1.0 INTRODUCTION

1.1 Salmonellosis

Salmonellosis, by definition, describes all the diseases caused by bacteria in the genus *Salmonella* which can be either enteric fever or non-typhoidal acute gastroenteritis. It is a serious public health problem in many geographical areas where outbreaks frequently occur especially in many developing countries but epidemics have also been reported from developed countries (Pang, 1995).

1.1.1 Classification and Characteristics

The taxonomy of the *Salmonella* is exceedingly complex. At present, the genus *Salmonella* is recognised to consist of only 1 species. This single *Salmonella* species has been named *Salmonella enterica* (Le Minor, 1984) and it is comprised of 7 subspecies. The subdivision into these subspecies is dependent on biochemical reactions of the strains with dulcitol, lactose, O-nitrophenol-β-D-galactopyranoside (ONPG), salicin, D-tartrate, mucate, gelatinase, sorbitol and potassium cyanide (KCN). This biochemical subdivision has been supported by DNA/DNA hybridization and serological methods. The formal subspecies epithets are *Salmonella enterica* subsp. *enterica*(1), subsp. *salamae*(II), subsp. *arizonae*(IIIa), subsp. *diarizonae*(IIIb), subsp. *houtenae*(IV), subsp. *bongori*(V), and subsp.

indica(VI). Each subspecies in turn is divided into serovars/serotypes according to the O-antigen (somatic) and H-antigen (flagellar) specification. Majority of the *Salmonella* strains (99.7%) isolated from humans belong to subspecies 1 which includes *Salmonella typhi* (Le Minor, 1988).

Salmonella typhi is a gram-negative, facultatively anaerobic, non-spore forming bacilli. It has 3 major antigens: H-antigen (flagellar), O-antigen (somatic) and Vi-antigen (capsular). Most strains ars motile by means of peritrichous flagella but non-motile strains also exist. *S. typhi* produces acid from glucose, maltose and sorbitol without the production of gas, does not ferment lactose, sucrose, rhamnose and few other sugars. Nitrite is produced from nitrates and hydrogen sulfide production is observed. Optimum growth temperature is 37°C and they are able to grow on a wide range of relatively simple media that distinguishes it from other members of the *Enterobacteriaceae* family (Holt, 1984).

1.1.2 Disease Syndromes

Enteritis-salmonellosis in man is characterized as a self-limiting, local infection and occurs particularly as a food-borne disease. The typical signs and symptoms of *Salmonella* induced acute gastroenteritis are profuse, non-bloody diarrhoea, with rapid dehydration and abdominal cramps, nausea and vomiting, generally with or without mild fever. Clinical signs begin suddenly and relatively short after the infection and persist for 2 to 5 days (Tshäpe and Prager, 1995).

Many *Salmonella* serovars such as *S. typhimurium*, *S. enteritidis* and *S. heidelberg* have been observed as pathogens of gastroenteritis in human being whereas only a small number of serovars such as *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, *S. dublin*, *S. choleraesuis* remain associated with systemic salmonellosis known as typhoid fever.

Typhoid fever is acquired by ingestion of food or water that has been directly or indirectly contaminated by excreta (primary faeces) of infected persons including carriers (Weissfeld *et al.*, 1994). The onset of typhoid fever is characterized by a long incubation period ranging from 1 week to 1 month after initial ingestion of the bacteria. Once ingested, *S. typhi* makes its way through the digestive system to the ileum of the small intestine where it is thought to gain access to the submucosa by invasion of M cells (Kohbata *et al.*, 1986) or through direct penetration of intestinal epithelial cells (Yobuchi *et al.*, 1986; Yokoyama *et al.*, 1987). Once inside the body, *S. typhi* enters the blood stream, survives in mononuclear phagocytes (macrophages), and invades and multiplies within the liver and spleen. Eventually *S. typhi* moves from the liver to the gall bladder and is shed in bile into the intestine often resulting in intestinal ulceration, which may be fatal if left untreated (Huckstep, 1962; Salyers and Whitt, 1994; Owen, 1994). In some people *S. typhi* persists in the gall bladder and these people can shed bacteria in their faeces for a number of years and are known as chronic carriers (Salyers and Whitt, 1994).

1.1.3 Diagnosis, Treatment and Prevention.

Diagnosis of *Salmonella* infection is made by isolation of the organism from blood, faeces, urine, bone marrow aspirate and bile of patients. The culture method is often supplemented by a variety of serological tests, including the classical Widal test. A variety of immunological tests based on indirect haemagglutination, latex agglutination, counter immuno-electrophoresis, fluorescent antibody, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay, have also been used to complement the culture and Widal tests. However, these methods have been associated with several problems, including time factor (it takes several days before results can be obtained from the culture method), insensitivity owing to the multiplicity of serotypes, low specificity, a high percentage of false positives or negatives owing to cross reactivity, low number of organisms in clinical specimens and the need for enrichment procedures. Many of the problems encountered can be overcome by using molecular-based approaches (Pang,1995).

