

CHAPTER 1

GENERAL INTRODUCTION

Access to safe drinking water is still a major problem in developing countries such as South Africa. It has been estimated that more than 12 million people do not have access to an adequate water supply and about 21 million people are without safe sanitation (DWAF, 1994). The presence of pathogenic organisms is a major concern when considering the safety of drinking water. The pathogens that may be transmitted through contaminated drinking water are diverse. Some of the pathogens that are known to be transmitted through contaminated water lead to severe and sometimes life threatening diseases (WHO, 2003). Waterborne diseases have proven to be the biggest threat to human health worldwide. The most well known waterborne diseases such as cholera, dysentery and typhoid are the leading causes of morbidity and mortality (WHO, 2003). The causative agents of waterborne diseases might be bacterial, viral or protozoal in nature (WHO, 2003). *Salmonella* is one of the most important water transmitted bacteria because of the severe diarrhea and typhoid fever they cause.

Salmonella species are ubiquitous enteric bacteria (Baudart *et al.*, 2000). These gram negative rods are the etiologic agents of food-borne salmonellosis and also the agents that cause typhoid and paratyphoid fevers. Although food products, including shellfish, are the most common sources of salmonellosis, *Salmonella* is a prime example of a water- and shellfish-transmitted pathogen. *Salmonella* has been frequently identified in aquatic and marine environments (Martinez-Urtaza *et al.*, 2004). *Salmonella* are widely distributed in the environment. They are the most predominant bacteria in

wastewater and can cause typhoid fever, paratyphoid fever, and gastroenteritis, besides diarrhea (Britton, 1994).

Wastewater is any water that has been adversely affected in quality by any anthropogenic influence (Muyima *et al.*, 1997). It therefore includes liquid waste discharged from domestic houses, industrial, agricultural or commercial processes. It is known that domestic wastewater contains not only inorganic and organic impurities but also microorganisms that decompose organic matter. Wastewater from the natural drainage patterns or sewers enters natural bodies of water such as ground water, rivers, lakes and oceans (Muyima *et al.*, 1997).

In addition to sewage from human and industrial activities, coastal waters receive water directly from rivers, which can carry enteric bacteria originating from their inland reservoirs (Martinez-Urtaza *et al.*, 2004). The presence of enteric bacteria in aquatic environments represents a source of contamination for the organisms present in the environment. *Salmonella* is a large genus of bacteria including more than 2,300 serotypes, and diagnosis in the majority of laboratories relies on costly and laborious culture screening with both nonselective and selective media (Baudart *et al.*, 2000).

Both human and animal excreta are sources of *Salmonella*, and many potential routes are available for the transmission of these enteric pathogens (Baudart *et al.*, 2000). The ability of *Salmonella* to be transmitted by any of these routes depends largely on its resistance to environmental factors, which control its survival, and its capacity to be carried by water. This survival capacity may depend on species and pollution sources (Baudart *et al.*, 2000).

Most studies have focused on the determination of *Salmonella* strain in some polluted areas (Baudart *et al.*, 2000). It was recently shown that the annual bacterial loads of this pathogen in rivers and coastal areas can be very important. However, information on the diversity and occurrence of *Salmonella* strains is scarce, and as a consequence, the ecology of these species remains unknown. This is partly due to the laborious methods required for the detection, isolation, and identification of *Salmonella* strains (Baudart *et al.*, 2000).

Serotyping offers a very precise and reliable method for differentiating isolated strains. Still it remains time-consuming and requires the use of more than 150 serum specific samples (Baudart *et al.*, 2000). Serotyping is not accessible to many laboratories, and this may partly explain why little information is available on the diversity of strains in natural ecosystems. Furthermore, some strains cannot be identified due to the untypeability of the isolate (rough strains). With the development of molecular biological methods, it is now desirable to use alternative methods which provide a higher power of discrimination and allow a more rapid identification attainable by non specialized laboratories (Baudart *et al.*, 2000).

Bacteria are one of the earliest forms of life on earth and are able to exist in almost all environments throughout the biosphere (Atlas and Bartha, 1993). To explore microbial diversity, molecular biological methods have been used to successfully identify individual types of bacteria in natural samples without culturing them. This is accomplished based on a study of DNA sequences of genes common to a genus. Microbial diversity is defined as the heterogeneity of a system given by the variety of different types of microorganisms occurring together in a biological community. The use

of molecular approaches for describing microbial diversity has greatly enhanced the knowledge of population structure in natural microbial communities. It is widely accepted that culture-based techniques are inadequate for studying bacterial diversity from environmental samples, as many bacteria cannot be cultured using current and traditional techniques (Atlas and Bartha, 1993).

The starting point for the molecular approach and related procedures is the extraction of intact genomic DNA of sufficient quality and quantity to permit the amplification of the genes of interest in a Polymerase Chain Reaction (PCR). There are two strategies based on rRNA and rDNA studies to identify bacteria in sample material (Ward *et al.*, 1990). The first approach is based on the recovery of rRNA and rDNA that is transcribed into cDNA, cloned and sequenced. The alternative approach is based on the recovery of high molecular weight DNA directly from sample material followed by the amplification of rDNA in a PCR reaction, cloning and sequencing (Ward *et al.*, 1990).

The genus *Salmonella* has been grouped into more than 2400 serotypes according to the Kauffman-White Scheme (Mansfield and Forsythe, 2001). This is based on the antigenic diversity of the flagellar and somatic antigens (H and O). The O antigens are located on the lipopolysaccharide (LPS) molecules which project from the outer leaflet of the outer membrane of the gram-negative bacterial cell (Mansfield and Forsythe, 2001). LPS molecule is divided into three regions, that is, a hydrophobic lipid A region linked to an oligosaccharide core region and a hydrophilic O-polysaccharide or O-chain. The O-chain region is composed of repeating subunits of differing chain lengths. Polysaccharide chains with the same lengths co-migrate in SDS-PAGE gels to form a 'ladder' pattern (Mansfield and Forsythe, 2001).

Rapid analysis of diversity of complex microbial communities has remained an elusive but important goal in microbial ecology. Bacterial diversity can be examined on several levels. One of the methods of examining bacterial diversity is when genomic DNA is amplified by PCR, followed by restriction digestion of amplified samples to identify differences in the genomes of species. Molecular approaches reveal differences not only in community composition but also in community organization by measuring the number (richness) and relative abundance (structure or evenness) of species (Dunbar *et al.*, 2000). The richness and evenness of biological communities reflect selective pressures that shape diversity within communities. Measuring these parameters is most useful when assessing treatment effects for example physical interference, pollution, nutrient addition, predation, climate change, etc. on community diversity. Diversity statistics can also indicate the ability of a community to recover from stress and utilize resources efficiently (Dunbar *et al.*, 2000).

One of the molecular biological methods for studying the diversity of microbial communities is analysis of PCR products, generated with primers homologous to relatively conserved regions in the genome or known genes, and genomic DNA as a template using denatured gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis. These approaches allow separation of DNA molecules that differ by single bases and hence have the potential to provide information about variations in target genes in a bacterial population. DNA fingerprinting techniques provide patterns or profiles of the genetic diversity in a microbial community. Several fingerprinting techniques have been developed and used in microbial ecology studies such as bioremediation, biodiversity of species and parental tests. The separation of or detection

of small differences in specific DNA sequences can give important information about the community structure and diversity of microbes carrying an essential gene. These techniques are important in separating and identifying PCR-amplified genes that might have the same size but different nucleotide sequences (Muyzer, 1999). An advanced method, terminal restriction fragment length polymorphism (T-RFLP) analysis, measures the size polymorphism of terminal restriction fragments after PCR- gene amplification. It combines at least three technologies, including comparative genomics (RFLP), PCR and electrophoresis (Muyzer, 1999).

Denaturing gradient gel electrophoresis (DGGE) and a similar method TGGE (thermal-GGE) are procedures by which fragments of DNA of the same length but of different sequences can be resolved electrophoretically (Muyzer, 1999). Separation is based on the different electrophoretic mobilities of denatured double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of a denaturing reagent or a linear temperature gradient (Muyzer, 1999).

In the past two decades, these molecular tools exemplified by 16S rRNA analyses have facilitated the study of natural microbial populations without cultivation which has made quantitative assessment of microbial diversity now conceivable (Ramsing *et al.*, 1996; Teske *et al.*, 1996). Many variations of the 16S rRNA approach are currently used for defining microbial diversity. These include analysis of PCR amplified 16S rDNA sequences and digestion with restriction endonucleases to obtain restriction fragment length polymorphism (RFLP) of whole 16S rDNA amplicons (Teske *et al.*, 1996). However, no single method allows definitive assessment of the bacterial diversity. Therefore, the use of a polyphasic approach involving a combination of molecular

biology techniques, microbiological methods and geochemical techniques or microsensors, is necessary to obtain a better understanding of the biodiversity of the microorganisms and their interaction in the natural environment (Ramsing *et al.*, 1996; Teske *et al.*, 1996). Thus, bacterial biodiversity can only be accurately determined using molecular taxonomic method.

The emergence of antimicrobial resistant bacterial pathogens has become a major public health concern (Cheng *et al.*, 2004). The use of antimicrobials in any venue, including disease treatment and growth promotion in domestic livestock, can potentially lead to widespread dissemination of antimicrobial-resistant bacteria (Cheng *et al.*, 2004). In recent years, testing of *Salmonella* isolates from different environments has shown an increasing proportion of multidrug resistant *Salmonella* species. Of particular concern is the isolation of ceftriaxone and ciprofloxacin resistant *Salmonella*, because of the importance of these two agents in treating *Salmonella* infections in children and adults (Cheng *et al.*, 2004).

Conventional antimicrobial agents, such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole, had been the drugs of choice in the treatment of salmonellosis before 1980 (Cheng *et al.*, 2004). However, multidrug resistance, with rates of resistance to these antimicrobial agents of more than 50%, has been reported in many areas of the world. Extended-spectrum cephalosporins and fluoroquinolones have been suggested as alternative agents in the treatment of infections caused by multidrug resistant *Salmonella* serotypes. However, since 1991, cases of infections caused by *Salmonella* serotypes resistant to extended-spectrum cephalosporins or fluoroquinolones have been increasingly reported (Cheng *et al.*, 2004). Hence, this study was aimed at

assessing the diversity of *Salmonella* isolates from selected drinking water and wastewater sources in Gogogo, Nkonkobe and Amalinda areas in the Eastern Cape Province. Specific objectives include:

- To isolate *Salmonella* species from drinking water and wastewater sources around the Eastern Cape Province.
- To evaluate the diversity of the *Salmonella* species using protein and lipopolysaccharide profile analysis and DNA fingerprinting.
- To determine the antibiotic susceptibility profile of the *Salmonella* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Relevance of bacterial diversity studies

Studies on microbial diversity are essential because microorganisms are important for a sustainable biosphere. They are able to recycle nutrients, produce and consume gases that affect global climate, destroy pollutants and can be used for biological control of plant and animal pests. People over the ages have been highly successful in applying processes carried out by microorganisms to solve problems in agriculture for example soil infertility, food production, environmental quality, and industry (Morris *et al.*, 2002).

In the mid-1900s, the publications of R. H. MacArthur and G. E. Hutchinson encouraged studies in the field of ecology into intense research and debate about the significance of biodiversity (Morris *et al.*, 2002). These and other co-workers claimed that biodiversity is a measure of important ecological processes such as resource partitioning, competition, succession, and community productivity and is also an indicator of community stability (Morris *et al.*, 2002). This new wave of biodiversity research concerned plant and animal communities. In the 1960s, following in the footsteps of plant and animal ecologists, microbiologists began investigating the impact of biodiversity on the function and structure of microbial communities (Morris *et al.*, 2002).

Genetic variation among individuals within a population has long been recognized as the starting block for adaptation and evolution among microorganisms as well as among other organisms. Likewise, the consequences of phenotypic variability for the accuracy of disease diagnosis and for establishing taxonomic relationships among microorganisms have been well studied. Interest in microbial biodiversity has been

further encouraged by (i) creation of the Diversitas international research program in 1991 to promote scientific investigations into the origins and conservation of biodiversity and the impact of biodiversity on ecological functions, (ii) the Biodiversity Treaty issued from the United Nations Conference on Environment and Development in 1992 in Rio de Janeiro, Brazil, and (iii) subsequent initiatives launched by science foundations, scientific societies, and research institutions in a wide range of countries. Microbial biodiversity has also received particular attention in areas such as medical, food biotechnology and where microbial activity has important implications for earth's climate and for the bioremediation of polluted sites. Nevertheless, in spite of the research devoted to microbial biodiversity and to biodiversity in general, the consequences of biodiversity on the ecological processes cited above are still the object of debate and analysis (Morris *et al.*, 2002).

The study of microbial diversity is thus important to solve new and emerging disease problems and to advance biotechnology. New technologies, particularly in nucleic acid analysis, computer science, analytical chemistry, habitat sampling and characterization place the study of microbial diversity on the cutting edge of science (Kapur and Jain, 2004). In the past ten years, due to advances in molecular methods and techniques, knowledge of microbial diversity has increased dramatically not only from a phylogenetic and taxonomic perspective but also from an ecological basis. Now it is known that microorganisms exist in every conceivable place on earth, even in extreme environments (Kapur and Jain, 2004).

Although the number of different human genes has turned out to be smaller than expected, the diversity of genes among microbial species is surpassing expectations

(Kapur and Jain, 2004). These microbial gene sequences yield information about biochemical functions, ecological niche, taxonomy and evolutionary relationships, whereas the location of a gene on a genome often implies its role in metabolic and regulatory networks. DNA sequences provide the basis for the current classification of microbial species; they are beginning to elucidate the evolutionary and ecological relationships among diverse species. New tools are accessing microbial diversity to provide novel genes and biosynthetic pathways. These genes, when introduced into a host strain, can bring about an unclear biochemical transformation from an unculturable microbe into a commercializable biocatalyst (Kapur and Jain, 2004).

Though the negative effects of bacteria such as the diseases they cause are well known, their often subtle functions explain why their biodiversity might positively affect humans (Kapur and Jain, 2004). The most important ecological function of bacteria is bioremediation, a process by which contaminated regions are restored by means of bacterial biogeochemical processes. It is an economical, versatile, environment friendly and efficient treatment strategy, and a rapidly developing field of environmental restoration. Bioremediation utilizes the microbial ability to degrade and detoxify chemical substances such as petroleum products, aliphatic and aromatic hydrocarbons, industrial solvents, pesticides and their metabolites, and metals (Kapur and Jain, 2004). The presence of a large number of diverse bacterial species in nature expands the variety of chemical pollutants that can be degraded as well as the extent to which pollutant sites can be decontaminated (Kapur and Jain, 2004). The use of microorganisms for degradation of pollutants is now being increasingly applied as the technology of choice for clean-up or restoration of polluted sites as it can be self sustaining and inexpensive

(Kapur and Jain, 2004). The use of techniques such as polymerase chain reaction (PCR) in amplification sequencing of 16S ribosomal RNA genes, random amplification of polymorphic DNA (RAPD) sequences, and nucleic acid hybridisation experiments provide a more reliable approach to determining bacterial biodiversity (Kapur and Jain, 2004).

2.2 Classical versus Molecular Techniques for Biodiversity Studies

Different techniques have been used to study the diversity of microorganisms. Historically, microbial taxonomy has been conducted using a variety of physical and biochemical tests that allow the grouping of microbial isolates into genera and species. This approach requires laboratory cultivation of the microbes in order to separate the various isolates into monocultures. This approach (classical taxonomy) has been used to identify and characterize many culturable bacteria (Hawksworth and Colwell, 1992). However, typically less than one percent of the bacteria can be cultivated in the laboratory. Indeed, only 3 000 to 4 000 species of bacteria have been described (Hawksworth and Colwell, 1992), even though it has been estimated that the number of bacterial species world-wide is close to three million (Colwell, 1997). In culture dependent techniques, bacteria are isolated from environmental samples and cultured in selected medium. Nucleic acids are then extracted from the bacterial culture. The biggest drawback in exploring bacterial biodiversity is the issue of viable but non-cultivable organisms. Diversities in bacterial communities are normally determined by phenotypic characterization of isolated strains (Bakonyi *et al.*, 2003). A problem is that phenotypic methods can be used only on bacteria which can be isolated and cultured. Many advances

have been made in microbiological culture techniques, however it is still not possible to culture a majority of bacterial species using the standard laboratory culturing techniques. Conventional characterization of microbial strains therefore has been subjected to debate, as it is dependent on the ability of the strains to grow under specific environmental conditions (Bakonyi *et al.*, 2003).

Classic microbiological methods are indirect and many produce artificial changes in the microbial community structure. Most bacteria might be excluded when phenotypic diversity is estimated (Bakonyi *et al.*, 2003). The isolated bacteria may account for only a minor proportion of the total bacterial diversity in the environment, while our knowledge about the dominant part is very scant. This has led to the current uncertainty regarding the true extent of bacterial life. The medicinal and industrial properties of known bacteria have led many to hypothesize that uncultured species have many positive applications to human existence (Bakonyi *et al.*, 2003).

In contrast, culture independent methods employ direct extraction of nucleic acids from environmental samples. It often involves the amplification of DNA or synthesis of cDNA from RNA extracted from environmental samples (Colwell, 1997). Alternatively, the amplified products may be cloned and sequenced to identify the bacterial species present in the sample. Also, the direct extraction of nucleic acids from environmental samples accounts for the very large proportion of microorganisms that are not readily cultured in the laboratory and these microorganisms may be responsible for the majority of the biodegradation activity of interest (Colwell, 1997).

