fully evaluate the usefulness of PCR in detection of DNA in clinical specimens and to design a DNA probe for *S. typhi*. According to studies done by Barry *et al.* (1990) working on *Aeromonas* sp, the V6 region exhibited greater variation between species than any of the other variable regions in the 16S rRNA gene. In another study conducted by Zuraina *et al.* (1992) it was reported that the V2 region appeared to be more specific for *Salmonella* compared to that of V6, but no conclusive results were reported . As a result, a more systematic and thorough evaluation was required in order to fully validate the results.

4.2 PCR Optimization

The objective of this part of the study was to amplify two regions, V2 and V6, of fifteen species of *Salmonella*. Results from agarose gel electrophoresis of PCR-amplified products (Figures 7 and 8) showed that both regions V2 and V6 were amplified using primers TP1-TP2 and TP3-TP4 respectively. The region V2 appeared to be more specific for certain salmonellae than V6 because the number of PCR-amplified products of V2 was less than V6 at all the different PCR conditions tested. Figure 9 shows that at 65°C annealing temperature, only the V6 region amplified. Repeated PCR amplification of the V2 region at 65°C annealing

temperature failed to yield any results. Due to the apparent specificity of the V2 region, further work in this study was concentrated only on the V2 region.

In PCR, primer concentrations of between 0.1 and 0.5 μM are generally considered optimal. High primer concentrations may promote mispriming and accumulation of non-specific products and increase the probability of generating a template independent artifact termed as primer-dimer (Innis and Gelfand, 1990). For reasons of economy, the lowest primer concentrations that provide maximal sensitivity and specificity was used (0.3 μM). Figure 10 shows that repeated amplification of the V6 region using diluted primers showed increased yield of the PCR-product and the disappearance of primer-dimers. For any PCR reaction, the production of large amounts of primer artifact is something to be avoided, because excess primer artifact reduces the amplification efficiency of the intended target (Mullis, 1991; Chou *et al.*, 1992).

The specificity and fidelity of PCR are increased by using lower dNTP concentrations. Low dNTP concentrations minimize mispriming at non-target sites and reduce the likelihood of extending misincorporated nucleotides (Erlich *et al.*, 1991). The presence of chelators in the primer stocks may disturb the apparent magnesium optimum. Optimization of the Mg^{2+} concentration is usually performed in order to regulate primer specificity.

Typical denaturation conditions are 95°C for 30 seconds or 97°C for 15 seconds. However higher temperatures may be appropriate, especially if the target is G+C rich. A higher denaturation temperature or too long a denaturation step may lead to unnecessary loss of enzyme activity, since the half-life of *Taq* DNA polymerase is more than two hours at 92.5°C, 40 minutes at 95°C, and 5 minutes at 97.5°C (Innis and Gelfand, 1990).

An increase in specificity was noticed in the results when higher annealing temperatures were used. Annealing temperature in the range of 55°C to 72°C generally yielded the best results although in this study it was found that at 65°C annealing temperature, there was no amplification for any sample for the V2 region. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and also reduces misextension of incorrect nucleotides at the 3'-end of primers. Although higher annealing temperatures will increase specificity of PCR (Innis and Gelfand, 1990), too high an annealing temperature will lead to a loss of sensitivity. Because the best results were obtained at 63°C annealing temperature, this temperature was fixed and subsequent optimization was done at this temperature.

In this study, PCR amplification at different concentrations of $MgCl_2$ showed that higher concentration of $MgCl_2$ promotes amplification while low concentration led to failure of amplification. The magnesium ion concentration should be calculated as a function of nucleotide concentration. For most PCR

protocols, a range of magnesium concentrations between 0.5 and 3.0 mM above the total nucleotide concentration is usually tested. Generally, excess Mg^{2+} will result in the accumulation of non-specific products, whereas too little Mg^{2+} will reduce PCR yield (Saiki *et al.*, 1988). The magnesium concentration may affect primer annealing, strand dissociation temperatures of both templates and PCR product, product specificity, formation of primer dimer artifacts, and enzyme activity and fidelity (Innis and Gelfand, 1990).

Because the best results obtained as yet were at 63°C annealing temperature and 3.5 mM $MgCl_2$, these conditions were fixed and the effect of various DNA concentrations on PCR was determined and was found that at 6.25 ng/µl of DNA all inconsistency in PCR amplification including those that appeared for *S. paratyphi* B was successfully eliminated. This showed that higher template DNA was important in optimizing the PCR conditions.

To obtain specific conditions for *S. typhi*, other gram negative bacteria such as *Escherichia coli, Vibrio cholerae, Burkholderia pseudomallei* and *Shigella sonnei* were also tested under all the PCR optimizing conditions and used as negative controls. Initial PCR amplification results show faint amplification for the V6 region of *E. coli* but not for the other gram negative bacteria. This was partly the reason for concentrating on the V2 region for all the subsequent PCR optimization work. All the PCR optimization conditions were targeted to eliminate

amplification for all of these bacteria and were included as negative controls for all the optimization conditions stated above.