Typhoid fever is usually treated by antibiotics. Typical regimes for treatment of *S. typhi* infection include amoxycillin, cotrimoxazole and chloramphenicol (Varnam and Evans, 1991). Unfortunately, antibiotic resistance (multiple resistance) is increasing in frequency among *S. typhi* isolates and is becoming a serious global problem. Some of the regions where these resistant strains have spread to include the Indian subcontinent, Latin America, Egypt, Nigeria, Greece and many more. Despite the existence of these resistant strains,

chloramphenicol still remains the most suitable first-line drug against typhoid fever, with the third generation cephalosporins and quinolones forming important alternatives (Pang *et al.*, 1995).

The main prevention against typhoid fever now are the oral vaccine Ty21a and the Vi-polysaccharide vaccines. Both vaccine provide up to 65-70% protection, with immunity lasting for 3-7 years (Levine *et al.*, 1989). New vaccines derived from engineered *S. typhi* strains, CVD 908 and CVD 908-*htr* A are also under development and initial testing showed that they are well tolerated and highly immunogenic after a single oral dose (Levine *et al.*, 1995). In view of the emergence of multiple antibiotic resistant strains and variable efficacies of the currently available vaccines, there may be a need for novel approaches, based on molecular informations, especially in relation to studying the genome structure of *S. typhi* and other pathogenic *Salmonella* spp. Vaccine development will require a better understanding of *Salmonella* genetics and immunogenic epitopes, as well as the physiological responses of *Salmonella* to various conditions *in vivo* during infection (Calva and Puente, 1995).

1.2 Laboratory Diagnosis of Typhoid Fever

1.2.1 Isolation and Culture

Laboratory diagnosis of typhoid fever is essentially based on culture of the pathogen, mainly from blood, and detection of antibodies, mainly using the Widal test. The positive rate of blood culture exceeds 80% if the specimen is obtained early during the first 7-10 days of the illness (Tsang and Chau, 1992). Though various culture media such as bile-containing media and culture techniques using mononuclear cell fraction, blood clot or whole blood with lysis-centrifugation have been suggested to be superior to conventional blood culture method, the latter is adequate for this purpose and is more practical (Levine *et al.*, 1995). Culture of duodenal bile obtained by the string capsule method has been reported to give positive rates approaching that of blood cultures but is relatively inconvenient to the patient (Avendano *et al.*, 1986). Stool culture is positive mainly after the 2nd week of the illness and the positive rate is low, especially after antibiotic treatment. Selective media and tedious manipulations are required. Bone marrow culture (with high positive rate) and urine culture (low positive rate) are rarely performed for the purpose of diagnosing typhoid fever (Tsang and Chau, 1992).

1.2.2 Serology

For the detection of anti-*S. typhi* O and H antibodies, the Widal test (bacterial agglutination test) is still the most widely used method. The specificity and sensitivity of the test is however greatly influenced by the timing of blood collection, the history of previous exposure due to the endemicity of typhoid fever or other salmonellosis in the locality, and the quality of the diagnostic reagent used in the test (Pang and Puthucheary, 1983). The test is of high diagnostic value when both the anti-O and anti-H antibody titres reach the diagnostic level, which is a 4-fold or greater increase in titres of O or H agglutinins, and that a single high titre is usually not significant in diagnosis . Unfortunately in a considerable proportion (up to 60%) of patients only either O or H antibody titres reach the diagnostic level (Tsang *et al.*, 1981). Serological methods other than the Widal test with the use of purified antigens have been suggested to overcome the problems with Widal test, which is neither specific nor sensitive enough (Sadallah *et al.*, 1990). Examples are ELISA or passive haemagglutination (pHA) tests with the use of purified lipopolysaccharides (LPS) or proteins (crude extract or purified porins) prepared from *S. typhi*, or synthetic trisaccharides corresponding to O factor 9 of *S. typhi*. The reported results appear to be promising and superior to the conventional Widal test (Sadallah *et al.*, 1990).

1.2.3 Antigen Detection

Detection of specific *S. typhi* antigen directly in clinical specimens is an appealing approach for rapid diagnosis of typhoid fever. Reported results concerning detection of Vi or O antigens of *S. typhi* from patient's blood or urine with the use of polyclonal antibodies by coagglutination or ELISA were however conflicting. A high false positive rate was noticed. The low specificity of this method might be explained by the possibility of these polyclonal antibodies cross-reacting with O antigens or other gram-negative bacteria (Sadallah *et al.*, 1990; Chaicumpa *et al.*, 1988). Others reported successful detection of protein antigens of *S. typhi* from patient's urine with a low false positive rate. In a preliminary study, Tsang and Chau (1987) reported that using monoclonal antibodies to Vi and to O factor 9 of *S. typhi,* they were unable to detect these antigens from patient's urine. Detection of *S. typhi*-specific DNA sequences by polymerase chain reaction (PCR) directly from clinical specimens might become practical for the rapid diagnosis of typhoid fever in the future. However, detection of *S. typhi*-specific antigens or DNA sequences is mainly used for rapid identification of the pathogen from primary culture before pure growth is obtained (Tsang and Chau, 1992).