The application of molecular techniques has greatly advanced the study of microbial ecology. Molecular methods have given a new perspective on biodiversity,

community structure and allowed the detection and identification of many novel microorganisms. Molecular techniques have shown that more than 99% of microorganisms in the environment remain uncultured and uncharacterized. Recent progress in molecular microbial ecology has revealed that traditional culturing methods fail to represent the scope of microbial diversity in nature, since only a small proportion of viable microorganisms in a sample are recovered by culturing techniques (Rondon *et al.*, 2000).

2.3 Molecular techniques used to study bacterial diversity

2.3.1 Protein based techniques for studying bacterial diversity

Analysis of proteins extracted from bacteria isolated in environmental samples can be employed as a fingerprinting method to type the bacterial diversity in the samples (Ehlers *et al.*, 1998). A bacterial strain under standardized growth conditions will produce a particular set of proteins, expressed by the genome of that particular strain. Thus, protein electrophoresis is said to be used as an indirect fingerprinting of that particular bacterial genome (Bosch and Cloete, 1993). This analysis gives an understanding of how genetic information is transformed into the diversity of function and form in microbial communities (Ehlers *et al.*, 1998). The resulting protein profiles help to monitor the response of microbial communities under stressful conditions and hence the deterioration or the enrichment of species diversity in those communities (Ehlers *et al.*, 1998).

There are several techniques based on the separation of bacterial proteins on polyacrylamide gels. These include sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), isoelectric focusing and two-dimensional PAGE (Bosch and

Cloete, 1993). Extraction of cell wall proteins, followed by SDS-PAGE separation, has been mainly used and found to be a reliable and rapid way of characterizing large numbers of strains. This method relates differences in cell wall protein profile to adaptation to different ecological niches. The sensitivity and accuracy of this method can be compared to that of DNA based methods (Bosch and Cloete, 1993).

Extracted protein molecules are separated on the basis of their molecular weight by electrophoresis and produce a complex banding pattern. These bands are then stained and analyzed using different software programs. The results are interpreted as the relatedness of microorganisms studied (De Angelis *et al.*, 2001). This method has been used in studying the diversity of *Acinetobacter* isolates from activated sludge systems (Maszenan *et al.*, 1997).

Protein electrophoresis is a sensitive technique, yielding valuable information on the similarity and dissimilarity amongst bacterial cultures during taxonomical studies. This method could therefore be used to determine the similarity and dissimilarity between different environmental samples containing microorganisms. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell soluble proteins prepared under standard conditions produce a complex banding pattern called protein electrophoregram or electrophoretic protein pattern which is reproducible and can be considered as a fingerprint of the sample investigated. The dendograms from the resulting protein profiles after SDS-PAGE of different bacterial samples could possibly lead to the better understanding of the diversity of microorganisms (Ehlers *et al.*, 1998).

Two-dimensional electrophoresis (2DE) of proteins is currently one of the highest-resolution analytical techniques available for the study of protein expression

patterns (Duffes *et al.*, 2000). This technique has already been used for studying minocycline-susceptible and -resistant *Mycobacterium smegmatis*. Comparative proteome analysis of *Mycobacterium tuberculosis* virulent and nonvirulent vaccine strains was carried out with the help of two-dimensional electrophoresis (Duffes *et al.*, 2000). Two-dimensional electrophoresis can be an important resource in identifying proteins involved in bacteriocin resistance. Thus, two-dimensional electrophoresis is a powerful tool to highlight the biochemical mechanisms governing development of cell resistance and will help in the design of new efficient molecules or mixing of molecules with different cell targets (Duffes *et al.*, 2000).

2.3.1.1 Application of classical and molecular techniques on the study of *Salmonella* diversity

Salmonellae are Gram-negative, flagellated, facultatively anaerobic bacilli possessing three major antigens, that is, H or flagellar antigen, O or somatic antigen and Vi antigen (possessed by only a few serovars) (Amann *et al.*, 1995). H antigen may occur in either one or both forms, called phase 1 and phase 2. The organisms tend to change from one phase to the other. O antigens occur on the surface of the outer membrane and contain specific sugar sequences on the cell surface. Vi antigen is a superficial antigen overlying the O antigen; it is present in a few serovars, the most important being *S. typhi* (Amann *et al.*, 1995).

Antigenic analysis of *Salmonellae* using specific antisera offers clinical and epidemiological advantages. Determination of antigenic structure permits one to identify the organisms clinically and assign serogroups, each containing many serovars. H antigen

also provides a useful epidemiologic tool in determining the source of infection and its mode of spread. The genus *Salmonella* is composed of two species, "*Salmonella enterica*" and *S. bongori* (Amann *et al.*, 1995). The primary basis for the typing of "*S. enterica*" is a serotyping scheme, the White-Kauffmann-Le Minor scheme in which 2,375 serovars have been recognized on the basis of the antigenic properties of the cell wall lipopolysaccharide (O antigen), the phase-1 flagellar protein (H1), and the phase-2 flagellar protein (H2) (Amann *et al.*, 1995).

The flagellar protein or flagellin constitutes the subunit of the helical filament that forms the flagellar organelle (Amann *et al.*, 1995). *Salmonella* flagellin consists of extremely conserved terminal regions and a variable central region. This central region of the molecule carries the antigenic determinant. For the phase-1 flagellin, 63 antigens have been distinguished. For the phase-2 flagellin, 37 antigens have been described. Some of these antigens are defined by a single factor (antigen i, d, or r); others are defined by several subfactors for example, antigens l,v; l,w; g,m; and e,n,x (Amann *et al.*, 1995).

The antigenic specificities of phase-1 and phase-2 flagellins are encoded by the flagellin genes *fliC* and *fljB*, respectively. These flagellar genes are found at two different locations on the chromosome. At one location is the gene *fliC*, at another location is an operon containing the genes *hin*, encoding the Hin recombinase; *fljB*, encoding phase-2 flagellin; and *fljA*, encoding a repressor for *fliC*. The Hin recombinase catalyzes the reversible inversion of a 993-bp segment of the chromosome containing a promoter. In one direction, the promoter directs transcription of the *fljB* and *fljA* genes. Phase-2 flagellin and a repressor are produced thus repressing *fliC* expression. In the other

direction, the promoter and the gene cause repression of the *fliC* gene and phase-1 flagellin is expressed (Amann *et al.*, 1995).

2.3.2 Nucleic acid-based techniques

The application of nucleic acid techniques has greatly advanced the detection and identification of microorganisms in natural environments (Gillespie *et al.*, 1997). Advances in nucleic acid analysis have resulted in the development of molecular techniques to analyze bacterial DNA. Perhaps the greatest advantage of identification techniques based on DNA structure is that these methods focus on the unique nucleic acid composition of the bacteria rather than on phenotypic expression of products that are encoded by DNA (Gillespie *et al.*, 1997). Consequently, techniques based on DNA structure and sequence for the identification of bacteria should theoretically be subject to less variability than currently used procedures involving phenotypic characterization (Gillespie *et al.*, 1997).

However, successful application of molecular techniques relies on effective recovery of nucleic acids from environmental samples. A variety of methods have been developed and used to directly recover nucleic acids from environmental samples but only few methods have been developed for recovering mRNA from environmental samples (Amann *et al.*, 1995). Since RNA is not stable, recovery of intact mRNA from environmental samples is a great challenge. The development of molecular techniques for nucleic acids has led to many new findings in studies of microbial ecology (Amann *et al.*, 1995). As a basic approach to study microbial communities, 16S rDNAs are amplified by PCR from total nucleic acids extracts. The PCR products are cloned, sequenced and

compared by a blast search in the database. This approach can avoid the limitation of the traditional culturing techniques for assessing the microbial diversity in natural environments (Hurt *et al.*, 2001).

2.3.2.1 RNA based techniques

2.3.2.1.1 rRNA sequencing based technique

The use of rRNA sequence-based analysis of microbial populations has allowed study of complex communities in the environment without the requirement for laboratory cultivation of organisms. The rRNA approach has been used to detect microbial populations and to determine the structure of microbial communities in various environments without isolating the component microorganisms (Watanabe *et al.*, 1998). Extensive sequencing of the ribosomal RNAs has been particularly informative. Sequencing divergence among these individual RNAs has defined the outline of a natural classification of microorganisms (Watanabe *et al.*, 1998).

This approach has revealed astonishing diversity in many environments. Several studies have analyzed over 100 rRNAs sequences from a single environmental sample, and yet, remarkably, very little repetition has been seen among sequences, and virtually all previous studies have revealed novel groups with few or no known cultivated members (Watanabe *et al.*, 1998). Most of these studies have identified new genera and even kingdoms of microorganisms, strongly suggesting that the information about the total diversity of microbes in the environment is very little (Watanabe *et al.*, 1998). 5S rRNA molecules were directly extracted from mixed samples. The 5S RNA molecules belonging to different community members were separated electrophoretically and a

comparative sequence analysis yielded phylogenic placing of the species (Wagner *et al.*, 1993). However 120 nucleotide long 5S rRNA is small molecule and the requirement for electrophoretic separation of the different 5S rRNA molecules limits this approach to less complex ecosystem. For this reason, study of large rRNA molecules was suggested for experiments in microbial ecology (Wagner *et al.*, 1993). The 16S rRNA has been the common target for determinative hybridization probes. Selected regions within the large rRNA molecules were used in hybridization reactions with synthetic deoxy-oligonucleotides. The rRNA of microbial species and subspecies can be distinguished by complementary oligonucleotides to the most variable regions of 16S ribosomal gene cluster. Some regions of the rRNA are invariable in all species and these can be used as universal primers or probes. These probes have been used to measure total rRNA abundance in the environment and also to assess differences in cellular rRNA contents (Amann *et al.*, 1990).

2.3.2.2 DNA based techniques

2.3.2.2.1 Extraction of DNA from microorganisms

A number of techniques have been used in assessing microbial communities on the basis of their gene diversity (Johnston *et al.*, 1996). DNA is the representative molecule of the genetic information of microorganisms, which provides information concerning the structure of a community. Use of conventional methods has limited the understanding of the entire communities which can be studied further using DNA techniques. DNA extraction methods that have been developed circumvent the bias imposed by traditional

microbiological methods. These methods, allow for the extraction of the entire genomic DNA and hence the recovery of information at a molecular level (Johnston *et al.*, 1996).

DNA extraction can be performed directly. The direct extraction involves the rupture of cell envelopes by mechanical methods and cleavage of disulfide bonds by sodium dodecyl sulphate (SDS) (Johnston *et al.*, 1996). Treatment of homogenate with phenol-chloroform followed by centrifugation of the DNA homogenate separates the DNA from the protein moiety. After centrifugation the DNA sample can be purified by ethanol precipitation. The major advantage of this method is that it eliminates the bias of unreleased cells; hence recover DNA from the rest of the cell components (Johnston *et al.*, 1996). The extracted DNA can be used to study the genome of the microbial strain using different techniques that involve DNA melting profiles and reassociation analysis.

2.3.2.2.2 DNA melting profiles and reassociation analysis

Genetic information of a microorganism is based on its DNA genome, and different microbes carry different proportion of nucleotide bases and different sequence of the bases in DNA molecules (Torsvik *et al.*, 1998). Studying the whole microbial community would thus be difficult if one relies on the extraction of individual bacterial genome. One limitation is that genetic information gathered would reflect the DNA population of a single bacteria and hence not the reflection of entire community. The genetic structure may be assessed by measuring the base composition and complexity of the total community DNA (Torsvik *et al.*, 1998). The measurement of the distribution of nucleotide sequences indicates the DNA composition of a particular bacterial species, and is measured as percentage of guanine + cytosine (%G+C) (Ritz *et al.*, 1997). The

percentage of guanine + cytosine has been determined for a wide variety of microorganisms and has been used to assess the relationship of bacteria in the environment. This base composition has been determined by thermal denaturing and the results interpreted by the analysis of the melting curves of DNAs (Ritz *et al.*, 1997 and Torsvik *et al.*, 1998).

The principle of the reassociation technique is based on the rate at which the thermally denatured DNA reassociate at a temperature below its melting point (Torsvik *et al.*, 1998). The reassociation kinetics is used as an indication of the complexity of the DNA. DNA from a bacterial community is a mixture of DNAs from different bacterial species present in different proportions. Low reassociation rates in such communities are indicative of a diverse composition of microorganisms, due to the differences in (%G+C). This technique in association with other DNA-based techniques has been used to compare the microbial diversity of natural and perturbed environments (Torsvik *et al.*, 1998).

2.3.2.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an *in vitro* method for replicating target DNA sequences so that they can be synthesized in large amounts (Atlas and Bej, 1994). It has been mainly used in the detection of microorganisms in different types of environments (Atlas and Bej, 1994). It is simple, rapid, sensitive and precise nature of application has allowed PCR to be the most widely used methods in many aspects of research (Atlas and Bej, 1994). However, the PCR method only detects a limited number of serovars at a time, and many different genetic markers are still to be developed or verified for

identification of various serovars (Cai *et al.*, 2002). Polymerase chain reaction results in an exponential amplification of the gene of interest and significantly increases the probability of detecting a rare sequence change or mutation. This method can be used to compare differences in genes between species and between classes and organisms. Amplified genes can be sequenced and compared to study how related or diverse species are in a studied environment (Cai *et al.*, 2002). The use of the PCR has been preferred over traditional methods, which are based on culturing the microorganisms. Due to its effectiveness, PCR has been combined with other molecular techniques for better understanding of the genomes of microbial communities in the environment (Cai *et al.*, 2002).

2.3.2.4 Fluorescent In Situ Hybridization (FISH)

The technique of Fluorescent In Situ Hybridization (FISH) has been successfully used to identify bacteria in complex environments (Glockner *et al.*, 1999). It has been used ideally to deduce the community composition from 16S rRNA gene libraries (Glockner *et al.*, 1999). A combination of PCR and direct retrieval of rRNA sequences followed by FISH allow the specific detection and identification of uncultured bacteria. Fluorescent rRNA targeted oligonucleotide probes are being used as an alternative to fluorescent antibodies in the identification of bacteria. Use of appropriate probes allows identification of microorganisms according to their phylogenetic range, from kingdom to species level. This technique has also been used to indicate the growth rate of cells, since the signal from the probe is directly proportional to the amount of target (Ritz and Griffiths, 1994).

DNA hybridizations have been used to quantify the extent to which communities are similar (Ritz and Griffiths, 1994). DNA from one community can be used as a template and the DNA from the other one is radiolabelled and used as a probe. The degree of similarity is then measured by the rate to which the probe anneals to the target DNA (Ritz and Griffiths, 1994). Fluorescent oligonucleotides have been used to analyze the spatial distribution of sulphate reducing bacteria in multispecies biofilms (Wagner *et al.*, 1993).

2.3.3 DNA fingerprinting technique

Nucleic acid based community analysis techniques have been developed to describe the microbial community without culturing microbes (Meharg, 2002,). The DNA extracted from environmental samples contains DNA fragments from all members of the community regardless of their growth specifications. The variable regions of the genome can be amplified using primers targeting flanking conserved regions and analyzed by gel electrophoresis after digestion with restriction enzymes. The patterns generated are referred to as DNA fingerprints (Meharg, 2002,). The different DNA fingerprinting techniques that have evolved from this principle include Restriction Fragment Length Polymorphism (RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Pulsed Field Gel Electrophoresis (PFGE), Amplified Ribosomal-DNA Restriction Analysis (ARDRA) etc. DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample after restriction digestion (Meharg, 2002).

DNA Fingerprinting is a process which involves the electrophoretic analysis of DNA fragments generated by restriction enzymes. Fragments are separated by size to create a DNA profile which is unique to each individual, while there is a small chance

that two identical profiles may come from different individuals, DNA fingerprinting can reveal with certainty if two samples are the same. It is also highly useful in forensic science at placing certain individual at the scene of a crime, similar to, but more precise than, a traditional fingerprinting (Meharg, 2002).

DNA fingerprinting, also called DNA Profiling, makes use of fragments of DNA that exhibit variability (caused by mutations) in the nucleotide base sequences from individual to individual (Meharg, 2002). When these segments of DNA are cleaved using restriction enzymes DNA fragments of various lengths are produced. If the DNA of an individual has mutations within restriction sites the DNA will not be cut at those sites and that individual's DNA fragments will be different in length from other DNA fragments produced by the same restriction enzymes. When the DNA fragments of an individual are separated by electrophoresis, transferred to a membrane using the Southern blotting technique, and mixed with specific radioactive single-stranded DNA probes, a unique pattern of bands is produced when the membrane is subjected to autoradiography. These bands can be used to identify that individual's DNA (Meharg, 2002).

2.3.4 Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) is a convenient method for the comparison of bacterial strains. The procedure involves the isolation of DNA, digestion of DNA with restriction endonucleases, size fractionation of the resulting DNA fragments by electrophoresis, DNA transfer from the gel to a nitrocellulose membrane, preparation of radiolabelled or chemiluminiscent probe, and hybridization to membrane-bound DNA (Babalola, 2003).

This method visualizes and compares only specific gene(s) from certain species, using radioactive or fluorescent probes (Grattard *et al.*, 1993). Although rRNA genes have been extensively analyzed by this method, other conserved genes may also be analysed. When the rRNA operon is used as a probe, the method is known as ribotyping (Grattard *et al.*, 1993). To analyze the heterogeneity of bacterial community using RFLP, total DNA is extracted from the bacterial strain. PCR is used to amplify the gene of interest. Usually rRNA genes are amplified from genomic DNA since they are highly conserved to bacterial communities. The amplified DNA molecules can thus be isolated and separated by gel electrophoresis. Restriction endonucleases are used to digest the isolated genomic DNA and to cleave specific fragments, which are separated by gel electrophoresis. The band patterns of the DNA fragments in the gel can be compared for similarity of microorganisms in the community population. Closely related strains have similar DNA fragments. This information is used to draw a phylogenetic tree of a bacterial community study (Burlage, 1998).