Among the other important factors that were optimized were *Taq* concentration and the number of thermal cycles performed. The number of thermal cycles for all the optimization steps was fixed at 35 cycles because no significant difference in results was observed when a range of 25 cycles to 45 cycles was performed in the thermal cycler. To optimize enzyme concentration, the PCR reaction was usually tested empirically at a variety of concentrations between 0.5 and 5 U/100-µl reaction (Innis and Gelfand.,1990). Best result in terms of sensitivity and specificity was obtained when 0.025 units of *Taq* DNA polymerase was used in a 20 µl reaction mixture and thus the enzyme concentration was fixed at this value. When enzyme concentrations are high, nonspecific priming will occur while concentrations that are too low will result in poor amplification efficiency

In an attempt to improve reaction specificity, the hot-start technique, where an essential component of the reaction e.g. polymerase, Mg^{2+} or primers was kept separate from the target until an elevated temperature was reached, was employed in all PCR reactions. In a typical PCR reaction, the non specific extension products synthesized during the setup period may have deleterious effects on reaction specificity and sensitivity. The use of a hot start can lead to enhanced sensitivity for some systems, especially those for which nonspecific product formation is a problem (Chou *et al.*, 1992).

The optimal condition used for PCR amplification gave a clean, single band (Figure 15). The optimal condition was: dNTP, 1mM; primers, TP1, 0.38 μM / TP2, 0.33 μM; *Taq* DNA polymerase, 0.025 Unit; target DNA, 6.25 ng/μl and $MgCl_2$ 3.5 mM in a final volume of 20μl reaction mix. The thermal profiles were:

denaturation step : 94°C for 1 minute,

annealing step : 63°C for 1 minute,

extension step : 72°C for 1 minute.

These PCR conditions were found to be the most optimum and therefore yielded the best results in terms of specificity, sensitivity and reproducibility. The PCR results for the V2 region after optimization showed amplification for *S. blockley, S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhimurium, S. wandsworth* and all *S. typhi* isolates. Although the optimized PCR conditions did not yield a *S. typhi*-specific product, there was a significant reduction in the number of amplified products, showing that all other possible non-specificity has been eliminated. One possible explanation for the results could be that these species are closely related among salmonellae and the other is that there is limited heterogeneity in the V2 region among the different *Salmonella* species, thus making the detection of a *S. typhi*-specific product unlikely with this set of primers.

In this study the PCR-amplified V2 region was about 120 bp, which is in accordance to the size reported by Barry *et al.* (1990) working on *Aeromonas*

salmonicida. Agarose gel electrophoresis also showed that the negative controls used in all the PCR reactions were clean and that all the amplified products were of good intensity. Because carryover contamination is mainly through aerosols created by handling of PCR products, initial attempts to control contamination included physical separation of pre- and post amplification products (Kwok and Higuchi, 1989). Several other sophisticated techniques available for inactivation of amplification products include photochemical, enzymatic, and chemical modifications of the nucleotide bases or sugars of the amplification products, where the altered strands were prevented from acting as templates for *Taq* DNA polymerase extension reactions. However, irradiating the reaction tubes with UV light following amplification was found sufficient in this study.

4.3 PCR amplification for clinical samples

To determine if the PCR optimized conditions would work for clinical samples, PCR was performed using DNA from a range of serum samples. Because the target microorganism is *Salmonella* which is predominantly intracellular, serum would probably contain less microorganism compared to whole blood or purified leukocytes. However, patients with even low titres of circulating bacteria could be detected by PCR. A collection of serum samples diagnosed using conventional methods such as bacterial culture and Widal test was used to determine if the optimized PCR conditions would be applicable in diagnosis.

All the Widal and culture positive samples were positive while the Widal and culture negative samples were negative showing that the optimized conditions were applicable for the serum samples. The fact that some Widal positive, culture negative samples were PCR positive suggests the possibility that PCR may be more sensitive than culture. Related to this is the fact that although isolation of *Salmonella* species from various body specimens provides a conclusive diagnosis, it is a time consuming process and sometimes can give false negative result owing to prior antibiotic therapy. Also confirmative results require both acute and convalescent phase serum samples that are rarely collected. Nevertheless the optimized PCR conditions were found to work quite adequately for clinical samples and seemed rather promising.

4.4 Southern and Slot-blot Hybridization Using PCR-amplified V2 region as probe

Because the specificity and speed of a hybridization reaction is determined by temperature, salt concentration, pH, probe concentration and probe size (Towner and Cockayne, 1993), the greater the stringency, the more successful it is for a specific hybridization to occur. In this study, Southern and slot-blot hybridizations were conducted to determine which of the two would be a more specific method when short oligonucleotides were used as probes.