The diagnosis of typhoid fever is a particularly challenging one as diagnostic methods have to deal with various types of clinical specimens (e.g. blood, stool, urine etc) characterized by low numbers of organisms and a multiplicity of serotypes. In terms of applications of molecular technology, DNA-based assays are

becoming popular alternatives to immunological and conventional culture methods for detection of *Salmonella* in various specimens due to their specificity and sensitivity (Pang, 1995). The most popular molecular approach to diagnosis of typhoid fever has been based on the development of genus -specific DNA probes together with procedures for amplification of target DNA using PCR.

1.2.4 DNA Detection

Nucleic acid probes are nucleic acid sequences that are used to detect complementary sequences within a sample. In other words, they are segments of DNA or RNA that have been labeled with enzymes, antigenic substrate, chemiluminescent moieties, or radioisotopes and can bind with high specificity to complementary sequences of target nucleic acid (Tenovar and Unger, 1993). The DNA probe approach to identification is unique because the focus of the method is the nucleic acid content of the organism rather than the products which the nucleic acid encodes. Following the detection of *Escherichia coli* in stool samples by DNA/DNA hybridization, various other bacterial pathogens have also been detected using DNA probes which are commercially available. DNA probes have been used to detect many common bacterial (Echeverria *et al.*, 1985; Levine *et al.*, 1987), viral, and protozoan enteric pathogens (Meloni *et al.*, 1989; Samuelson *et al.*, 1989). In general, nucleic acid sequences that are used as DNA probe for micro-organisms fall into five main categories:

1. DNA sequences that code for antigens,
2. DNA sequences that code for toxins,
3. DNA sequences identified by differential hybridization using total DNA probes from related species against a DNA bank made from the micro-organism of interest,
4. unique plasmid- borne DNA sequences,
5. ribosomal RNA (rRNA) sequences.

rRNAs in general have been the main targets for the generation of DNA markers for micro-organisms and have been used as targets for DNA probes for a number of micro-organisms (Barry *et al.,* 1990).

Rubin *et al.* (1985, 1988) reported the development of a DNA probe based on the Vi capsular antigen which was specific for *Salmonella typhi*. Although this probe was highly specific and sensitive for the detection of *S. typhi* among bacterial isolates, it could not be used to directly detect the presence of the organism in blood due to sensitivity limitations. As such, the successful use of the probe depended upon a pre-enrichment procedure where bacteria had to be grown overnight on nylon filters (on nutrient agar plates) before hybridization with the probe. This process of concentration was inevitable because patients with typhoid fever usually have less than 15 *S. typhi* cells per ml of blood, and the probe cannot detect fewer than 500 bacteria. In addition to the Vi DNA probe, other probes have been developed to detect other genes from other *Salmonella* spp (e.g.Hd flagellin gene) but most have only had limited testing on clinical samples (Rubin, 1990).

Olsen *et al.* (1991) have successfully isolated a *Salmonella*-specific DNA hybridization probe from *S. typhimurium*. The probe was evaluated in a colony hybridization assay, in which 185 strains of 93 different *Salmonella* serovars were correctly identified as belonging to *Salmonella*. They extended the evaluation of this *Salmonella*-specific DNA probe to more than 300 strains of *Salmonella* spp. (Aabo *et al.*, 1992).

The advent of recombinant DNA technology and, more specifically, the development of polymerase chain reaction (PCR) by Saiki *et al.* (1985) has opened the way to another approach in the laboratory diagnosis of infectious diseases (Hopkin and Wakefield, 1990). The PCR approach has been used successfully in the laboratory diagnosis of a variety of viruses and microorganisms that cause infectious diseases, including dengue virus (Deubel *et al.*, 1990), *Borrelia burgdorferi* (Debue *et al.*, 1991), *Chlamydia pneumoniae* (Campbell *et al.*, 1992), *Helicobacter pylori* (Clayton *et al.*, 1991), *Mycobacteria leprae* (Woods and Cole, 1989), *Treponema pallidum* (Wicher *et al.*, 1991), human immunodeficiency virus (HIV-1) (Guatelli *et al.*, 1991), and *Neisseria meningitidis*, as well as other bacterial species known to be found in the cerebrospinal fluid (Greisen *et al.*, 1994).