Restriction Fragment Length Polymorphism technique is regarded as the most sensitive technique for strain identification. Several bacterial strains have been widely studied using this technique. Kabadjova and co-workers (2002) established a rapid PCR-RFLP based identification scheme for four closely related *Camobacterium* species. Manceau and Horvais (1997) used RFLP analysis of rRNA operons to assess phylogenetic diversity among strains of *Pseudomonas syringae* pv tomato. They successfully established the close relationships existing between *P. syringae* and *P. viridiflava* species.

2.3.5 Single Strand Conformation Polymorphism

Single Strand Conformation Polymorphism (SSCP) is a PCR based technique that is used to analyze DNA molecules of the same size but different nucleotide composition (Tebbe *et al.*, 2000). It has been used for the rapid detection of low numbers of pathogenic bacteria in natural environments. All PCR-based methods are sensitive enough to allow identification of individual taxa. This approach has high potential and sensitivity in mutation analysis. It has been developed for the detection of mutations mainly in human genes (Schweiger and Tebbe, 1998).

The method involves amplification of a fragment of bacterial 16s rRNA gene by PCR, using universal primers. The PCR products are then digested with lambda exonuclease and separated by electrophoresis. One advantage of this method is that it can bypass the reannealing and heteroduplex formation of DNA strands which tend to increase the number of bands in the gel and hence giving wrong analysis of results (Schweiger and Tebbe, 1998).

2.3.6 Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) is considered to be a very powerful technique (Muyzer *et al.*, 1993). It can be used for detecting single base changes and polymorphisms in genomic DNA, cloned genes and PCR products. This technique has a range of applications in the study of human genetics and clinical research. Changes of the base composition in the genome of an organism may lead to certain diseases. DGGE can assist in direct detection and localizing those single base mutations that may cause the diseases (Muyzer *et al.*, 1993).

PCR amplified DNA fragments of equal lengths differing by single base change can be detected (Diez *et al.*, 2001). They are separated by electrophoresis on the basis of their melting points in a polyacrylamide gel. The gel contains a linear gradient of DNA denaturants such as urea and formamide. Separation is based on the electrophoretic mobilities of partially melted DNA molecules in the gel. DNA molecules that may be identical, but otherwise differing by a single base change has slightly different melting properties. Thus, migration through the polyacrylamide gel will differ. The mobility of each molecule slows down at a position in the DGGE gel that corresponds to its melting point. Partial denaturing of the molecule slows down its rate of migration. Since the sequences of the melting domain determine its melting temperature, sequence variants of particular fragments will stop migrating at different positions in the gel (Diez *et al.*, 2001). DGGE bands can be sequenced and analyzed. Polymorphisms can be recognized by the appearance of bands in some of the DNA samples, which are not present in others. A shift of a band in one or more samples compared to others can also indicate polymorphism. Rapid comparison data for many communities and specific phylogenetic information are derived from the sequenced bands (Diez *et al.*, 2001).

The melting of the DNA molecule is determined by two factors, the hydrogen bonds between complimentary base pairs, and bonds between neighboring bases of the same strand (Muyzer *et al.*, 1993). Sequences with abundant GC (guanine-cytosine) content melt at high temperatures compared to sequences rich in AT (adenine-thymine) content. A single point mutation can alter the migration pattern of wild type DNA. For example, mutation that substitutes a G for an A increases the molecule's resistance to melting hence its migration will be higher compared to the wild type DNA. Complete

melting of the DNA molecule is prevented by incorporating a GC clamp at one end, thus creating a high melting domain (Muyzer *et al.*, 1993). The high detection rate and sensitivity of this method has made it an ideal choice in diagnostic laboratories. It has been used in the diagnosis of genetic disorders. Muyzer and co-workers (1993) have successfully used this method in the profiling of complex microbial populations.

There are variations to this method, which involve changes in the denaturants. In Temperature Gradient Gel Electrophoresis (TGGE) technique, the chemical denaturant gradient is replaced by a gradient of increasing temperature (Heuer *et al.*, 2000). When the chemical denaturant is at a constant concentration, the method is termed Constant Denaturant Gel Electrophoresis (CDGE). In this approach, different gel conditions are required for each PCR fragment to be analyzed and it has been mainly limited to the analysis of known mutations. Denaturing gel electrophoresis (DGE) including denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are used to investigate mixed microbial communities (Gilbride *et al.*, 2006).

With DGE, the amplified rRNA gene fragments which are usually limited to 500 bp are separated on the basis of sequence differences instead of size variations (Gilbride *et al.*, 2006). Individual bands can be excised and sequenced to determine the phylogenetic composition. DGE has been shown to be a powerful tool for monitoring the diversity of particular microbial systems (Gilbride *et al.*, 2006). It has been used to discern microbial community composition, to observe population shifts and to follow the succession of bacterial populations over time. However, the limited DNA sequence information obtained from these relatively short fragments can minimize the specificity of phylogenetic identification. In addition, single stranded DNA formed during PCR

amplification from the 16S rRNA gene can contribute to an overestimation of sequence heterogeneity in single GGE bands (Gilbride *et al.*, 2006).

2.3.7 Pulsed Field Gel Electrophoresis

Since the introduction of molecular fingerprinting, there has been a great deal of effort directed towards developing molecular methods suitable for use in research laboratories. Pulsed Field Gel Electrophoresis involves the use of restriction enzymes to generate a limited number (10 to 20) of high molecular weight restriction fragments. These fragments are then separated by agarose gel electrophoresis with programmed variations in both the direction and the duration of the electric field. The resulting electrophoretic patterns are highly specific for strains from a variety of organisms and also provide an opportunity to examine multiple variations throughout the genome of the organism so as to identify specific strains and accurately link them with disease outbreaks (Gautom, 1997).

Pulsed field gel electrophoresis has great value in epidemiological analysis, in the differentiation of pathogenic strains and in monitoring of their spread among communities. The technique has been successfully employed in tracking diseases caused by a number of different bacterial pathogens. Pulsed Field Gel Electrophoresis has also permitted the characterization of isolates indistinguishable by phage typing, ribotyping, plasmid analysis and randomly amplified polymorphic analysis (Gautom, 1997).

Pulsed Field Gel Electrophoresis is proving to be a useful technique in detecting an outbreak caused by a single strain and trace it to its source. The critical stage during the preparation for PFGE is incubation of the agarose plugs with restriction enzymes.

Restriction enzymes recognize specific nucleotide sequences and cut the double stranded DNA wherever that sequence occurs. Restriction digestion is important during PFGE because the length of the fragments of DNA resulting from this digestion will provide a pattern that differs between two bacterial strains. The restriction enzymes used in this process recognize up to 8bp at the site of cleavage. If cells originate from identical bacterial isolates, their chromosomes will have the same nucleotide sequence. Therefore when a restriction enzyme cleaves the DNA wherever it recognizes its specific sequence, the segments released from both chromosomes should be identical (Hentea, 2004).

Since the DNA fragments released by enzymes that recognize up to 8 base pairs (bp) sequences will be very large, a special kind of electrophoresis is needed to separate them. The agarose plugs are placed in the wells of agarose gel, in an electrophoresis chamber and voltage is applied. The principle behind this procedure is the ability of the DNA to travel through a gel sieve and give a specific band pattern. The agarose gel is basically chains of sugar residues cross linked to each other (Hentea, 2004). This technique has become a standard technique among public health agencies due to its accuracy and reproducibility. However, current PFGE protocols involve time consuming, tedious procedure for the purification of intact genomic DNA trapped in agarose, lengthy restriction enzyme digests and extended electrophoresis times. These time-consuming steps preclude the use of current PFGE procedure in monitoring the rapid evolution of events during ongoing outbreaks (Gautom, 1997).

2.3.8 Amplified Restriction Fragment Length Polymorphism (AFLP)

The AFLP method is based on the detection of genomic restriction fragments using PCR amplification (Vos *et al.*, 1995). This method can be used for DNA of any origin or complexity. The technique involves three steps, that is, restriction digestion of the DNA and ligation of oligonucleotide adapters, selective amplification of sets of restriction fragments and gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and the restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments are amplified and visualized after PCR. The method allows the specific co-amplification of high numbers of restriction fragments. The number of fragments that can be analyzed simultaneously, however, is dependent on the resolution of the detection system. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels. The AFLP provides a novel and very powerful DNA fingerprinting for DNAs of any origin or complexity (Vos *et al.*, 1995). The combination of different restriction enzymes and the choice of selective oligonucleotides for primers for PCR make AFLP a useful new system for molecular typing of microorganism (Lin *et al.*, 1996).

Fingerprints are produced without prior sequencing using a limited set of generic primers. The number of fragments detected in a single reaction can be tuned by selection of specific primer sets. The AFLP technique is robust and reliable because stringent

reaction conditions are used for primer annealing. The reliability of the RFLP technique is combined with the power of the PCR technique (Vos *et al.*, 1995).

2.3.9 DNA sequencing

Several DNA sequencing methods are available (Erikson, 2004). The DNA sequencing methods that so far have been developed are based on two fundamental different strategies, namely, direct or indirect sequencing. Direct sequencing involves a variety of synthesis, degradation and separation techniques. The development of improved sequencing methods has led to recent important advances in understanding gene structure and function. A number of methods for sequencing the bases in a DNA molecule have been developed since Sanger and Coulson presented the first technique in 1975 (Erikson, 2004). The sequencing techniques fall into two categories, that is, chemical and enzymatic techniques. In 1977, Sanger and co-workers developed two enzymatic methods that depend on primed synthesis with DNA polymerase to generate fragments of different lengths (Erikson, 2004). In 1980, Maxam and Gilbert developed a method that uses chemical reactions to cleave terminally labeled DNA at specific nucleotide producing fragments of varying lengths. The DNA sequencing techniques rely on electrophoresis on polyacrylamide gels to separate fragments that share a common end but vary in length. However they also require at least one additional preparative gel electrophoresis step to isolate the fragment to be sequenced (Erikson, 2004). Regardless of which method is used, DNA has to be isolated before it can be sequenced. The DNA amount in a cell is too small to be used directly as a source for sequencing (Erikson, 2004).

2.3.9.1 Sequencing of DNA according to Sanger

The Sanger sequencing method which is also called the “plus and minus” technique was discovered by Sanger and Coulson in 1975 (Rosenblum *et al.*, 1997). Two years later Sanger and his collaborators described a new more efficient method, which has been fundamental to the field of DNA sequencing. The Sanger method is now known as the chain termination method, the dideoxynucleotide method or simply known as the Sanger sequencing method (Rosenblum *et al.*, 1997). The method emphasizes on the inability of DNA polymerase to synthesize DNA using 2, 3- dideoxynucleoside triphosphates (ddNTP). Four reactions are set up including primed single stranded DNA template, DNA polymerase, dideoxynucleoside 5-triphosphate and the four deoxynucleoside triphosphates being labeled with ^{32}P . When a dideoxynucleoside is incorporated at the 3' end of the growing primer chain, chain elongation is terminated at G,A,T,C due to the lack of a free 3-hydroxyl group. Each of the four elongation reactions contains a population of extended primer chains, all of which have a common 5' end determined by the annealed primer and a variable 3' end terminating at a specific nucleotide. The base pairs can be visualized after autoradiography of the gel (Rosenblum *et al.*, 1997).

2.3.9.2 Maxam and Gilbert Sequencing Technique

Maxam and Gilbert discovered a chemical method for sequencing DNA in 1977 (Blomstergren, 2003). They exposed a ^{32}P - labeled DNA molecules to reagents that first modify a base. The backbone of the DNA molecule is weakened at these positions and could therefore easily be broken. The removal of bases was limited to one residue for every 50 to 100 bases, while the cleavage of the backbone was performed to completion.

Four different reactions, affecting different bases were performed and the resulting fragments were separated and analyzed on polyacrylamide gels followed by autoradiography (Blomstergren, 2003).

2.3.9.3 Automated sequencing method

Sanger's dideoxy DNA method is the most commonly used method for DNA sequencing, particularly in large scale genomic sequencing. Automated DNA sequencing makes use of fluorescent dyes for the detection of the electrophoretically resolved DNA fragments. Two different methods of automated DNA sequencing have evolved that is the dye-labeled primer sequencing, in which the fluorescent dyes are attached to the 5' end of the primer oligonucleotide, and dye-labeled terminator sequencing, in which the dyes are attached to the terminating dideoxynucleoside triphosphate (Rosenblum *et al.*, 1997).

Dye-labeled primer sequencing has benefited from the use of DNA polymerases which do not discriminate between deoxy- and dideoxynucleotides (Rosenblum *et al.*, 1997). These polymerases provide synthesis of DNA fragments followed sequencing of the fragment which has very even peak heights. Base-reading is easy and reliable, and the ability to determine heterozygotes can be based on peak heights as well as the presence of two bases at a position. The major disadvantage of the dye primer method is the requirement for four separate extension reactions and four dye-labeled primers for each template (Rosenblum *et al.*, 1997).

The major advantages of dye-labeled terminator sequencing are convenience, since only a single extension reaction is required for each template, and the synthesis of a labeled primer is unnecessary, allowing the use of preferred hybridization sites. In

addition false terminations, in which the DNA fragments are terminated by a deoxynucleotide rather than a dideoxynucleotide, are not observed (Rosenblum *et al.*, 1997).

2.4 Lipopolysaccharide analysis methods

2.4.1 Distribution of lipopolysaccharides in bacteria

The envelope of *Salmonella* contains a complex lipopolysaccharide (LPS) structure (Gomes, 2000). The lipopolysaccharide moiety may function as an endotoxin and might be important in determining virulence of the organisms. This macromolecular endotoxin complex consists of three components, an outer O-polysaccharide coat, a middle portion and inner lipid A coat. Lipopolysaccharide structure is important for many reasons. First, the nature of the repeating sugar units in the outer O-polysaccharide chains is responsible for O antigen specificity. *Salmonella* lacking the complete sequence of O sugar repeat units are called rough because of the rough appearance of the colonies. They are usually avirulent or less virulent than the smooth strain that possesses a full complement of O-sugar repeat. Second, antibodies directed against the middle coat or R core may protect against infection since a wide variety of Gram-negative bacteria share a common core structure or may moderate their lethal effect. Third, the endotoxin component of the cell wall may play an important role in the pathogenesis of Gram-negative bacteria. Endotoxins evoke fever, activate the serum complement, kinin and the clotting system, affect myocardial function and alter lymphocyte function. Circulating endotoxin might be responsible for septic shock that can occur in systemic infections (Gomes, 2000). These macromolecules composed of a hydrophilic heteropolysaccharide (comprising the core

oligosaccharide and O-specific polysaccharide or O-chain) which is covalently linked to lipid A, which anchors these macromolecules to the outer membrane (Shnyra *et al.*, 1993).

The innermost, hydrophobic region, lipid A, is responsible for the major toxic and beneficial properties of bacterial endotoxins (Hamidi *et al.*, 2005). Lipid A is the least variable part of the molecule among the different species of a genus, and its structure generally consists of a diglucosamine backbone substituted with variable number (usually four to seven) of ester- or amide-linked fatty acids. Phosphate and other substituents are linked to carbons at the C-1 and C-4' positions of the glucosamine disaccharide. 2-keto-3-deoxyoctonate unit links the lipid A to a core oligosaccharide composed of 10 sugar residues divided into two regions: a best conserved inner core part and a distal outer core. The core is linked to a third outermost region of a highly immunogenic and variable O-chain polysaccharide or O-antigen. The latter region of the LPS molecule is responsible for bacterial serological strain specificity and is present only in smooth-type bacteria. The so-called rough-type bacteria produce lipopolysaccharides lacking O-antigens (Hamidi *et al.*, 2005)

2.4.2 Lipopolysaccharides extraction methods

Lipopolysaccharides can be isolated from Gram-negative bacteria by different methods, the most efficient and commonly used one being the hot phenol-water extraction procedure introduced by Westphal and Lüderitz (Westphal and Luderitz, 1954). It was later modified by different authors, and specific methods were developed for rough-type endotoxin extractions (Hamidi *et al.*, 2005). However, each method requires several days

for the extraction and purification of endotoxins and further steps to isolate the lipid A moiety. New methods have been described to extract LPS from small quantities of cells, such as by mini phenol extraction or using an RNA-isolating reagent, but these methods still require 2 or 3 days (Hamidi *et al.*, 2005).

In early experiments, acid hydrolysis was used to isolate lipid A from endotoxins, splitting the acido-labile ketosidic bond of Kdo (Hamidi *et al.*, 2005). In 1963 the method was modified by a mild hydrolysis treatment using acetic acid. Excessively long and strong hydrolytic conditions, which are sometimes necessary to release the lipid A-polysaccharide, result in dephosphorylation and O-deacylation of lipid A. Such modifications strongly diminish the biological activities of the molecule. Mild hydrolysis conditions, such as pH 4.5 with sodium acetate buffer often proved to be efficient for lipid A isolation and are usually improved by adding SDS when the hydrolysis kinetics are too slow (Hamidi *et al.*, 2005).