The slot-blot hybridization technique employed in this study as compared to the Southern hybridization differed in three main aspects, which are summarized in Table 7 below:-

Table 7 : The different conditions of Southern and slot-blot hybridization performed in this study

	Southern hybridization	slot-blot hybridization (I)	slot-blot hybridization (II)
DNA transfer	Capillary	Vacuum	Vacuum
Labeling of probe	ECL kit	^{32}P	^{32}P
Hybridization wash conditions	42°C	42°C	45°C,52°C and 55°C

Results of Southern hybridization (Figure 16) showed that although the results obtained were consistent with PCR amplification and that the hybridized samples had high degree of homology to the probe, the probe itself did not bind specifically to *S. typhi*. In the case of the two slot-blot hybridizations conducted at different stringency conditions in terms of different hybridization wash temperatures showed that high target DNA concentration (500 ng/µl) was important for effective binding and confirmed the absolute specificity of the probe at higher stringency conditions.

The high specificity of the probe in slot-blot hybridization as compared to Southern hybridization could be due to the method of probe labeling employed in the slot-blot technique. Studies on labeling methods of short oligonucleotides or

probes below 300 bp showed the radioactive labeling to be superior than the chemiluminesence reaction of the ECL kit. Another explanation for the specificity of the probe in slot-blot hybridization is the stringency of the wash determined by the temperature which was greatly increased compared to the temperature of wash in Southern hybridization which was fixed at 42°C, according to the manufacturer's instructions. In this study, the absolute specificity of the probe was demonstrated using higher stringency conditions with a slot-blot hybridization and hence the slot-blot was found to be a more effective method as compared to Southern particularly when using short oligonucleotides as probes.

4.5 DNA sequencing of the PCR-amplified product of the V2 region

In contrast to standard identification protocols, direct 16S rRNA gene sequence determination is rapid and results in a definitive and unambiguous identification. This approach allows the prompt recognition and characterization of previously unrecognized species as analysis of 16S rRNA sequence enables the study of phylogenetic relationships among bacteria (Woese, 1987). Unlike phenotypic and biochemical features, the 16S rRNA sequence of a species is a stable property which is specific for microorganisms at a species level. The characterization of a novel 16S rRNA gene sequence with the appropriate phenotypic data including the sequences of all established species belonging to the

genus of the isolate in question allows an isolate to be established as a genetically distinct and unique taxon and subsequently increase our understanding of microbial diversity.

Analysis of sequences result obtained in this study, showed the V2 region to be identical for both *S. typhi* (1106) and *S. typhimurium* in agreement with the work of Subramaniam, G (unpublished data).The single gap detected at position 68 in the PCR product of the V2 region obtained in this study as compared to the results of Chang *et al.*, 1997, can be attributed to the differences in *S. typhi* and *S. typhimurium* strains that were used and it was necessary to introduce it in order to obtain a proper alignment. The high homology in this otherwise "variable" region could be the reason for PCR amplification for all *S. typhi* isolates as well as *S. paratyphi* A, B, and C, *S. typhimurium, S. blockley* and *S. wandsworth.* Although the V2 region of *S. paratyphi* A, B and C, *S. blockley* and *S. wandsworth* was not sequenced, it is very likely that the V2 region of these species could also be very similar to that of *S. typhi.*

In addition, it also appears that there exists more heterogeneity in the V6 region. The sequence results in this study is also supported by the ones obtained by Barry *et al.*, 1991. They found that within the V2 region, the DNA sequence is almost identical between *A. salmonicida* and related species, differences ranging from one to four base pairs whereas the V6 region shows differences of two to forty base pairs. However even this "variable" region is quite invariant within

closely related organisms e.g. *Mycobacteria* species, making it an unsuitable target for the generation of DNA probes (Rogall *et al.*, 1990). As a result, a more hypervariable region was sought and Barry *et al.* (1991) found significant sequence heterogeneity within the eubacterial 16S/23S spacer region at the genus and species level. However conflicting results were reported by Gardes *et al* .(1991), working with closely related symbiotic fungi.

Ribosomal RNA in general has been the main target for the generation of DNA markers for microorganisms, and this region has been used as the target for DNA probes for a number of microorganisms (Barry *et al.*,1991). By developing a series of DNA probes directed towards rRNA which is often present in microbes in upwards of 10,000 copies/cell, it is possible to choose probes that can detect rRNA sequences which are present only within a given sub-species, species or genus (Kohne *et al.*, 1986). In this study, primers, which were based on sequences available from the *E. coli* 16S rRNA gene that can be used to amplify unknown sequences from a wide range of eubacteria have been used. The simplicity of the procedure, allows the generation of primary sequence data very rapidly. Because the sequence analysis of the V2 region for closely related species of the genus *Salmonella* does not differ significantly, it was difficult to generate a suitable probe for *S. typhi* based on this region although it appeared to be quite specific by DNA-DNA hybridization technique at higher stringency conditions used in a slot-blot hybridization. However, more work has to be directed into this area before it could be used as a DNA probe for *S. typhi* similar to the probe derived from the V6

region that has been shown to be successful for *Aeromonas* species and thus could be used to trace contaminants in clinical, food or veterinary samples.

4.6 Suggestions for further work

The optimized PCR conditions established in this study were found to be quite promising when used on DNA from serum samples. This suggests the possible use of the 16S rRNA as a genus-specific clinical probe , but further investigation is needed to identify species- specific probes as diagnostic tools for *Salmonella* spp.