1.3 Polymerase Chain Reaction (PCR)

The PCR is a powerful technique that was first reported in 1985 in the prenatal diagnosis of sickle cell anaemia (Saiki *et al.,* 1985). This approach relies upon sequential amplification of specific DNA sequences present in a specimen through the use of a thermostable DNA polymerase. Double stranded bacterial DNA extracted from clinical specimen is denatured by heat and the primers allowed to anneal. Primer extension is then carried out by DNA polymerase. This cycle of denaturation, annealing and extension is then repeated up to 25-30 times in a thermocycler, resulting in a millionfold amplification of the original DNA (Figure 1).

1.3.1 PCR Optimization

The PCR is a very dynamic biochemical reaction. PCR with the thermostable *Taq* DNA polymerase is a robust technique that works well with most targets. However, adjustments that will improve specificity and yield have to be made to some of the reaction parameters. These adjustments include $MgCl_2$, primers, dNTPs, buffers and the enzyme concentrations, annealing and extension time and temperature (Saiki *et. al*., 1989). The PCR principle requires a three step-cycling process that is repeated for a certain number of cycles.

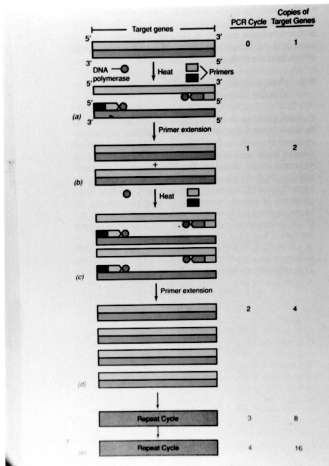

Figure1 : The polymerase chain reaction for amplifying specific DNA sequences (Brock *et al.*, 1991)

The process includes high temperature denaturation of double stranded template DNA, annealing of the oligonucleotide primers and primer extension by polymerase enzyme (Schochetman *et al.,* 1988) resulting in a million times the original amount of DNA or reverse transcribed RNA after 20-30 cycles within 3 hours. Each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle and this results in an exponential accumulation of the specific target fragments, approximately 2n, where n is the number of cycles (Saiki *et al.*, 1988).

Double stranded template DNA is denatured by briefly heating the sample to 90-95°C. Insufficient heating during the denaturation step is one of the most common causes of failure in the PCR reaction. This temperature is dependent on the length and Guanine+Cytosine (G+C) content of the fragment (Saiki *et al.*, 1989). The annealing temperature depends on the length and G+C content of the primers and extensive incubation at the annealing temperature can lead to the production of nonspecific products. The incubation time at the extension temperature depends on the length of the DNA segment being amplified and excessive incubation time will lead to the production of nonspecific amplified products (Saiki *et al.,* 1988).

A recent approach to improve PCR specificity termed " Hot-Start" describes the manual addition to the reaction tube of an essential reagent (e.g., DNA polymerase, magnesium chloride, template DNA, etc.) at elevated temperatures.

"Hot-Start" has been shown not only to improve specificity but also to minimize the formation of the "primer-dimers".

1.3.1.1 *Taq* DNA polymerase

Initially, Klenow DNA polymerase was used as the enzyme in the amplification reaction but this heat-labile enzyme had to be added in each subsequent cycle because the enzyme was inactivated after each cycle (Schochetman *et al.,* 1988). This enzyme was only able to amplify fragments not greater than 250 bp. However, the high temperature DNA polymerase from the hot-springs bacterium *Thermus aquaticus, Taq* polymerase is heat-stable and once added at the start of PCR is able to amplify sequences up to 2.0 kb long (Saiki *et al.,* 1988). The error rate of each PCR amplification vary considerably among DNA polymerases, the least accurate being those enzymes that lack a proof reading exonuclease activity (Kunkel and Eckert, 1989). The fidelity (nucleotide misincorporation frequency) of *Taq* polymerase depends upon the concentration of free Mg^{2+} and dNTPs, on whether the four dNTPs are balanced, on pH and on heat damage to the template DNA (Eckert and Kunkel, 1991)

The amount of polymerase is one of the more important factors to be optimized for a particular assay. For most assays, the optimum amount of enzyme will be between 0.5 and 2.5 units in a 50 μl reaction volume. Increased enzyme

concentrations sometimes lead to decreased specificity and reduced yield (Saiki *et al.*, 1988).

1.3.1.2 Primer

The PCR amplification involves one pair of oligonucleotide primers that flank the opposite strands of DNA sequence to be amplified. Depending on their purpose, useful primer lengths are 14 to over 40 bases long, with a G+C content ranging from 40-75%. General guidelines for primer design are similarly summarized by Rappolee (1990):

1. Primers should lie within highly conserved regions of the genome of the analyzed species.
2. 3' ends of primer should use conserved amino acids, with nondegenerate codon. (e.g. Trp and Met).
3. 3'ends of primer should avoid complementarity to prevent "primer dimer" formation and resultant waste of primers in the PCR
4. Primers should lack secondary structure
5. Primers should be specific to a single member of a gene family

Most PCR applications are mainly controlled through the design of the primers and the choice of primers combinations, concentrations and sequence modifications. Optimal concentrations of the primer vary widely: for most genomic targets the range of 0.1-1.0 μM give the best results. Excessive primer will reduce

the specificity and increase the probablity of " primer-dimer" formation that is often seen as an intense band with a size that is a sum of the two primers (Mullis, 1989).