2.4.3 Techniques involving the analysis of lipids

The use of lipid biomarkers for revealing the composition of microbial communities is well established. A comprehensive lipid analysis of microbial communities has formed a framework for improved understanding of the population structure in natural environments (Jahnke *et al.*, 2001). Extraction, identification and quantification of phospholipid fatty acids (PLFA) can provide valuable information on the total viable biomass in microbial communities (Macnaughton and Stephen, 2000). Lipids are essential components of membranes and are universally distributed in all cells (White and Ringelberg, 1998). The signature lipid biomarker analysis is used in combination with

other methods that are necessary for fine-scale taxonomic studies. These are mainly nucleic acid-based methods. The combination of lipid analysis and PCR-fingerprinting can bring an improved report on the physiological status and identify the major components of an active microbial community. Fatty acids composition of bacteria is affected by conditions, under which the bacteria are being cultured, thus its combination with other techniques is essential (Macnaughton and Stephen, 2000).

A combination of lipid biomarker analysis and nucleic acid based methods successfully reveal a phylogenetic relationship of *Brucella abortus* with members of the Alpha-2 subdivision of the class Proteobacteria (Moreno *et al.*, 1990). This method has also been used in the analysis of the community and trophic structure of the hyperthermophilic pink streamers from Octopus spring (Jahnke *et al.*, 2001).

Lipopolysaccharides (LPS) play an important role in the pathogenesis of gram-negative bacteria. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in conjunction with silver staining is a powerful tool, allowing the analysis of size heterogeneities of small quantities of LPS without LPS purification (Sledjeski and Weiner, 1991). Using this method, the LPS components of many gram-negative bacteria have been characterized. The LPS of many species of Enterics and of many *Vibrio* were found to have a ladderlike banding pattern (Sledjeski and Weiner, 1991).

Using this technique, lipid A binds to SDS and moves through the gel with the bound O chain moiety (Aucken and Pitt, 1993). Since O chains occur in different lengths, the result is a ladder profile in which the lowest rungs represent rough LPS while the higher rungs represent smooth LPS with increasing numbers of side chain repeating units.

The size of the repeating unit is reflected in the spacing between the rungs. It follows that isolates of the same species with different LPS electrophoretic profiles will also have serologically different O antigens. Therefore, this technique may allow the comparative typing with potentially similar discriminatory power as O serotyping. LPS profile typing method has been applied to differentiate and classify isolates of a variety of gram-negative species from a number of hospital outbreaks (Aucken and Pitt, 1993).

Analysis of S-form LPS preparations by SDS-PAGE followed by silver staining technique reveals numerous bands arranged in ladderlike pattern (Formsgaard *et al.*, 1990). Such bands reflect the number of repeating units present in the O chain of the LPS. The fastest migrating band is LPS lacks the repeating units, whereas the second band represents the core-plus-one repeating unit and so forth. Thus, silver-stained SDS-polyacrylamide gels are often used to distinguish between the R-form and S-form LPS. SDS-PAGE analysis of LPS derived from clinical isolates of polyagglutinable strains of *Pseudomonas aeruginosa* showed a rough LPS profile after silver staining which was characteristic of nontypeable *P. aeruginosa* strains (Formsgaard *et al.*, 1990).

Lipopolysaccharides of *Salmonella dublin*, *S. enteritidis*, *S. typhimurium*, *S. choleraesuis* and similar strains were analyzed to investigate the correlation between LPS and virulence plasmid of *Salmonella* (Kawahara *et al.*, 1989). All wild-type strains had smooth type LPS i.e. LPS with long O-specific polysaccharide. The virulence plasmid-cured strain of *S. dublin*, C524, exhibited a shorter O-specific chain than its parent strain, 5240. No distinct ladder bands were observed at high molecular weight region on the SDS-PAGE for C524 lipopolysaccharide. In the case of *S. enteritidis* neither *S. typhimurium* nor *S. choleraesuis*, alteration of neither chemical composition nor

electrophoretic profile of LPS was detected by deletion and reintroduction of their virulence plasmids. Those results suggest that certain regulatory factors of the chain length of O-specific polysaccharide are encoded on the virulence plasmid of *S. dublin* (Kawahara *et al.*, 1989).

2.5 Background on antibiotics

Antibiotics can be categorized as bactericidal or bacteriostatic. They are bactericidal if they kill the susceptible bacteria or bacteriostatic if they reversibly inhibit the growth of bacteria. Generally, the use of bactericidal antibiotics is preferred but many factors may impose the use of bacteriostatic antibiotics. However when bacteriostatic antibiotics are used the duration of the therapy should be sufficient to allow cellular and humoral defense mechanisms to eradicate the bacteria. In clinical laboratories a more common test for antibiotic susceptibility is a disk diffusion method. During this test the bacterial isolate is inoculated uniformly onto the surface of an agar plate. A disk impregnated with a standard amount of an antibiotic is applied to the surface of the plate and the antibiotic is allowed to diffuse into the adjacent medium. The result is a gradient of antibiotic surrounding the disk. Following incubation, a bacterial lawn appears on the plate. Zones of inhibition of bacterial growth may be present around the antibiotic disk, the size of which is dependent on the diffusion rate of the antibiotic, the degree of sensitivity of the microorganism and the growth rate of the bacterium. The zone of inhibition in the disk diffusion test is inversely related to the Minimum Inhibitory Concentration (Mayer, 2003).

2.5.1 Antimicrobial susceptibility pattern of *Salmonella* isolates

The emergence of antimicrobial resistant bacterial pathogens has become a major public health concern. The use of antibiotics in medical and agricultural sectors has resulted in widespread antibiotic resistance and in the development of genetic mechanisms efficient for the dissemination of antibiotic resistance gene cassettes, especially within and between species of gram-negative organisms. In recent years, testing of *Salmonella* isolates from various countries has shown that an increasing proportion is multidrug resistant (Cheng *et al.*, 2004). Of particular concern is isolation of ceftriaxone and ciprofloxacin resistant *Salmonella* species because of the continuous use of these two agents in treating *Salmonella* infections in children and adults respectively (Cheng *et al.*, 2004).

Resistance to antimicrobial agents in bacteria is mediated by several mechanisms including changes in bacterial cell wall permeability, energy dependant removal of antimicrobial agents via membrane-bound efflux pumps, modification of the site of drug action, and destruction or inactivation of antimicrobial agents (Cheng *et al.*, 2004). Bacteria can possess one or all of these mechanisms. Acquired antimicrobial resistance phenotypes most often develop via conjugative transfer of plasmids. Plasmids may carry class 1 integrons which are mobile DNA elements that are important in the proliferation of bacterial multidrug resistance (MDR), especially among the gram negative enteric species. Integrons primarily have been found located within transposons Tn 402 and Tn21 which in turn reside on broad-host range plasmids or the IncF plasmid. By incorporating into transposons and plasmids, integrons participate in the capture of resistance genes and dissemination of these genes among bacteria (Cheng *et al.*, 2004).

Molecular genetic techniques have been used to characterize antimicrobial-resistant *Salmonellae*, especially *Salmonella enterica* serovar *typhimurium* DT104. For instance, variant *Salmonella* genomic island 1 (SG11) MDR regions, consisting of integrons encoding different resistance genes, have been found in the chromosomal DNA of *Salmonella* serovar *typhimurium* DT104 and Agona (Boyd *et al.*, 2002). The formation of these MDR clusters is hypothesized to favor expression of a large number of resistance genes and to enhance their transfer to other bacteria. Since class 1 integrons have become integrated into the chromosome in *Salmonella* serovars *typhimurium* DT104 and Agona, they are able to persist even in the absence of antimicrobial selection with no apparent fitness cost to the cell. This has led to a stable and widely disseminated clone of multidrug resistant *Salmonella* serovar *typhimurium* in the case of *Salmonella* serovar *typhimurium* DT104 (Cheng *et al.*, 2004).

For more than 40 years, chloramphenicol was the drug of choice for the treatment of *Salmonella enterica* serovar *Typhi*, the causative agent of typhoid fever (Kariuki *et al.*, 2004). However, the emergence of multidrug resistant serovar *Typhi* to ampicillin, chloramphenicol and cotrimoxazole was reported in South Africa, India, Arabian Gulf and the Philippines in the late 1980. This has led to the use of the fluoroquinolones as alternative drugs (Kariuki *et al.*, 2004). Among the first reports of clinical treatment failure due to serovar *Typhi* resistant to nalidixic acid and showing an increased ciprofloxacin MIC (0.125 µg/ml) resistance was first demonstrated in 1991 in a patient who had recently returned to the United Kingdom from India (Kariuki *et al.*, 2004). Thereafter, several cases of MDR serovar *Typhi* also resistant to nalidixic acid and fluoroquinolones have been reported in Bangladesh, India, Thailand, Vietnam and

Tajikistan, raising concern about further spread of the species to other regions where typhoid fever is endemic (Kariuki *et al.*, 2004). In addition, molecular characterization of the serovar Typhi outbreak strains have revealed that resistance to commonly used drugs including chloramphenicol, ampicillin and trimethoprim are encoded by plasmids of the HI incompatibility group (Mills-Robertson *et al.*, 2002).

2.5.2 The mode of action of antimicrobial agents used in this study.

Bacteria cause diseases that can sometimes lead to life-threatening conditions (Cheng *et al.*, 2004). Because bacteria are able to multiply rapidly, spread diseases quickly and can be devastating. Antibiotics show the ability to stop bacterial growth by blocking processes essential for cell division (Cheng *et al.*, 2004). Therefore, antibiotics can be defined as agents that are selectively toxic to bacteria, either killing them (bacteriocidal) or inhibiting their growth (bacteriostatic) with little or no harm to the patient. Toxic selectivity is very crucial in the activity of antibiotics, these compounds must act on structures found in bacteria, but not on the host (Cheng *et al.*, 2004).

In ensuring this principle three major processes in bacteria are targeted by antibiotics. The first is protein synthesis by binding of the antibiotic to the 30S subunit of the ribosome. By binding to the ribosome, the antibiotic prevents translation and the bacteria cannot synthesize proteins (Cheng *et al.*, 2004). The second function targeted is cell wall synthesis. Certain groups of antibiotics prevent cell wall formation by blocking cross-linking reactions of peptidoglycan necessary for stabilization of the cell wall and thereby causing the cell to rupture (Cheng *et al.*, 2004). The most common family of antibiotics that affect cell wall synthesis is the β -lactams of which penicillin and

ampicillin are members of. The third class of antibiotics interacts with the genomic material of an organism. For instance Novobiocin and Levofloxacin interfere with DNA gyrase and prevent replication of DNA necessary for cell division and therefore survival of the bacteria (Cheng *et al.*, 2004).

After isolation of pure colonies, the susceptibility of bacterial isolates to a variety of antibiotics can be tested. The basic quantitative measures of the *in vitro* activity of antibiotics are Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). The MIC is the lowest concentration of an antibiotic that inhibits visible growth either on plate or turbidity in broth culture under standard conditions (Cheng *et al.*, 2004). The MBC is the lowest concentration of the antibiotic that kills 99.9% of the original inoculum within a given period (Mayer, 2003). In other words the zone of inhibition around a disk impregnated with antibiotic is a measure of antibiotics inhibitory activity (Cheng *et al.*, 2004).

2.5.2.1 Neomycin

Neomycin is used to treat external infections in humans and is given orally to cattle, sheep, pigs, goats and poultry in cases of bacterial gastrointestinal infections. It is an aminoglycoside and is active against bacteria that grow aerobically. It is considered to be inactive against anaerobes, and its activity against facultative bacteria *in vitro* is lower in anaerobic environments than in the presence of oxygen (Brown, 1988).

Neomycin is produced by *Streptomyces fradiae* (Brown, 1988). The antibiotic is a complex consisting of neomycin A, neomycin B and neomycin C, generally containing

more than 90% neomycin B, the remainder being mainly neomycin C. Neomycins B and C both contain three amino sugars attached by glycosidic linkage to the central hexose. Neomycin A, more appropriately referred to as neoamine, is a hydrolysis product of either neomycin B or neomycin C and usually makes up less than 1% of the mixture. Neomycin A has a bicyclic ring system with four amino groups. Neomycin B has a total of six amino groups and consists of neamine and neobiosamine B, a disaccharide of D-ribose and neosamine B. Neomycin C is a stereoisomer and consists of neamine and neobiosamine C, a disaccharide from D-ribose and neosamine C (stereoisomer of neosamine B). When tested by antibiotic dilution techniques against aerobic and facultative bacteria, the activity of neomycin B is generally greater than that of neomycin C, which is greater than that of neamine (Brown, 1998).

The bacteriocidal effects of aminoglycosides are caused by disruption of cellular transport mechanisms as a result of the formation of abnormal cell membrane channels by abnormal proteins. Neomycin reacts with 30S ribosomal subunits of prokaryotic cells by electrostatic attraction (Prescott *et al.*, 2000). Since it is a polycation, neomycin binds to negatively charged bacterial surface anions such as the lipopolysaccharide of gram-negative bacteria, teichoic acids of gram-positive bacteria and polar portions of phospholipid in both types of bacteria (Brown, 1998). Active transport is required for neomycin to traverse the membrane so that it can reach the ribosomal target site. Such transport mechanisms are lacking in a number of the anaerobes studied to date, and for this reason they are generally not sensitive to the aminoglycosides. Aminoglycosides are moved across the cytoplasmic membrane by the membrane potential after non-specific association with a transporter in the cytoplasmic membranes. Ribosomes and nucleic

acids act as binding sites for transported aminoglycosides and contribute significantly to total cell uptake. In the presence of sufficient concentrations of neomycin, transport may cause some release of cell components, including potassium, amino acids and nucleotides, from exposed bacterial cells through the damaged cell wall (Brown, 1998).

Aminoglycosides have additional effects on microorganisms, including interference with the cellular electron transfer system, induction of RNA breakdown, disruption of polysomes into inactive monosomes, inhibition of translation, blocking of initiation of DNA replication, effects on DNA metabolism and damaged cell membranes (Prescott *et al.*, 2000). Most of these effects are likely to be due to the mistranslation of mRNA.

2.5.2.2 Chloramphenicol

Chloramphenicol (Cm) was first discovered in 1947 as an antibiotic in cultures of the *Streptomyces venezuelae* and is now synthesized chemically. It is one of the most effective and inexpensive treatments, for the cases of Rocky Mountain Fever (Stallings, 2001). This important antibiotic inhibits protein biosynthesis by binding reversibly to 50S prokaryotic ribosomal subunits at a site that blocks the peptidyl transferase step in protein biosynthesis. Resistance to chloramphenicol due to the enzymatic inactivation of the antibiotic is the result of chloramphenicol acetyltransferase (CAT) activity (Shaw and Leslie, 1991).

Chloramphenicol has a very broad spectrum of activity because it functions by inhibiting bacterial protein synthesis (Cocolin *et al.*, 2001). It is active against Gram

positive bacteria, Gram negative bacteria and anaerobes. It is not active against *Pseudomonas aeruginosa* or *Enterobacter* species. It has some activity against *Burkholderia pseudomallei*, but is no longer routinely used to treat infections caused by this organism (Cocolin *et al.*, 2001). The original indication of chloramphenicol was in the treatment of typhoid, but the worldwide problem of multidrug resistant *Salmonella typhi* has minimized the usage of this antibiotic except when the organism is known to be sensitive. Chloramphenicol may be used as a second line agent in the treatment of tetracycline resistant cholera (Cocolin *et al.*, 2001).

Chloramphenicol is bacteriostatic, that is it stops bacterial growth by inhibiting the enzyme peptidyl transferase. While chloramphenicol and the macrolide class of antibiotics function ultimately in the same manner of affecting the 50S ribosomal subunits, chloramphenicol is not a macrolide. There are three mechanisms of resistance to chloramphenicol: that is reduced membrane permeability, mutation of the 50S ribosomal subunit and elaboration of chloramphenicol acetyltransferase. It is easy to select for reduced membrane permeability to chloramphenicol in vitro by serial passage of bacteria, and this is the most common mechanism of low level chloramphenicol resistance (Cocolin *et al.*, 2001). High level resistance is conferred by the *cat*-gene. This gene codes for an enzyme called chloramphenicol acetyltransferase which inactivates chloramphenicol by covalently linking one or two acetyl groups derived from acetyl-S coenzyme A, to the hydroxyl groups on the chloramphenicol molecule. The acetylation prevents chloramphenicol from binding to the ribosome. Resistance conferring mutations of the 50S ribosomal subunit are rare. Chloramphenicol resistance gene maybe carried on a plasmid that also codes for resistance to other drugs (Cocolin *et al.*, 2001).

2.5.2.3 Doxycycline

Doxycycline is a member of the tetracycline antibiotics group and is commonly used to treat a variety of infections (Chopra and Roberts, 2001). Tetracyclines were first isolated from the *Streptomyces species*. The tetracyclines, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. The tetracyclines reversibly bind to the 30S ribosome and inhibit binding of aminoacyl-t-RNA to the acceptor site on the 70S ribosome. Tetracyclines (tetracycline, doxycycline) bind to the 30S subunit and block polypeptide chain elongation by preventing the attachment of charged aminacyl-tRNA. Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites (Chopra and Roberts, 2001).

2.5.2.4 Streptomycin

Streptomycin is an antibiotic that belongs to the group called aminoglycosides (Kingston, 2004). It is the first class to be discovered in this group and was the first antibiotic to be used for remedy of tuberculosis (Kingston, 2004). Streptomycin was first isolated in 1943 in the laboratory of Selman Abraham Waksman by Albert Schatz (Kingston, 2004). It is derived from the actinobacterium *Streptomyces griseus*. Streptomycin stops bacterial growth by damaging cell membrane and inhibiting protein synthesis. Specifically, it binds to the 16S rRNA of the bacterial ribosome, which prevents the release of the polypeptide chain. An adverse effect of this medicine is ototoxicity (Kingston, 2004).

2.5.2.5 Kanamycin

The antibiotic kanamycin is used extensively in genetic engineering as a marker (Onaolapo, 1994). There is a well known-resistance towards antibiotics of a particular type. A mutation to resistance to an antibiotic may cause resistance to some or all members of the antibiotic family (Onaolapo, 1994). Kanamycin is a member of the aminoglycoside antibiotics. Cross-resistance between kanamycin and other aminoglycosides including streptomycin, gentamycin and tobramycin was found to vary markedly between isolates (Onaolapo, 1994). All of the antibiotics mentioned are used to treat bacterial infections. The aminoglycoside antibiotic neomycin was found to cross react with kanamycin B in inhibiting RNase P ribozyme, 16S ribosomal RNA and tRNA maturation (Onaolapo, 1994). Kanamycin inhibits the binding of EF-G to the ribosome (Chopra, 2002).

2.5.2.6 Cotrimoxazole

Cotrimoxazole is an approved sulphanamide drug (Kim *et al.*, 2004). It consists of a mixture of two drugs, trimethoprim and sulphamethoxazole, which both prevent bacteria from reproduction. They interfere with folate metabolism in the bacterial cell by blocking the biosynthesis of tetrahydrofolate. Cotrimoxazole is also used to treat Shigellosis, urinary tract infections, isosporiasis and bronchitis. Intravenously cotrimoxazole is used for treating Shigellosis, Salmonellosis and severe or complicated urinary tract infection (Kim *et al.*, 2004).

2.5.2.7 Sulfamethoxazole

Sulfamethoxazole is a sulfonamide bacteriostatic (Kim *et al.*, 2004). It is most often used as part of a synergistic combination with trimethoprim in 5:1 ratio with cotrimoxazole, which is also known as bactrim or septrin. It can be used as an alternative to amoxicillin-based antibiotic to treat sinusitis. They are structural analogs and competitive antagonists of para-aminobenzoic acid (PABA). They inhibit normal bacterial utilization of PABA for the synthesis of folic acid, an important metabolite in DNA synthesis (Kim *et al.*, 2004). Sulfonamides interfere with folate metabolism in the bacterial cell by competitively blocking the biosynthesis of tetrahydrofolate (Kim *et al.*, 2004)

Tetrahydrofolate acts as a carrier of one-carbon fragments and is necessary for the ultimate synthesis of DNA, RNA and bacterial cell wall proteins (Kim *et al.*, 2004). Unlike mammals, bacteria and protozoa usually lacks a transport system to take up preformed folic acid from their environment. Most of these must synthesize folates, although some are capable of using exogenous thymidine, circumventing the need for folate metabolism. Sulfonamides competitively block the conversion of pteridine and p-aminobenzoic acid to dihydrofolic acid by the enzyme pteridine synthetase (Kim *et al.*, 2004). The effects seen are usually bacteriostatic in nature. Folic acid is not synthesized in humans, it's a dietary requirement. This allows the selective toxicity to bacterial cells (or any cell dependent on synthesizing folic acid) compared to human cells (Kim *et al.*, 2004).

CHAPTER 3

MATERIALS AND METHOD

3.1 Sampling

Water samples from Gogogo and Tyume rivers, and untreated wastewater samples from Fort Hare wastewater treatment plant in Alice, Amalinda wastewater treatment plant in East London, Schornville wastewater treatment plant in King Williams Town were collected using sterile 1L Nalgene bottles. The samples were then transported on ice in cooler boxes to the laboratory of the Department of Biochemistry and Microbiology, University of Fort Hare, Alice for further processing. Water samples were collected by carefully removing the cap of the bottles to prevent contamination of the inner surface. Holding the bottle at the bottom and plunging it below the water surface allowed the water to enter the bottle. The mouth of the bottle was placed opposite the water current. If there was no current, it was created artificially by pushing the bottle forward. The bottle was filled leaving about 2.5cm of empty space to allow mixing during laboratory analysis. It was then immediately closed and kept in a cooler box. All the water samples were analysed within 12-24 h after collection.

3.2 Isolation and identification of *Salmonella* species

Five hundred milliliters (500 ml) of water samples were centrifuged in sterile 50ml centrifuge tubes at 3500×g for 25 minutes using the Beckman type centrifuge (TJ-6 Centrifuge, Scotland). The supernatants were discarded, and 20 ml of the supernatant and pellets were left for further processing. The cell suspensions were used for *Salmonella* species isolation. The suspensions were enriched in tetrathionate broth (Merck,

Darmstadt, Germany) in a 1:1 ratio (sample: broth) and incubated for 18-24 hrs at 35°C on an orbital incubator (Stuart Scientific U.K.). After incubation period, a loopful of the enriched culture was transferred onto XLD agar (Biolab Diagnostics, Saarchem, S.A) and incubated at 35°C for 18 - 24 hrs. Red colonies with black spots in the center that appeared on the agar were randomly picked and purified. A single colony was streaked onto a nutrient agar plates, incubated overnight at 35°C and colonies were Gram stained. The isolates were further confirmed as *Salmonella* species by API assay (Bio Mérieux, Lyon, France).

3.3 Antimicrobial susceptibility test of isolates

The susceptibility of *Salmonella* isolates to antimicrobial agents was determined using Kirby-Bauer disk diffusion method (Bauer *et al.*, 1996). A bacterial lawn was prepared by transferring a bacterial colony in 2.5 ml normal saline using a sterile inoculating loop, and spreading onto Muller-Hinton agar (Biolab Diagnostics, Saarchem, S.A). The excess inoculum was withdrawn off with a Pasteur pipettes. Plates were allowed to dry at room temperature. Disks containing predetermined amounts of antibiotics were dispensed onto the bacterial lawn and the plates were immediately incubated at 37°C for 18-24 hours. After the incubation period, the diameters of the inhibition zones were measured. The following antibiotics and sulfonamides were used: neomycin (NEO), 10 µg/disk; chloramphenicol (CHL), 30 µg/disk; doxycycline (DXT) 30 µg/disk; sulphonamides (SMX) 50 µg/disk; kanamycin (K) 30 µg/disk; streptomycin (S) 300 µg/disk and cotrimoxazole (TS) 25 µg/disk. The test was done in triplicates.

The recorded zones of inhibition were compared with the standard of the United States National Committee for Clinical Laboratory Standards (NCCLS) now known as CLSI. The isolates were classified in three different categories as follows: resistant (R), sensitive (S) and intermediate (I). An isolate was considered resistant if the disc has no discernable zone around it or if it has an inhibition zone less or equal to the disc diameter. It was considered sensitive if it had a clear zone or if it had an inhibition zone greater than the disc diameter. It was considered intermediate if the zone of inhibition was cloudy (Bauer, 1996).

3.4 Isolation of DNA and restriction digestion analysis

Genomic DNA from forty *Salmonella* isolates cultured in nutrient broth was isolated according to Neumann *et al.* (1992). Cells were harvested by centrifugation at 1000×g for 20 min., using a JA20 angle rotor (Beckman centrifuge). The pellets were resuspended in 400 µl Tris-EDTA (TE) buffer, containing 0.01M Tris-HCl pH 7.4, 0.001M EDTA, (Saarchem, Gauteng, S.A) by vortexing. Fifty microliters of 10% Sodium Dodecyl Sulphate, (SDS) (Saarchem, Gauteng, SA) was added to the suspensions. Samples were digested with 50 µl (20mg/ml stock solution) proteinase K (Merck, Darmstadt, Germany) for 1 hr at 37°C. DNA was extracted twice with equal volumes of phenol: chloroform (1:1) (Merck, Darmstadt, Germany) by centrifugation at 1000×g for 1 min. It was re-extracted with equal volume of chloroform followed by centrifugation at 1000×g for 10 min. The supernatants were treated with 5 µl RNase A (5mg/mL in RNaseA buffer containing 0.5M NaCl, 0.01M EDTA) and the samples were incubated at 37°C for 30 min. DNA was precipitated with 5M ammonium acetate, final concentration of 0.5M and

2 volumes of isopropanol by incubation at 4°C overnight. The DNA was centrifuged for 10 min at 10 000xg and washed twice with 70 % ethanol. The pellets of DNA were dried at room temperature and dissolved in 50-100 µl TE buffer.

Two-five micrograms of the DNA samples were digested with 20-50 units of each of the restriction endonucleases Xba1 and Nde1, and Bst Z1 (Promega, Madison. USA) in the presence of buffer D (Promega, Madison. USA) in a final volume of 20 µl. The digestion of the DNA samples was allowed to proceed at 37°C for 3 hrs to overnight. The digested DNA samples were separated on 5% Acrylamide/Bisacrylamide (30%/0.8% w/v stock solution) (Merck, Darmstadt, Germany) gel containing 0.04M Tris acetate and 0.002M EDTA pH 8. The gels were run at 90mV, 30mA for five and a half hours. When the bromophenolblue dye reached the end of the gel, the electrophoresis was stopped. The gels were stained with silver sequence DNA staining kit (Promega, Madison, US.). Images of the gels were taken using Biodoc-IT System with built-in CCD Camera (Transilluminator, UVP, Upland, CA). Dendograms were constructed using Jackard simple matching coefficient (found in the vegan package) as the input into the UPGMA clustering technique in the R statistical computing environment.

3.5 Extraction of protein and SDS-PAGE analysis

After identification of *Salmonella* strains using API 20E assay, a single colony of each of the forty *Salmonella* strains was inoculated into 200 ml of nutrient broth (Saarhem, Gauteng, S.A) in a 500 ml Erlenmeyer flask. The culture was incubated at 35°C for 48h at 180 rpm on an orbital incubator (Stuart Scientific U.K.). Cells were harvested by centrifugation at 1000×g for 30 min using a JA angle rotor (Beckman centrifuge). The

pellets were washed twice with millipore water and resuspended in Tris-HCl buffer, containing 0.05 M Tris HCl pH 7.5, 4% sodium dodecyl sulphate (SDS) and 20% (v/v) glycerol (Merck, Darmstadt, Germany). The ratio of buffer to cells was (1:1) buffer per gram of cell wet mass. The cell suspensions were heated in a water bath at 100 °C for 15 min and centrifuged at 4°C for 3 h (16000 ×g). The supernatants containing the proteins were dialyzed for 48h against distilled water. The dialysates were freeze dried and the crystallized protein dissolved in 100-150 µl of milli Q water. The proteins were analysed by SDS-PAGE. Gels containing 12% acrylamide (Merck, Darmstadt, Germany) and 0.8% bisacrylamide in 0.04M Tris HCl pH 8 and 0.5% SDS. As a standard, a broad range molecular weight marker (225-10KD) was used to calibrate the gels. The samples were run at 70 mV in vertical slab gel apparatus (Tall Mighty Small Vertical Slab Gel Unit, model SE 200 Hoefer Scientific instruments San. Francisco California) until the marker dye reached the bottom of the gel. The gels were stained with silver - Fast silverTm gel staining kit (Calbiochem Darmstadt, Germany) and destained in a solution containing 25% ethanol and 8% acetic acid. All protein extracts were run in duplicates. Gel images were taken using Biodoc-H system transilluminator (UVP, Upland, CA. U.S.). Dendograms were constructed using Jackard simple matching coefficient (found in the vegan package) as the input into the UPGMA clustering technique in the R statistical computing environment

3.6 Isolation and analysis of lipopolysaccharides

Lipopolysaccharides (LPS) were isolated from *Salmonella* strains according to the method of Yang and Lin (1998). *Salmonella* isolates were cultured separately in 500 ml

nutrient broth overnight at 35°C. Five hundred milliliters of overnight cultures of *Salmonella* strains were centrifuged at 1000×g in a Beckmann centrifuge for 30 minutes. The pellets were suspended in 0.1M phosphate-buffered saline, pH 7.4. The suspensions were stirred for 30 min on ice and centrifuged at 10 000×g for 10 min in an Eppendorf centrifuge (Hamburg, Germany). The cell pellets were treated with hot phenol buffered with sodium carbonate (0.1% NaHCO₃, 95% phenol) at 65°C for 15 min and cooled to 10°C for 15 min. The samples were centrifuged at 10 000×g for 10 min and the aqueous upper layer was poured into dialysis tubes and dialyzed against distilled water for 48 hours. After dialysis the samples were centrifuged at 10 000×g for 5 min and supernatants were lyophilized in Xerotec Freeze Dryer (Sussex, England). The LPS were collected in 50-100 µl of distilled water. Aliquots of the samples were loaded onto 12% PAAC/SDS gels using slab gel electrophoresis apparatus (Tall Mighty Small Vertical Slab Gel Unit, Hoefer Scientific Instruments San Francisco, USA) (Laemli, 1970). Twenty microliters of each sample of LPS was mixed with 20 µl of 5X sample buffer containing 0.5M Tris- HCl (Merck, Darmstadt, Germany), pH 8.8, 10% glycerol (Merck, Darmstadt, Germany), 10% SDS (Sodium dodecyl sulfate), (Saarchem,Gauteng,SA), 0.0001M Beta-mercaptoethanol (Merck, Darmstadt, Germany) and 0.04% Bromophenol blue (Sigma, Aldrich, St. Lois, Mo. US). Ten microliters of standard protein marker (Broad range Protein Molecular Weight Marker, Promega Madison,USA) was used for calibration of the gel. The lipopolysaccharides were run according to the method of Laemmli (1970). The electrophoresis was run at 60mV for 6hrs using power supply (LKB-Broma, Uppsala, Sweden). The gels were stained with silver - Fast silver[™] gel staining kit (Calbiochem Darmstadt, Germany). Images of the gels were taken using

Biodoc-H system (Tronsilluminator UVP, Upland, CA. U.S). Dendograms were constructed using Jackard simple matching coefficient (found in the vegan package) as the input into the UPGMA clustering technique in the R statistical computing environment.

3.7 Dendogram

The software used was R Development Core Team (2007). R: A language and environment for statistical computing (R Foundation for Statistical computing, Vienna, Australia, ISBN3-9000 51-07-0, URL

CHAPTER 4

RESULTS

4.1 Antibiotic sensitivity of *Salmonella* species

Forty purified and confirmed *Salmonella* strains were tested for antibiotic susceptibility. The results of the antibiotic sensitivity profile given in Tables 1 show the percentages of the isolates against different antibiotics, based on their reaction to the drugs as resistant, intermediate or susceptible. The antibiotic sensitivity test of the *Salmonella* isolates against neomycin revealed a very high level of susceptibility. Ninety percent of the isolates tested manifested susceptibility to neomycin while 10 % was intermediate (Table 1). The classification of *Salmonella* isolates sensitivity against neomycin was determined in accordance with NCCLS derived performance standards as follows: Resistant $\leq 12\text{mm}$, intermediate 13-16mm and susceptible $\geq 17\text{mm}$ (NCCLS, 1999).

The sensitivity tests of *Salmonella* strains against chloramphenicol showed a high level of susceptibility, with 72.5% of isolates being susceptible, 22.5% were intermediate and 5% were resistant. When doxycycline was used to determine *Salmonella* strains sensitivity, the strains were categorized as susceptible ($\leq 14\text{mm}$), intermediate (15-18mm) and resistant ($\geq 19\text{mm}$) in accordance with zone size interpretive standards for doxycycline (NCCLS, 1999). As seen from Table 1, the doxycycline sensitivity test revealed that 57.5% of isolates were resistant, 37.5% were intermediate and 5% were susceptible, which shows that more than 50% of species were not affected by the antibiotic. Also, of the forty *Salmonella* isolates tested for sensitivity towards Sulphomethoxazole 92.5% were resistant towards the sulphonamide and only 7.5%

showed intermediate effect while none was susceptible. A high degree of susceptibility was observed for kanamycin. Only 5% of them showed resistance and 5% were intermediate. Results of streptomycin on *Salmonella* species revealed a high level of susceptibility (85%), absence of resistant strains and 15% intermediate strains. Similarly, the *Salmonella* isolates showed a high level of susceptibility towards cotrimoxazole (85%) with 10% exhibiting intermediate effect and only 5% were resistant.

Table 1: The effect of tested antimicrobials on *Salmonella* species.

Antibiotic	Antibiotic concentration on the disc (mg)	Antibiotic sensitivity (Percentages)		
		Resistant	Intermediate	Susceptible
Neomycin	0.03	0	10	90
Chloramphenicol	0.01	5	22.5	72.5
Doxycycline	0.03	57.5	37.5	5
Sulfonamides	0.05	92.5	7.5	0
Kanamycin	0.03	5	5	90
Streptomycin	0.3	0	15	85
Cotrimoxazole	0.025	5	10	85

4.2 DNA profile of the *Salmonella* strains

We have studied the genetic diversity of forty *Salmonella* isolates from Amalinda, Shornville, Fort Hare wastewater plants, and different sites of Tyume and Gogogo rivers in Eastern Cape Province. Restriction digestion of genomic DNA of the 40 *Salmonella*

strains with EcoR1 and SMA 1 revealed different band patterns. The DNA profiles of *Salmonella* strains were resolved into 18 different bands ranging in size from 500 bp to 12000 bp (Figure 1).