1.3.1.3 Magnesium chloride

$MgCl_2$ has important effects on PCR specificity. Optimum concentration may be varied from approximately 0.5 mM to 5 mM and depends on the sequence being amplified and the nature of the primers. Mg^{2+} influences enzyme activity, increases T_m (melting temperature) of dsDNA, and forms soluble complexes with dNTP, which is essential for dNTP incorporation. Since nucleotides chelate divalent cations, the Mg^{2+} concentration in PCR reactions must be a few millimolar units higher than the nucleotide concentration. Mg^{2+} and other salts also influence the hybridization of primers to the template DNA.

For many optimization strategies, it is suitable to modify neither Mg^{2+} nor dNTP concentrations before all the other reaction components are optimized (Rolfs *et al.*, 1992). High concentrations of $MgCl_2$ will inhibit the PCR reaction (Gelfand, 1989).

1.3.1.4 Deoxynucleotide triphosphate (dNTP)

Optimal dNTP concentrations depends on length of amplification product, $MgCl_2$ and primer concentration and reaction stringency. Generally, a 10 mM stock solution, equimolar with each of the four dNTPs, is suitable for multi-tube assays. Imbalanced dNTP mixtures reduces *Taq* fidelity. Nucleotides are remarkably resistant to heat and have a half-life of more than 40 thermal cycles in a PCR amplification (Innis and Gelfand, 1990).

1.3.1.5 Buffers

A number of different buffers for PCR are currently in use. However, the important factors involved in optimizing PCR buffers are:

1. pH- *Taq* appears to have a pH optimum of 7.0-7.5 at 72°C. Normally a Tris buffer which is pH 8.5-9.0 at 25°C is used because the pH of Tris buffers decreases by 0.03 pH units for each degree of increase in temperature.
2. Detergents- *Taq* is a highly hydrophobic protein and tends to precipitate from aqueous solution. The addition of non-ionic detergents (Triton X-100, NP-40, or Tween-20 at a final concentration of 0.01%) helps to maintain full activity, both in storage solutions and in the amplification reaction.

3. Salts- There is no absolute requirement for salts other than Mg^{2+} in PCR. Some reports indicate that even low concentrations of KCl are inhibitory (Innis and Gelfand, 1990).

1.3.1.6 Template DNA

Template DNA, used are stored in TE (Tris-EDTA) or water. PCR does not require highly purified template DNA. Slightly degraded or sheared DNAs may be amplified more readily because they denature more easily, allowing better primer access. Only very small amounts of DNA should be used. Genes present in higher copy number (e.g.. mitochondrial DNA sequence) can be amplified from smaller quantities of template.

1.3.2 Limitations of PCR

1.3.2.1 Error rate of *Taq* Polymerase

Taq has a relatively high error rate. Base substitutions occur at a rate of about one per 9000 bp and frameshifts occur about one per 40 000 bp(Tindall and Kunkel, 1988). These mutation rates are highly template-specific but are several orders of magnitude higher than that of other DNA polymerases. The high error rate is probably due to the lack of a 3'-5' exonuclease activity in purified *Taq* (Lawyer *et al.*, 1989).

1.3.2.2 Contamination

PCR is a powerful technique; even the smallest amount of contaminating DNA can be amplified, producing a false positive result. To minimize the chance of contamination, separate areas should be used for reagent preparation, PCR, and analysis of the product (Schochetman *et al.,* 1988; Kwok and Higuchi,1989). Reagents should be aliquoted to minimize the number of repeated samplings and it is suggested to use positive displacement pipettes. It also helps to reduce contamination if 'premix' reagents are used for PCR reactions, with the DNA added last. The use of positive and negative controls is necessary for the detection of any contamination (Kwok and Higuchi, 1989). According to Sarker and Sommer (1990), contamination can also be limited by exposing the reaction mixture to UV light before adding template DNA.