The dendrogram obtained using Jackard simple matching coefficient as the input to the UPGMA clustering technique in the R statistical computing environment (software R development Core Team 2007) (Figure 2), revealed four similarity groups of strains (Table 3). Group 1 (Table 3) comprises 65% of all isolates and includes strains: 5, 6, 11, 12, 13 and 15 recovered from Amalinda wastewater plants' sludge tank, strains: 26, 32, 36, 37, 38, 39 and 40 isolated from Amalinda wastewater plant's sludge tank, strain 9, 24, 27, 31, 33 isolated from Shornville wastewater plant's sludge tank, strains: 7, 14, 16 and 30 recovered from Shornville wastewater plant's secondary clarifiers, strain 25 recovered from Fort Hare wastewater plant, strains 4 and 8 recovered from Gogogo river and strain 10 recovered from Tyume river. All 26 strains clustered as a tight and distinct group with genetic similarity ranging from 80 to 93%. They formed a well defined group, clearly separated from the rest of the isolates.

The second similarity group comprised the following strains: 1, 22, and 23 isolated from Shornville wastewater plant's sludge tank, strain 21 isolated from Shornville wastewater plant's secondary clarifier, strain: 28, isolated from Tyume River. The genetic similarity values of the five strains ranged between 60 and 69%. The third similarity group included strains with similarity values ranging from 42 to 51%, strains: 29 and 35 were recovered from Amalinda wastewater plant's secondary clarifier, strain: 20 were recovered from Amalinda wastewater plant's sludge tank, strain: 34 were

recovered from Shornville wastewater plant's sludge tank and strain 18 was recovered from Shornville wastewater plant's secondary clarifier.

The fourth similarity group comprised a tight and distinct cluster with similarity values ranging from 21 to 35%. The strains: 2 and 19 were isolated from Amalinda wastewater plant's sludge tank, strains: 3 and 17 were isolated from Fort Hare wastewater plant's sludge tank. They formed a small distinct group as seen from Figure 2 and 3.

Table 2: *Salmonella* isolates sources representations on SDS-PAGE gels.

Water Sources	Lanes on SDS-PAGE gels
<i>Amalinda Wastewater Plant</i>	
Sludge tank:	
Site 1	Lane 2
Site 2	Lane 5
Site 3	Lane 6
Site 4	Lane 11
Site 5	Lane 12
Site 6	Lane 13
Site 7	Lane 15
Site 8	Lane 19
Site 9	Lane 20
<i>Amalinda Wastewater Plant</i>	
Secondary clarifier	
Site1	Lane 26
Site 2	Lane 29
Site 3	Lane 32
Site 4	Lane 35
Site 5	Lane 36
Site 6	Lane 37
Site 7	Lane 38
Site 8	Lane 39
Site 9	Lane40

Shornville Wastewater Plant

Sludge tank

Site 1	Lane 9
Site 2	Lane 22
Site 3	Lane 23
Site 4	Lane 24
Site 5	Lane 27
Site 6	Lane 31
Site 7	Lane 33
Site 8	Lane 34
Site 9	Lane 1

Shornville Wastewater Plant

Secondary clarifier

Site 1	Lane 7
Site 2	Lane 14
Site 3	Lane 16
Site 4	Lane 18
Site 5	Lane 21
Site 6	Lane 30

Gogogo River

Site 1	Lane 8
Site 2	Lane 4

Tyume River

Site 1	Lane 10
Site 2	Lane 28

Fort Hare Wastewater Plant

Site 1	Lane 3
Site 2	Lane 17
Site 3	Lane 25

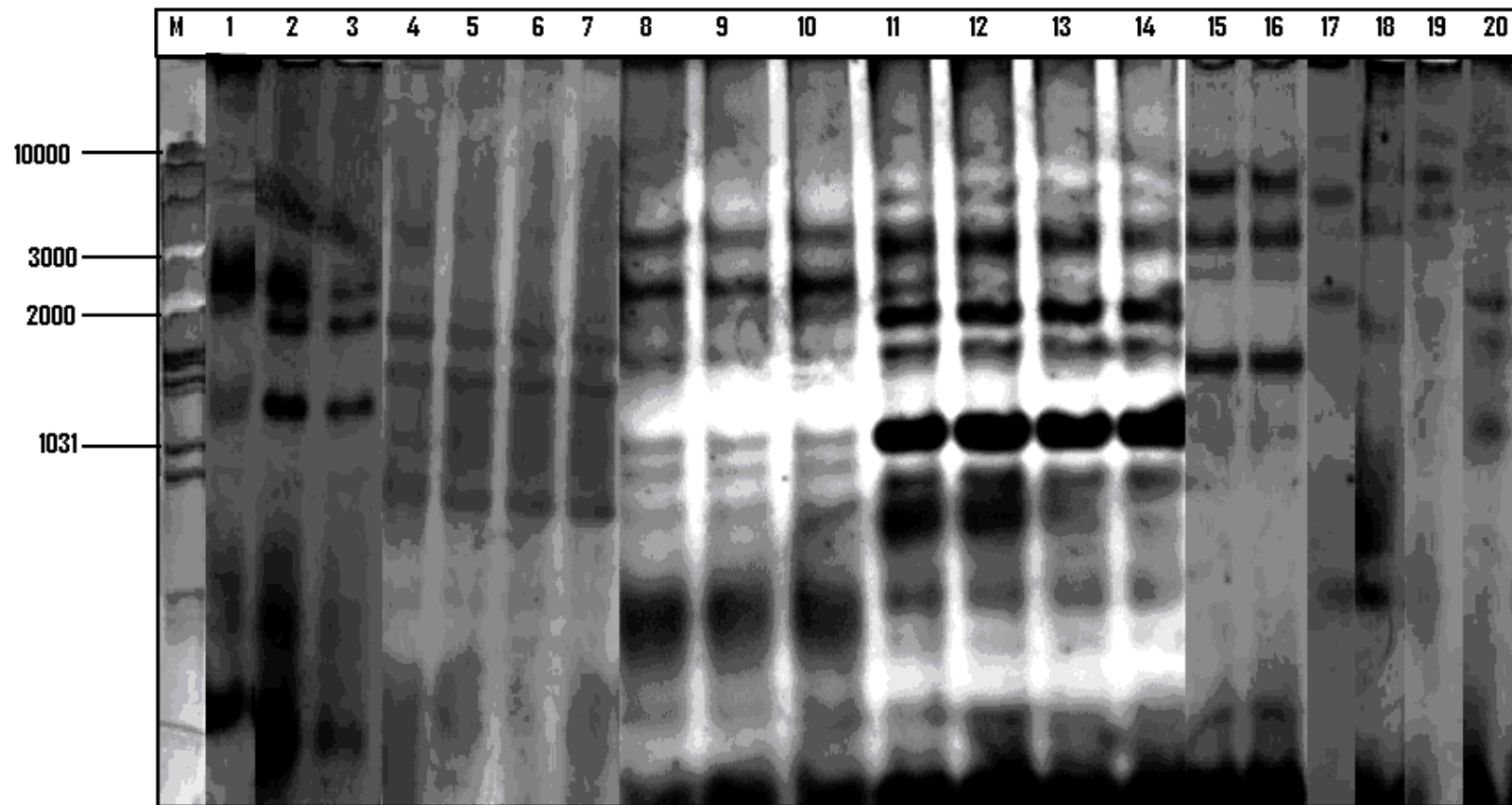


Figure 1: Lanes of digested DNA samples on the gel (Lanes 1-20).

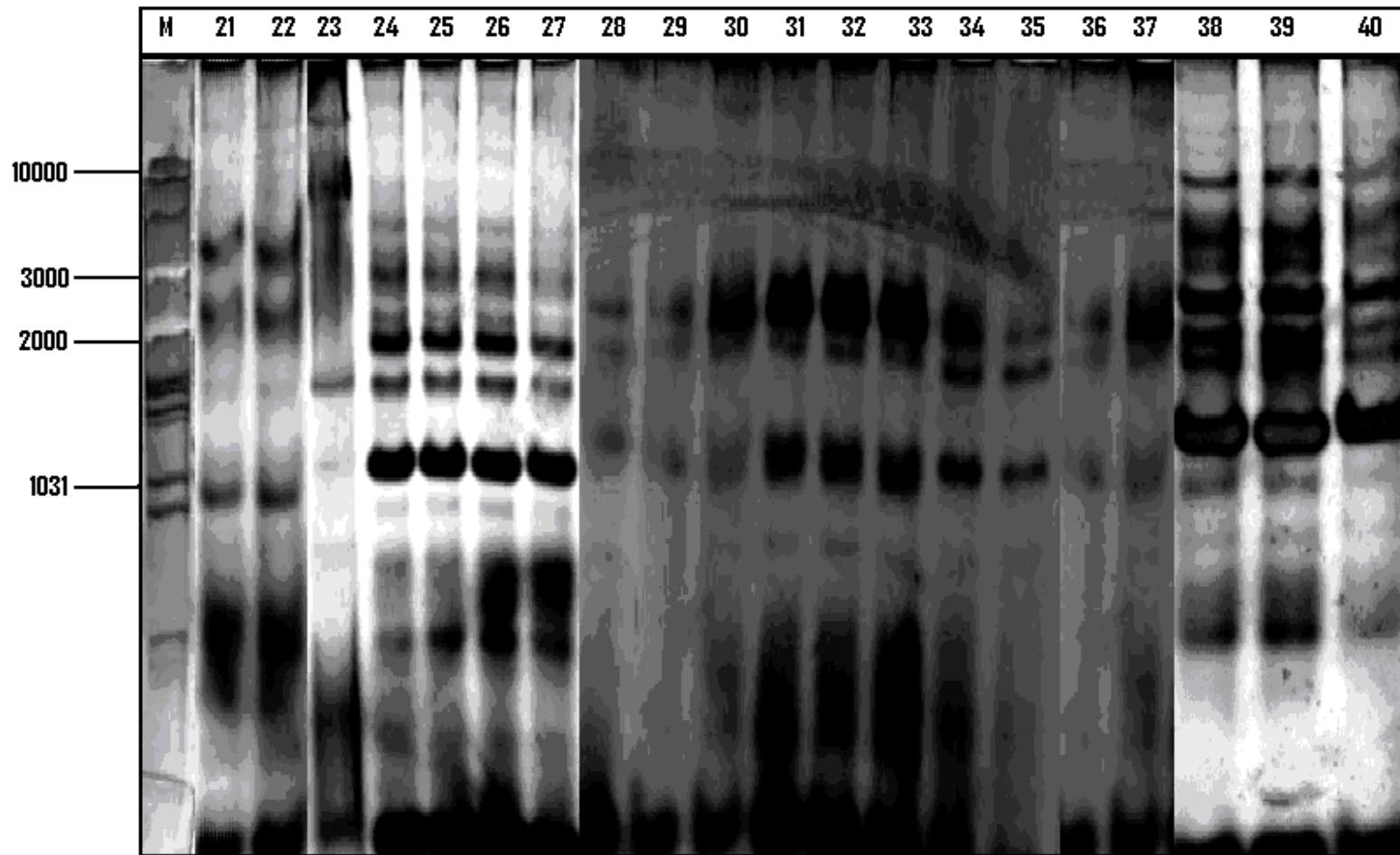


Figure 1 (continuation): Lanes of digested DNA samples on the gel (Lanes 21-40).

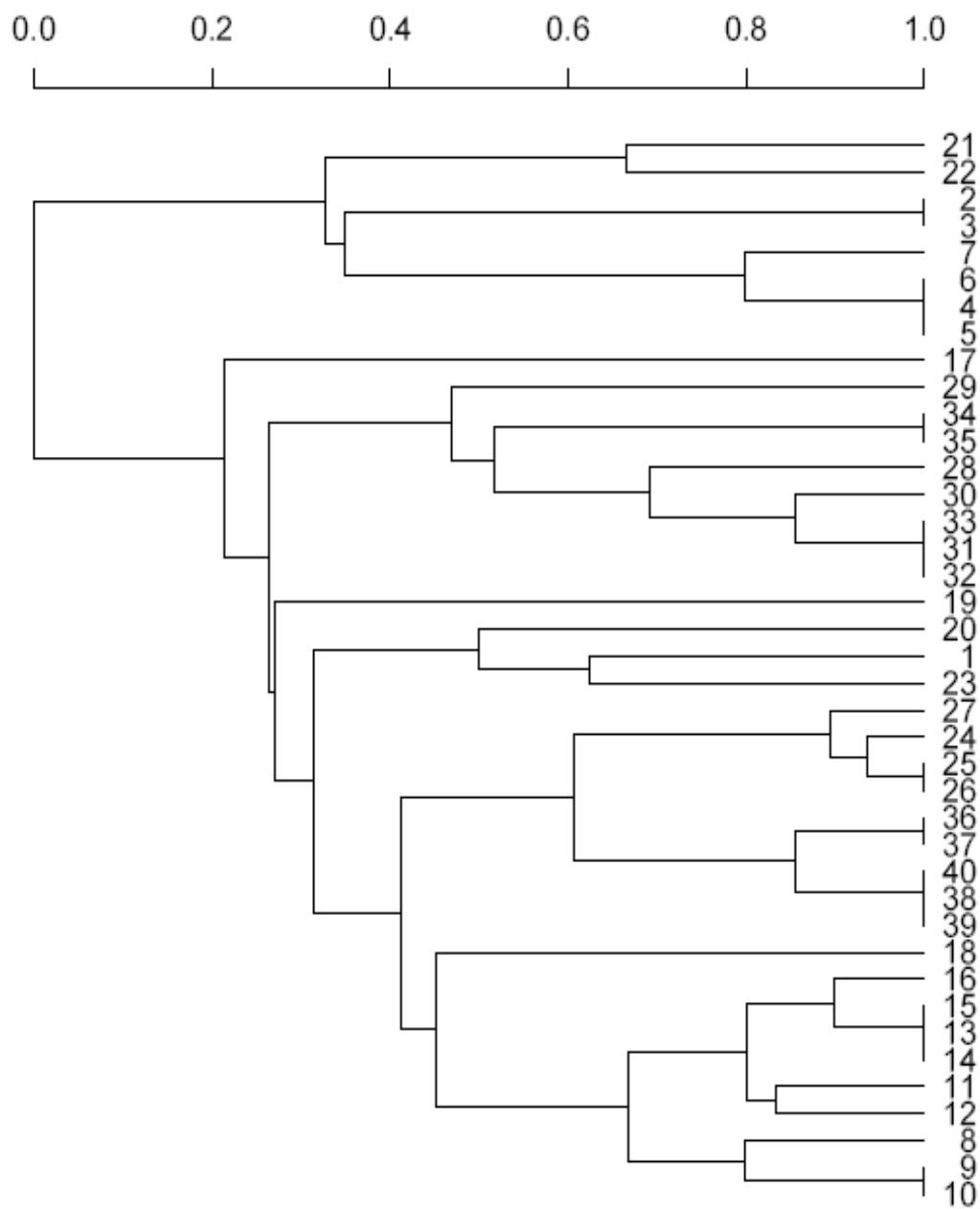


Figure 2: The DNA dendograms obtained for samples 1-40.

Table 3: Distribution of DNA groups according to the similarity coefficient (S_{sm}) based on restriction digest profile.

Group 1	Group 2	Group 3	Group 4
80-93%	60-69%	42-51%	21-35%
4, 5, 6, 7,8, 9, 10 11,12,13 14, 15, 16, 24, 25, 26, 27, 30, 31, 32, 33, 36, 37, 38, 39, 40	1, 21, 22, 23, 28	18, 20, 29, 34, 35	2, 3, 17, 19

4.3 Protein moiety of the *Salmonella* strains

The protein profiles of forty *Salmonella* isolates from Amalinda, Shornville, Fort Hare Wastewater Plants, Gogogo and Tyhume rivers in the Eastern Cape Province were evaluated (Figure 3). The protein patterns showed a total of 29 different protein bands ranging in molecular weights from 10 KD to 150 KD (Figure 3). The dendrogram obtained using Jackard simple matching coefficient as the input to the UPGMA clustering technique in the R statistical computing environment software (R Development Core Team, 2007) revealed two similarity groups: Group 1 with similarity values ranging from 50-70 % and Group 2 with percentage similarity in the range of 23-48 % (Figure 4 and Table 4).

Group one forms a distinct cluster of isolates including isolates from Shornville wastewater plant's sludge tank (strains 1, 22, 23 and 27); Amalinda wastewater plant's

sludge tank and clarifier (strains 2, 20, 36 and 38); and one strain isolated from Tyume river (strain 37). Since most of the isolates were recovered mainly from two wastewater plants – Amalinda and Shornville which are in close proximity, this might be the reason for the close relatedness of the strains. Group 2 comprises thirty strains with similarity coefficients ranging from 23 to 48 % (Figures 3; Table 4). The isolates were recovered from different water sources. Strains from Amalinda wastewater plant's sludge tank included strains 5, 6, 11, 12, 13, 15, and 19. Amalinda wastewater plant's secondary clarifier yielded strains 29, 34, 35, 37, 39 and 40 while Shornville wastewater plant's sludge tank yielded strains 9, 24, 31, 33 and 34. From Shornville wastewater plant's secondary clarifier came strains 7, 14, 16, 18, 21 and 30 while Fort Hare wastewater plant's sludge tank yielded strains 3 and 17 and its secondary clarifier yielded strains 25. Gogogo river yielded strains 8 and 4, and from Tyume river came strain 28 (Tables 2 and 4). The isolates in group 2 showed lower percentage of relatedness – 23 to 48 % as compared to the strains in Group 1 with similarity coefficient ranging from 50 to 70 % (Figure 4).

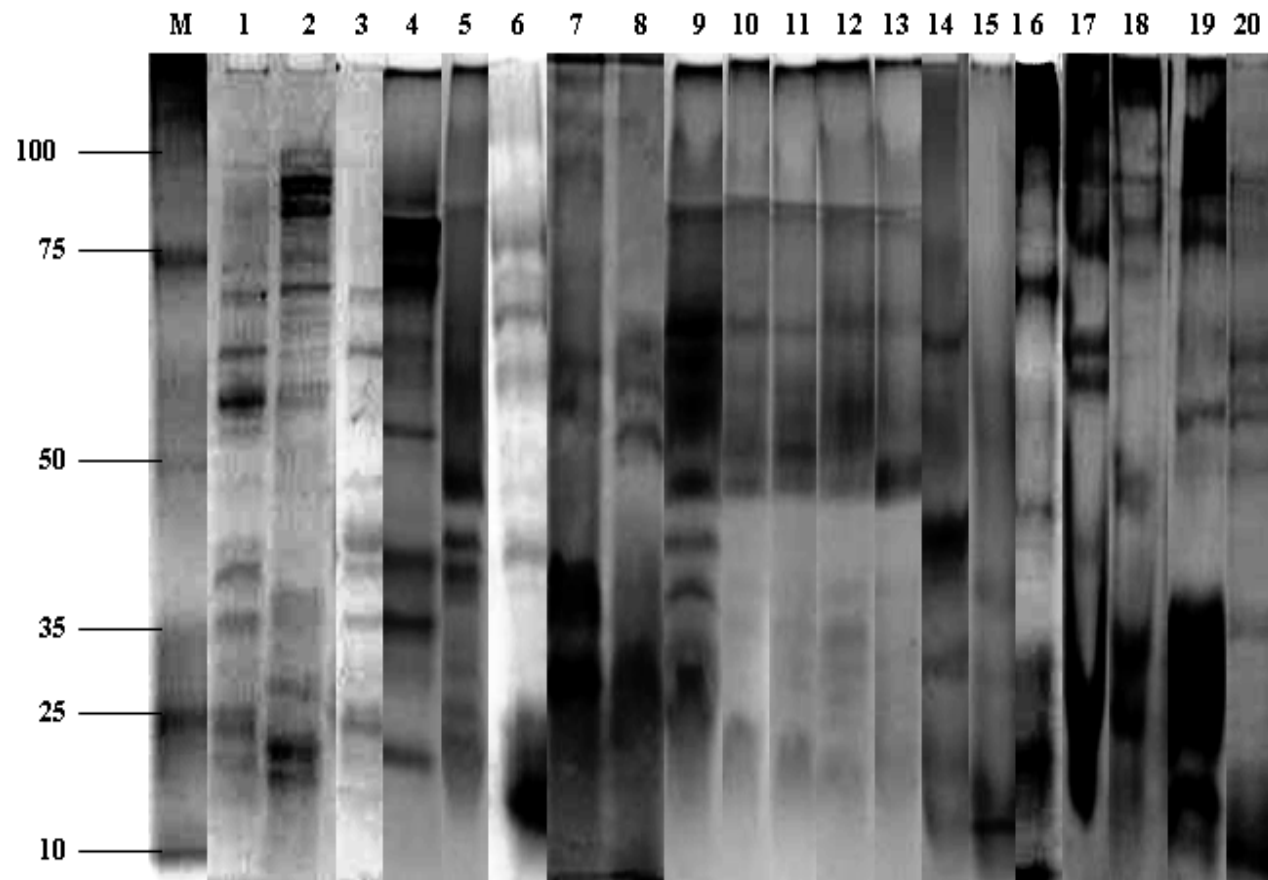


Figure 3: Lanes of protein samples on the SDS-PAGE gel (Lanes 1-20).

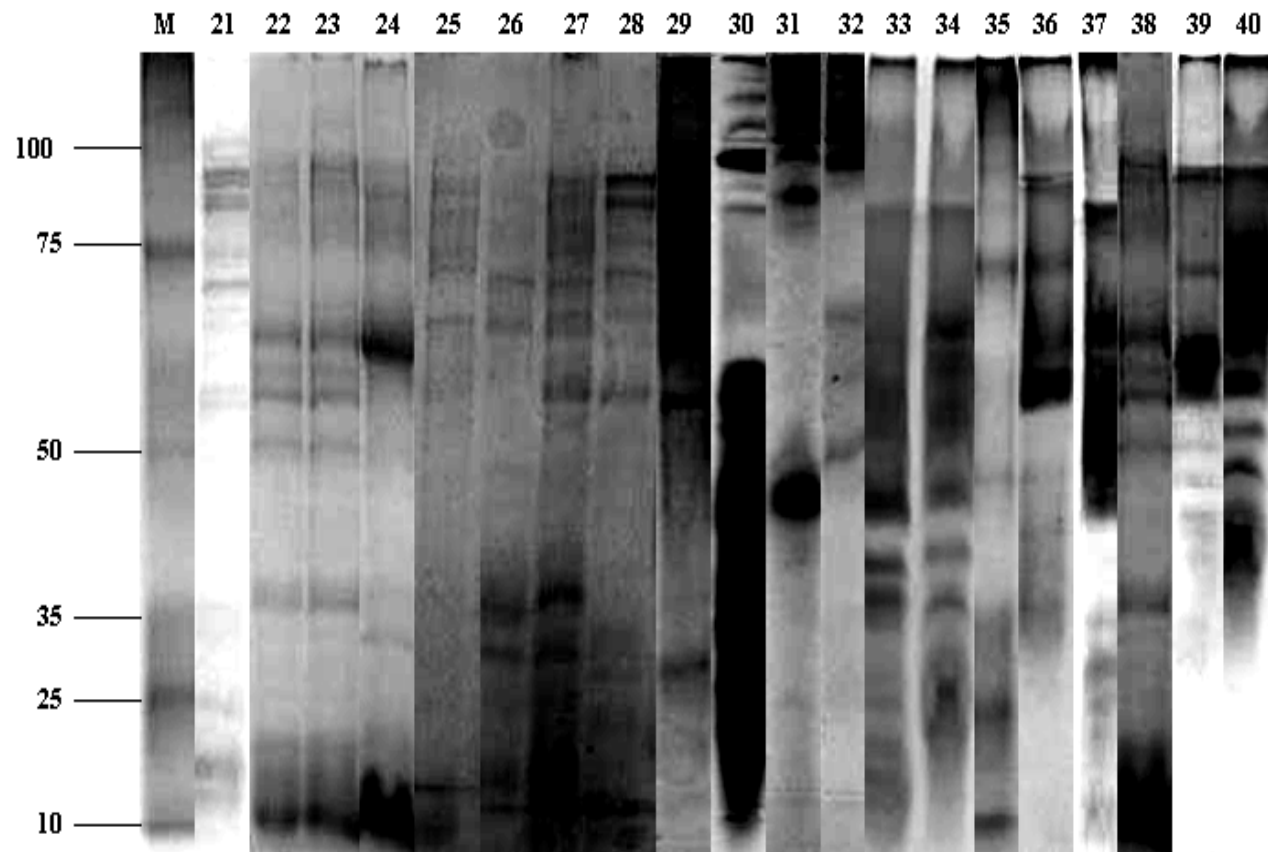


Figure 3 (continuation): Lanes of protein samples on the SDS-PAGE gel (Lanes 21-40).

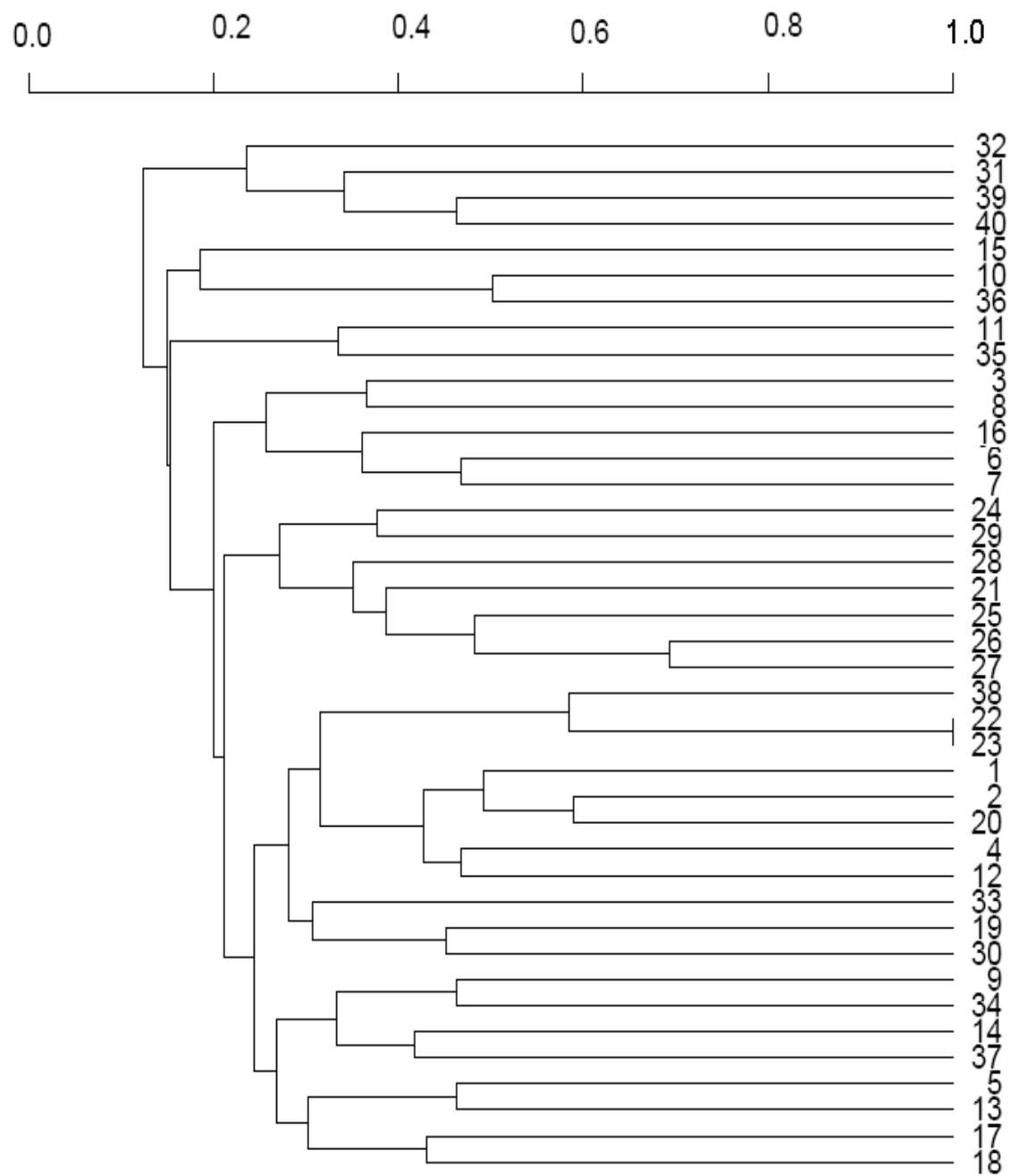


Figure 4: The Protein dendograms obtained for samples 1-40

Table 4: Distribution of protein groups according to the similarity coefficient (S_{sm})

Group 1	Group 2
50 -70 %	23 - 48 %
1, 2, 19, 20, 22, 23, 26, 27, 36, 38,	3, 4, 5, 6, 7, 8, 9, 11,12 13, 14, 15 16, 17, 18, 19, 21, 24, 25, 28, 29 30, 31, 32, 33, 34, 35, 37, 39, 40

4.4 Lipopolysaccharides profile of the *Salmonella* strains

Lipopolysaccharides from forty *Salmonella* strains were evaluated. The strains patterns were compared with a marker: lipopolysaccharides of *Salmonella enteritidis* (Sigma-Aldrich Co. GMBH, Germany). As seen in Figure 5, the lipopolysaccharides of the forty strains were resolved into 15 bands with different electrophoretic mobility. The dendogram obtained revealed three similarity groups (Figure 6). Twenty one strains (52.5% of all isolates) formed a distinct cluster with high sequence similarity values ranging from 67 to 87 % (Figure 6). Strains 6, 11, 12, 13, 15 and 20 were isolated from Amalinda wastewater plants sludge tank; strains 26, 39 and 40 were recovered from Amalinda wastewater plant's secondary clarifier; strains 9, 22, 27 and 31 were isolated from Schornville wastewater plant's sludge tank; strains 7, 14, 16, 21 and 30 were recovered from Shornville wastewater plant's secondary clarifier; strain 17 was isolated from Fort Hare wastewater plant; strain 8 was recovered from Gogogo river and strain 10 was recovered from Tyume river (Table 2).

The second similarity group included 16 strains which formed rather homogenous group with similarity values ranging between 37 and 63%. This group comprised the following: strains 2, 5 and 19 from the Amalinda wastewater plant's sludge tank; strains 29, 32, 35, 36 and 38 from Amalinda wastewater plant's secondary clarifier; strains 23, 24, 33 and 34 from Shornville wastewater plant's sludge tank; strain 18 from Schornville wastewater plant's secondary clarifier; strains 3 and 25 from Fort Hare wastewater plant's; and strain 4 from Gogogo river. Strains 1, 28 and 37 formed a distinct group, with similarity coefficient ranging from 29 to 34%. Strain 1 was isolated from Schornville wastewater plant's sludge tank, while strain 28 was recovered from Tyume river and strain 37 was isolated from Amalinda wastewater plant's secondary clarifier. The first similarity group comprising species with similarity coefficients of 67 to 87% includes more than 50% of the isolates which shows a very high relatedness between strains from different water sources- Amalinda wastewater plant, Schornville wastewater plant and Fort Hare wastewater plant indicative of a high stability in the pattern of the lipopolysaccharides. The difference between the three similarity groups may be indicative of the adaptation of the bacterial outer membrane to the different ecological niches of the bacterial strains examined.

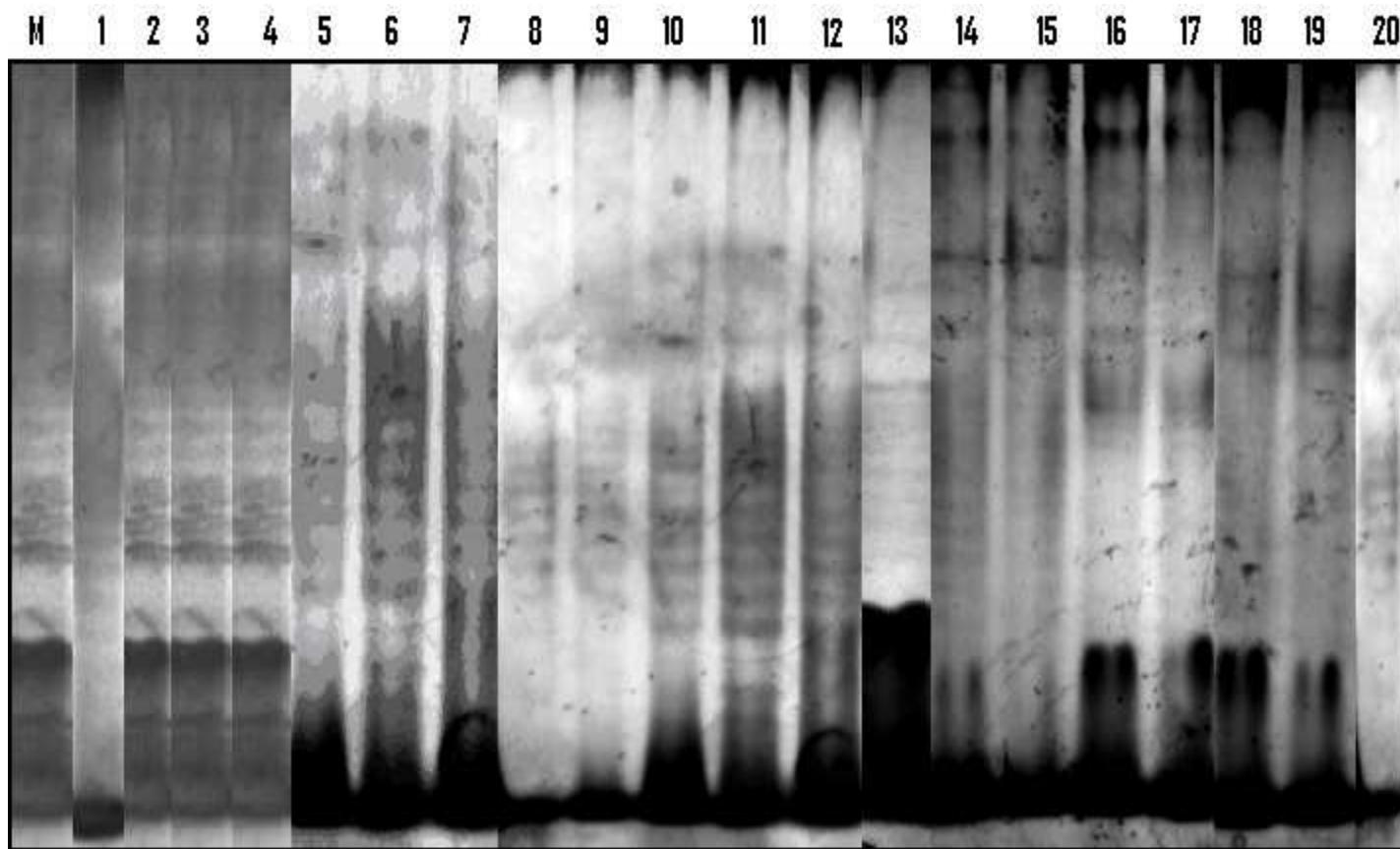


Figure 5: Lanes of lipopolysaccharides samples on the SDS-PAGE gel (1-20 samples)