1.3.3 *S. typhi* Detection by PCR

Some of the applications of PCR to the specific detection of the genus *Salmonella* are as follows. A PCR based test was developed by Song *et al.* (1993) for the detection of *Salmonella typhi* in the blood specimens of patients with typhoid fever using two pairs of oligonucleotide primers that were able to amplify a 343-bp fragment of the flagellin gene of *S. typhi*. Peripheral mononuclear cells from 11 of 12 patients with culture-positive typhoid fever were positive by PCR whereas

10 blood specimens of patients with other febrile illnesses were negative. In addition, PCR was positive for patients suspected of having typhoid fever but who were culture negative (Song *et al.*, 1993). It was estimated that the PCR could detect 10 cells of *S. typhi* as determined by serial dilutions of DNA from *S. typhi*. The same studies were carried out by Chaudry *et al.*(1994) for the detection of *S. typhi* in the blood of patients using primers that were designed to amplify a 486-bp fragment from the specific region of the dH flagellin gene of *S. typhi*. Hashimoto *et al.* (1995) devised a combination of PCR primers based on the *via*B region (Vi antigen) and by using a nested-PCR strategy, they were able to use it to specifically detect *Salmonella typh*i from clinical specimens including blood samples. In this study, 45 strains of *Salmonella* were analyzed as well as 23 strains from other genera. It was found that all *S. typhi* strain were PCR-positive as well as an Vi-positive *S. paratyphi C* and that the assay was able to detect *S. typhi* at the single cell level.

A study conducted by David *et al.* (1992) concluded that PCR can detect as little as 1 pg of DNA from pure *S. typhi* cultures. In another study carried out by Zhu and Lim (1994), unique PCR primers were designed based on the intergenic spacer region between 5S and 23S RNA gene sequences of *S. typhi* Rawlings (type strain) and was used to detect *S. typhi* from food samples. They were able to detect as low as 0.1 pg of *S. typhi* genomic DNA.

The numerous applications of PCR-based methods reported in the last decade showed the potential of this technique in the detection of *Salmonella* spp.(Table 1). Luk (1994) described a PCR-enzyme immunoassay for the detection of *S. typhi.* The PCR products were immobilized to 96-well plate microplates and detected using antibodies. Chevrier *et al.*(1995) developed a PCR-based test for the detection of *Salmonella* spp. The amplified product was analyzed by non-radioactive sandwich hybridization in Covalink microplates using biotinylated oligonucleotides. The hybrid molecules were detected by avidin conjugated with alkaline phosphatase and a chromogenic substrate. A multiplex PCR analysis was carried out by Way *et al.* (1993) utilizing *pho*P and Widjojoatmodjo *et al.* (1991, 1992) using *hin* and H-*li* primers to detect *Salmonella* species in environmental samples such as soil and water. This method was able to distinguish *Salmonella* species from other enteric bacteria. With the well known limitation of low numbers of organism in clinical specimens, recent approaches have attempted to combine enrichment with PCR amplification. For example, Widjojoatmodjo *et al.* (1992) developed a magnetic immuno-PCR assay (MIPA) for the direct detection of salmonellae in fecal samples. The MIPA sensitivity obtained was10^5 CFU/ml of faeces.

Despite the successful application of PCR , there are limitations and problems associated with this technology. First, if proper precautions are not taken, the technique is susceptible to` amplicon' contamination which can lead to false positives. Second, many types of clinical specimens contain compounds which

Table 1 : Examples of the applications of PCR-based detection and diagnosis in *Salmonella* spp.

Serovars	Targets	References
S. typhi	*rfb* gene	Luk (1994)
	flagellin gene	Song *et al.* (1994)
		Choudhry *et al.* (1995)
	Vi gene (*via*B)	Hashimoto *et al.* (1995)
	*Omp*C gene	Pang *et al.* (unpublished data)
	5S-23S spacer region of rRNA gene	Zhu *et al.* (1996)
S. typhimurium	*inv*A	Rahn *et al.* (1992)
		Stone *et al.* (1994)
	*ara*C	Aksenov *et al.* (1994)
Salmonella subsp. I	a 93 bp genetic sequence	Chevrier *et al.* (1995)
Salmonella spp.	*Salmonella* specific HindIII fragment	Nguyen *et al.* (1994)
	*him*A	Bej *et al.* (1990)
	*lam*B	Bej *et al.* (1990)
	*spv*R virulence gene	Mahon and Lax (1993)
	IS*200*	Cano *et al.* (1993)
	a 2.3 kb *Salmonella* specific fragment	Aabo *et al.* (1993)
	*pho*P/*pho*Q loci	Way *et al.* (1993)
	hin/H2 and *H-li* flagellin gene	Widjojoatmodjo *et al.* (1991, 1992)
		Cohen *et al.* (1993, 1994)
	A 469 bp genetic sequence	Luk *et al.* (1993)
	*rfb*gene	Baquar *et al.* (1993)
	IS*200*	Stone *et al.* (1994)
	*inv*E and *inv*A	

inhibit PCR. This includes hemoglobin and heparin in blood and bilirubin and bile salts in faeces. Third, PCR can be technically demanding and reproducibility and specificity are often encountered practical problems.

1.4 The 16S rRNA

Ribonucleic acid (RNA) plays a number of important roles in the expression of genetic information in the cell (Brock *et al.,* 1994). RNA is almost always single-stranded and is synthesized from the DNA template in a correspondingly different way (Watson *et al.,* 1991). Three major types of RNA are messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Ribosomes are essential constituents for eukaryotic and prokaryotic cell proliferation and are composed of protein and RNA moieties. In eubacteria, the RNA entity comprises of three distinct types: the 16S, 23S, and 5S rRNAs. The genes coding for these rRNAs exhibit an operon organization that is essentially consistent from one eubacterium to another. Briefly, the operon organization consists of a promoter region followed by a sequence coding for the 16S rRNA, a spacer or intergenic sequence (which in some instances may contain coding sequences for tRNAs), the 23S rRNA coding sequence, another short spacer sequence, and the sequence coding for 5S rRNA (Figure 2) (Barry *et al.,* 1991).This arrangement is present in the genome in multicopies, and the number of operons present can vary from one group of micro-organism to another.

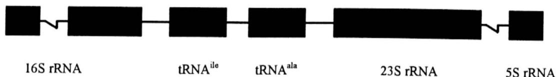

Figure 2 : Position of conserved regions within the rRNA operon. The boxed areas represent the various genes of the bacterial rRNA operon. Transcription of the operon is from left to right. Some bacteria have two tRNA genes as shown; others have either one ($tRNA^{glu}$, $tRNA^{ala}$ or $tRNA^{ile}$) or none. The dark lines represent the spacer regions that separate the various rRNA genes and the dark jagged lines represent breaks in the 16S and 23S genes.

1.4.1 The Use of the 16S rRNA Gene in Probe Construction

The sequence of the 16S rRNA varies in an orderly manner across phylogenetic lines and contains segments that are conserved at the species, genus, or kingdom level, and thus useful in the study of molecular evolution (Woese, 1987). Barry *et al.* (1990) have established a method that permits the rapid generation of DNA probes for a variety of eubacteria by PCR. Their approach uses the 16S rRNA gene which has been found to have variable, species-specific regions that can be utilized for the construction of specific probes. This gene complex

comprises a cluster of genes that have both constant and variable regions and which are flanked by conserved sequences. Limited sequence alignment of the eubacterial 16S rRNAs indicates that two regions, designated V2 and V6 (Figure 3), exhibit greater variation between species than other variable regions.

The generality of this method was shown using *S. typhimurium*, *Staphylococcus aureus*, *Clostridium perfringens*, *Klebsiella pneumoniae*, *Aeromonas salmonicida* and *Mycobacterium bovis*. *A. salmonicida* was examined in detail and a 20-mer oligonucleotide probe was synthesized based on comparison of sequences of the V6 region from various *Aeromonas* species. The probe was end-labelled and hybridized with equal amounts of DNA from four *Aeromonas* species, *E. coli*, *S. typhimurium*, and *C. perfringens*. The result obtained shows a strong positive signal only for *A. salmonicida*. They concluded that specific probes can be designed even with only a two base pair difference in the target sequence. (Barry *et al.*, 1990).

Greisen *et al.* (1994) used the same approach, with some modification, to develop probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid.

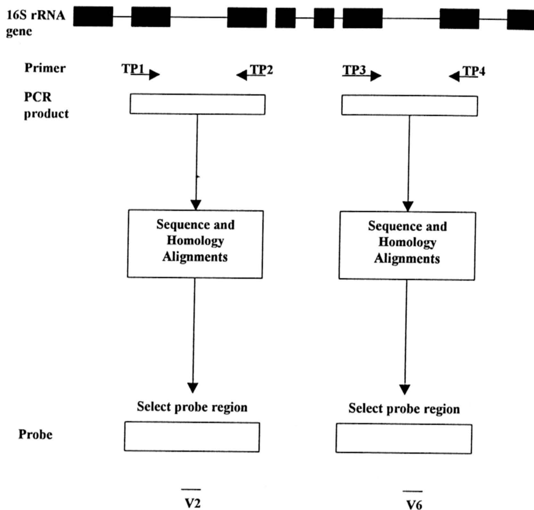

Figure 3 : Schematic representation of the regime for developing specific oligonucleotide probes for eubacteria. Boxes represent constant regions and bars represent variable regions of 16S rRNA genes.(From Barry *et al.*, 1990)

The eubacterial 16S rRNA sequences are of interest owing to the following reasons

1. rRNAs are of value in the study of molecular phylogeny and to develop probes (most current methods require knowledge of rRNA sequences);

2. the presence of relevant 16S rRNA sequences in large copy numbers in the bacterial genome could increase the sensitivity of probes, enabling usage of nonradioactive labels;

3. all known bacterial pathogens of humans belong to the eubacterial kingdom (Kenneth *et al.*, 1990); and

4. the presence of conserved and variable regions which may contain species-specific sequences.