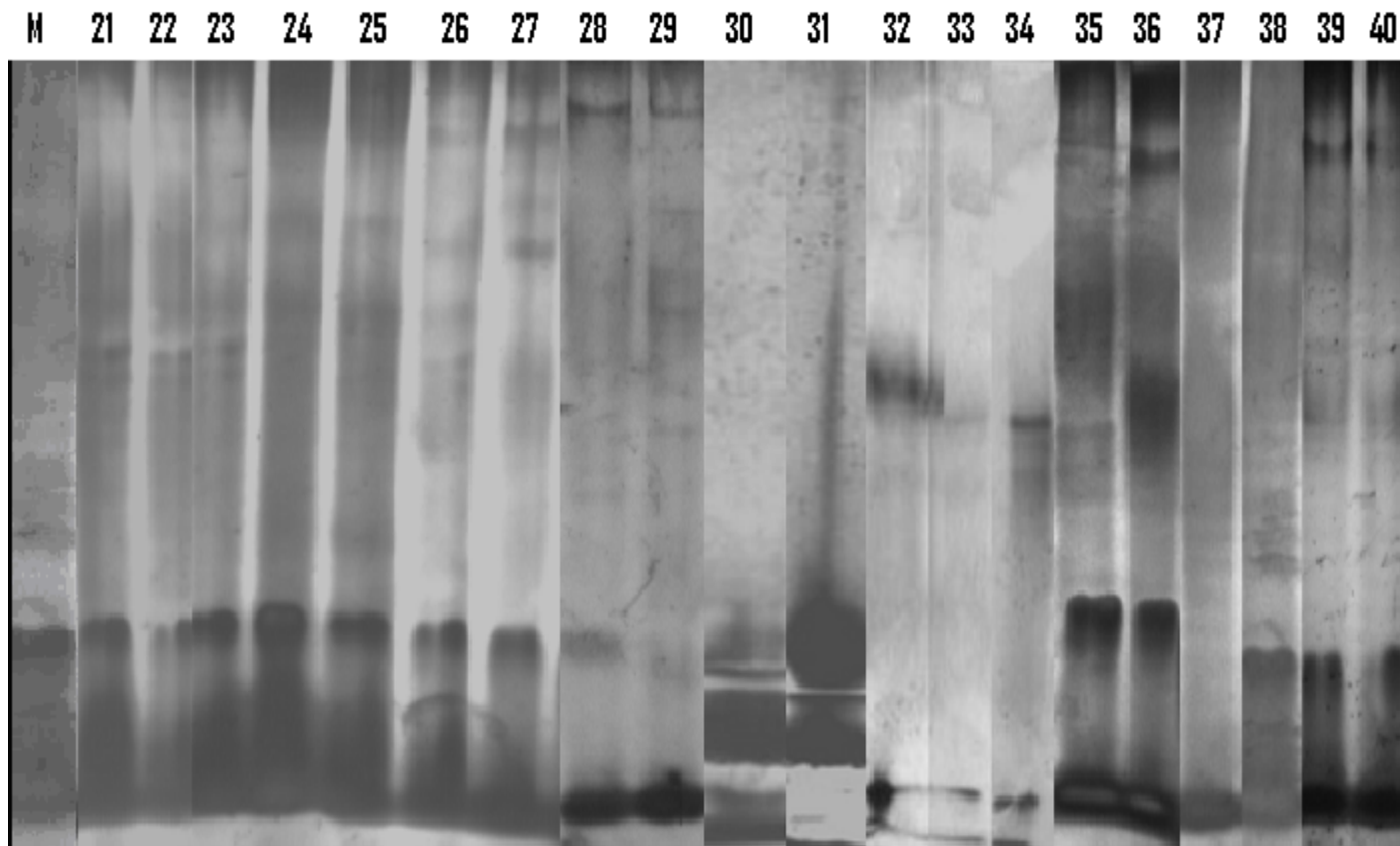


Figure 5 (continuation): Lanes of lipopolysaccharides samples on the SDS-PAGE gel (21-40 samples)

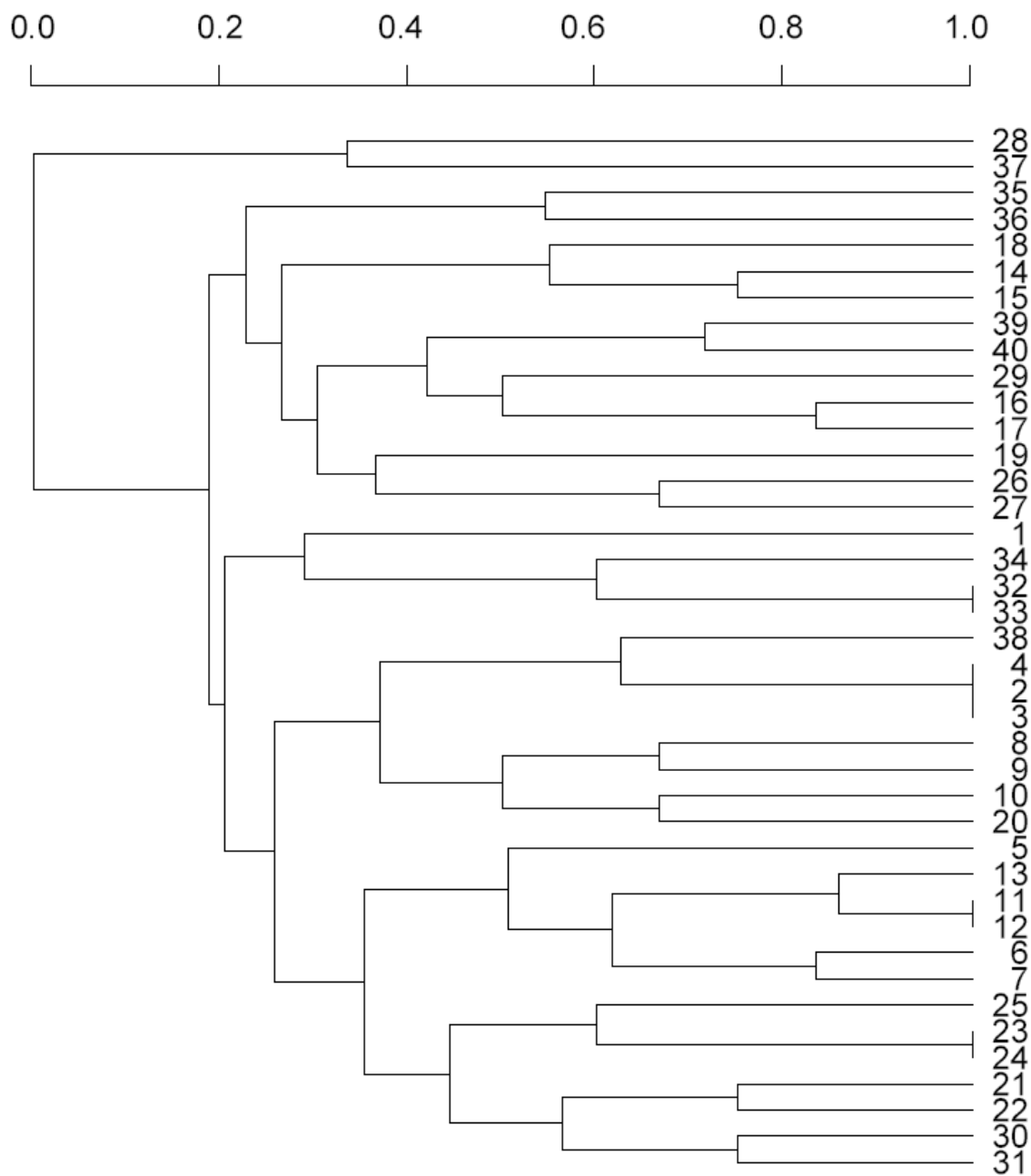


Figure 6: The Lipopolysaccharides dendograms obtained for samples 1-40

Table 5: Distribution of Lipopolysaccharides groups according to the similarity coefficient (S_{sm}).

Group 1	Group 2	Group 3
67-87%	37-63%	29-34%
6, 7, 8, 9, 10, 11, 12,	2, 3, 4, 5, 18, 19, 23,	1, 28, 37
13, 14, 15, 16, 17, 20,	24, 25, 29, 32, 33, 34,	
21, 22, 26, 27, 30, 31,	35, 36, 38	
39, 40		

CHAPTER 5

DISCUSSION

The aim of the present study was to examine the biodiversity of forty *Salmonella* isolates collected from domestic water sources and wastewater treatment plants in the Eastern Cape Province of South Africa. The outcomes of this research will certainly shed a new light on the diversity of *Salmonella* strains and on their environmental and epidemiological impact. The antibiotic susceptibility profile of the forty *Salmonella* strains was evaluated as well. This information is essential in providing the much needed data on the diversity of *Salmonella* species in the studied province since the information on the diversity of these species in the Eastern Cape Province is non existent to the best of our knowledge. However reports in other parts of South Africa and other countries are available. Moreover, antibiotic susceptibility data obtained on *Salmonella* strains in the areas covered is imperative in providing information on the antibiotics used in this study and reviewing their clinical use.

Our results on antibiotic susceptibility of *Salmonella* strains indicates that out of seven antibiotics and sulfonamides tested on forty *Salmonella* species, namely: neomycin, chloramphenicol, doxycycline, sulfamethoxazole, kanamycin, streptomycin and cotrimoxazole, five proved to have a substantial effect on these *Salmonella* strains. Between 72.5% and 90% of the strains proved to be susceptible towards neomycin, chloramphenicol, kanamycin, streptomycin and cotrimoxazole. Comparable observation was reported by Thong and coworkers (2002) who demonstrated the sensitivity of *Salmonella* isolates towards chloramphenicol, neomycin, kanamycin streptomycin and cotrimoxazole. These results agree with that of Obi *et al.* (2007), who reported similar

patterns of sensitivity of *Salmonella* isolates from HIV/AIDS patients in Limpopo, South Africa towards kanamycin.

The introduction of antibiotics for the chemotherapy of bacterial infection has been one of the most important medical breakthroughs since their discovery. However, the emergence of bacterial resistance to antibiotics undermines the therapeutic utility of existing agents and thereby necessitating more research in this field. Towards doxycycline and sulphamethoxazole, the *Salmonella* strains showed considerable resistance of 57.5% and 92.5% respectively. This indicates that the two drugs can hardly be considered for treatment of *Salmonella* infections as compared to neomycin, chloramphenicol, kanamycin, streptomycin and cotrimoxazole.

Using restriction digestions of genomic DNA, followed by SDS/PAGE electrophoresis and construction of dendograms, four similarity groups of *Salmonella* strains were identified based on their DNA profiles with different base pair lengths. We identified two different groups of the strains based on their protein moiety using SDS/PAGE electrophoresis and three different groups of *Salmonella* strains based on their lipopolysaccharide profiles. DNA profiles revealed four similarity groups showing 18 different bands with base pairs length varying from 500bp to 12000 bp. Most of the strains (approximately 65%) recovered from Amalinda wastewater plant, Shornville wastewater plant, Fort Hare wastewater plant, Gogogo and Tyume rivers showed a high percentage of genetic similarity ranging from 80 to 93%. The genetic variability of the strains between this group and the rest three groups ranging from 21 to 69% suggests a high degree of genomic rearrangements that could be associated with the acquisition of mobile genetic elements, insertion sequences and mutations, resulting from the different

environmental conditions of the water sources from where the strains have been recovered, as well as the environment of the areas whose wastewater were collected in the Amalinda, Shornville and Fort Hare wastewater plants.

Restriction digestion of a given DNA with specific enzymes provides a reproducible array of fragments which can be separated by agarose gel electrophoresis into categories of relatedness among strains or interpreted using criteria of Tenover *et al.*, 1995 as follows: (a) isolates are considered indistinguishable if the number and size of the bands are the same; (b) closely related isolates have patterns that differ by 2-3 band difference; (c) an isolate may be considered possibly related if the PAGE pattern corresponds to 4-6 band differences. Thus restriction digestion of genomic DNA followed by SDS-PAGE gel provides a valuable method for evaluating the relatedness of *Salmonella* species.

Ribeiro and coworkers (2007) used PFGE for studying genetic diversity of *Salmonella* strains isolated from salami. They observed low genetic similarity amongst strains indicating that the strains could be from different sources. The SDS-PAGE method was used to evaluate the similarities and differences between the protein profiles of the *Salmonella* species. The main advantage of this method is the relative ease as well as the quantity of samples that can be used. The second advantage of the method is that the results obtained by SDS-PAGE of whole cell proteins discriminate at much the same level as DNA: DNA hybridization (Ribeiro *et al.*, 2007).

Comparing DNA and protein profiles of the forty *Salmonella* isolates, it was observed that isolates 1, 22 and 23 fall in the close similarity group. Similarity coefficients for these isolates are in the range of 60-69% for DNA and 50-70% for

protein. Isolates 1, 22 and 23 were recovered from same wastewater treatment plant's sludge tank and this might be the reason for their close similarity. This indicates that the restriction digestion of DNA followed by gel electrophoresis and construction of dendograms methods we used allows us to group closely related isolates, recovered from the same water sources and also differentiates from those obtained from different water sources. Thong and coworkers (2002) reported that the DNA profiles of most of the *Salmonella* serotype Weltevreden isolates from various hospitals varied greatly indicating that the isolates belong to different clones.

Isolates 3, 17 and 19 have close similarity coefficients which fall in the range of 21 to 35% for DNA and 23 to 48% for their protein profile. Isolates 3 and 17 were recovered from the same wastewater plant, namely Fort Hare wastewater plant and they have a percentage similarity which falls in the same range with strain 19 which was isolated from Amalinda wastewater plant's sludge tank thus suggesting that a unique pattern for DNA, proteins or lipopolysaccharides does not exist for each genomic species. DNA and proteins for strains 10, 26, 27, 36 and 38 fall within close similarity groups. Similarity coefficients for these isolates are in the range of 80-93% for DNA and 50-70% for proteins, which shows a very high relatedness between strains from different water sources. Strains 21 and 28 have close similarity coefficients which fall in the range of 20-69% for DNA and 23-48% for proteins. The differences in the similarity coefficients of proteins and DNA of a number of *Salmonella* species from the same water sources shows that there exists substantial diversity between these strains. Though the similarity levels of the protein profiles of the forty *Salmonella* species varied appreciably, 25% of the strains formed a tight and distinct cluster with similarity values ranging from 50 to 70%.

Thus the SDS-PAGE protein patterns support the view that considerable phenotypic diversity can exist within the individual genomic species, although some strains such as strains 39 and 40, 10 and 32, 22 and 23 have identical patterns. These results are comparable to the results obtained by Maszenan and coworkers (1997), who noticed a considerable diversity in the protein profiles of *Acinetobacter* genomic species from activated sludge systems, but contrasted the findings of Franco *et al.* (1992), who noticed homogeneous outer membrane protein profiles among *Salmonella typhi* strains isolated from Peru and Indonesia.

Lipopolysaccharides are one of the components of the bacterial outer membrane. The differences in their profiles on SDS/PAGE gels may be indicative of the adaptation of the bacterial outer membrane to the different ecological conditions. Comparing the lipopolysaccharide and protein profiles for the 40 *Salmonella* isolates, we found that the similarity coefficients for strains 1, 2, 23, 36 and 58 fall in the range of 37 to 63%. Similarity coefficients are in the range of 50 to 70 % for their proteins and 37 to 63 % for their lipopolysaccharides. Strains 3, 4, 5, 18, 19, 24, 25, 29, 32, 34 and 35 have close similarity coefficients which fall in the range of 23-48% for their proteins and 37-63% for lipopolysaccharides. These results show that there is a considerable relatedness between the *Salmonella* species from different sampling sites. These observations corroborate the report of Mansfield and Forsythe (2001). They studied the lipopolysaccharide profile of 42 *Salmonella* isolates from food, animals and strains from the culture collection at the Nottingham and Trent University and Dynal Research and Development using SDS/PAGE and revealed 19 serogroups suggesting considerable relatedness between these isolates.

The similarity coefficients of the proteins of strains 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 21, 30, 31, 39 and 40 fall in the range of 23 to 48 % while the similarity coefficients of their lipopolysaccharides are in the range of 67 to 87 % which shows the diversity of the species in terms of proteins and lipopolysaccharide. Strains 10, 20, 22, 26, 27 have a close similarity coefficients of their proteins which fall in the range of 50 to 70 % and 67 to 87 % of their lipopolysaccharides. The similarity coefficients of the proteins of strains 28 and 37 fall in the range of 23 to 48% while the similarity coefficients for their lipopolysaccharides fall in the range of 29 to 63% which confirms their relatedness.

CONCLUSION

Antibiotics susceptibility test revealed that the forty *Salmonella* are susceptible to five antibiotics and significantly resistant to 2 antibiotics which is of clinical importance in the treatment of *Salmonella* outbreaks. Also, the restriction digest pattern, SDS/PAGE and dendrogram construction shows that there is a high similarity between the forty *Salmonella* strains studied. Sixty five percent of the strains based on DNA show similarity coefficient in the range of 80-93 which indicates a high genetic relatedness between the strains investigated. DNA and proteins of *Salmonella* show close relatedness between the strains with few exceptions. Lipid profile of the *Salmonella* strains has shown a considerable variability in terms of similarity coefficients. Our observations have proffered a veritable reference point on the diversity of *Salmonella* strains in the studied areas. Also, the methods used in this study have proven to be valuable tools for evaluating the relatedness of *Salmonella*. However, it was not possible to show a clear

correlation between isolates characteristics and the sources of the isolates in this study. Further studies on epidemiology of isolates and the correlation between strain characteristics and their sources should be considered.

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