It should be noted however, that although the variable regions of the 16S rRNA have been frequently used as the target for DNA probes to identify micro-organism, the major disadvantage these sequences have as candidates for DNA markers is that the "variable" regions can almost be identical when closely related micro-organisms are examined e.g. *Mycobacteria* species (Barry *et al.*, 1991). In an effort to identify target sequences with a greater variability, the 16S/23S region have been studied and found to have a significant sequence heterogeneity at the genus and species levels and therefore this intergenic region has also been used to generate species-specific probes (Barry *et al.*, 1991).

1.5 Hybridization

Double-stranded DNA can be denatured into two single strands of complementary sequence by heating or by treatment with alkali or other helix-destablizing agents such as formamide. When incubated under the appropriate conditions, the complementary strands will reassociate (or renature) to reform a duplex structure. The term hybridization refers to this formation of sequence-specific base-paired duplexes. Hybridization occurs not only between single-stranded DNA molecules but also between RNA molecules of complementary sequence. (Dyson., 1993).

1.5.1 Dot blots (Slot-blots)

The simplest type of hybridization analysis is carried out using dot blots (also called slot blots) which are used to measure the abundance of target sequences in a sample. Multiple samples are individually spotted onto a filter and the quantity of a specific sequence in each sample is determined after hybridizaton with a labeled probe by comparing the signal produced with control samples containing known amounts of the target. Slot blotting technique is simple and enables the simultaneous screening of many samples relatively rapidly. The analysis can be very sensitive; using radioactive probes of high specific activity, as little as 1 pg of target

can be detected in an overnight exposure of the hybridized filter (target is defined as sequences in the sample that are complementary to the probe).

1.5.2 Southern Blotting

Southern blotting is the name given to a technique, originally described by Southern, 1975 for the transfer of DNA from a gel to a filter. The term 'Southern blotting' is commonly used to encompass DNA transfer from any type of gel to any type of filter matrix. DNA that is separated by electrophoresis and transferred in this way can be analyzed by hybridization with a suitably -labeled nucleic acid probe.

A disadvantage of slot-blots relative to Southern blots is that there is less discrimination between correct hybridization and cross-hybridization. The signal seen in a slot-blot is the sum of all the hybridizing species within the sample, whereas in a Southern blot a band of strongly-hybridizing material can be picked out of a background smear. As a result hybridization backgrounds are higher in a slot-blot and it is important that the correct controls are included.

1.6 DNA Sequencing

Methods to determine the sequence of DNA were developed in the late 1970 (Sanger *et al.,* 1977) and have revolutionized the science of molecular genetics. The DNA sequences of many different genes from diverse sources have

been determined, and the information is stored in international databanks such as EMBL (European Molecular Biology Laboratory) and GenBank. Projects are already underway to map and sequence the entire genome of organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Homo sapiens*. Recent technological advances, especially in the area of automated sequencing, have removed much of the drudgery that used to be associated with the technique, and modern innovative computer software has greatly simplified the analysis and manipulation of sequence data.

The two original methods of DNA sequencing described in 1977 (Sanger *et al.*, 1977 and Maxam *et al.*, 1977) differ considerably in principle. The enzymatic (or dideoxy chain termination) method of Sanger (Sanger *et al.*, 1977) involves the synthesis of a DNA strand from a single-stranded template by a DNA polymerase. The Maxam and Gilbert (or chemical degradation) method (Maxam *et al.*, 1977) involves chemical degradation of the original DNA. Both methods produce populations of radioactively labeled polynucleotides that begin from a fixed point and terminate at points dependent on the location of a particular base in the original DNA strand. The polynucleotides are separated by polyacrylamide gel electrophoresis, and the order of nucleotides in the original DNA can be read directly from an autoradiograph of the gel (Sambrook *et al.*, 1989).

1.7 Objectives of this Study

The main objective of this study was to attempt to adapt the approach of Barry *et al.* (1990) based on the 16S rRNA gene to design a species-specific probe that would serve as a diagnostic tool for *Salmonella* species in detection studies. Thus, a study was conducted on two variable regions, V2 and V6 in the 16S rRNA gene of *Salmonella* species. Sȇveral experiments were carried out:

1. Amplification of two variable regions, V2 and V6 within the 16S rRNA gene of *Salmonella* species from various parts of the world by the use of polymerase chain reaction (PCR).
2. Optimizing PCR conditions to obtain a *S. typhi* specific product to be used as a diagnostic tool.
3. Slot-blot and Southern hybridization to verify the specificity and origin of the PCR product.
4. Sequencing the PCR product to confirm the origin of the product.
5. Optimizing PCR conditions for serum samples.

These experiments constituted the approach of Barry *et al.* (1990) to generate a species-specific probe from the V2 and V6 regions of the 16S rRNA gene of *Salmonella* species.