CHARACTERIZATION OF ANTIBIOTIC RESISTANCE IN *ENTEROBACTERIACEAE* ISOLATES FROM THE MHLATHUZE RIVER

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By

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A dissertation submitted in fulfillment of the requirements for the degree of Master of Science in the department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, South Africa.

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Supervisor: Dr J. Lin

DECLARATION

I declare that this dissertation hereby submitted to the University of Zululand for the degree of Master of Science has not been previously submitted by me for a degree at this or any other University, that it is my own work in design and execution, and that all material contained therein has been duly acknowledged.

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Signature

26/11/2003
.....
Date

DEDICATION

This work is dedicated to my loving mother, MaGonya, if it were not for your undying love and faith I would not have come this far. There is no doubt in my mind, you are the best Mom I could ever ask for. I will always love you!

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I thank God, the almighty and father of my personal Lord and Savior Jesus Christ, really in HIM all things are possible!

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May the good Lord richly bless you all!!!

ABSTRACT

The wide and indiscriminate use of antibiotics often results in the establishment of a pool of antibiotic resistance in the environment. In order to establish the state of bacterial resistance to antibiotics in the Mhlathuze River, 114 enteric bacteria were isolated from water samples collected from this river over a period of two years. The isolates were identified using the culture methods and confirmed by the API 20E system. The isolates were then tested for their susceptibility or resistance to a battery of 15 antibiotics. Those that showed multiple antibiotic resistance, 43 in total were screened for the presence of class1 integrons and the associated antibiotic resistance genes using the polymerase chain reaction (PCR).

The resistance of the enteric bacteria isolated over a period of one year showed that resistance to the older classes of antibiotics was high (94.7 % resistance to one antibiotic and 80.8 % resistance to two antibiotics). Furthermore, antibiotic resistance data of the environmental isolates showed a strong correlation (r= 0.97) with data obtained from diarrhea patients. PCR based methods demonstrated that class 1 integrons were present in more than 50% of the environmental bacterial isolates that were resistant to multiple antibiotics. This class of integrons is capable of transferring genes responsible for resistance to beta-lactam, aminoglycoside, sulfonamide and quaternary ammonium antimicrobial agents. Conjugate plasmids were also isolated, but from a small percentage of isolates. This study showed that the Mhlathuze River (i) is a medium for the spread of bacterial antibiotic resistance genes (ii) acts as a reservoir for these genes and (iii) due to socio-economic pressures may play a role in the development and evolution of these genes along this river system.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. *Introduction*

Antibiotics are chemotherapeutic compounds, naturally produced by microorganisms that have the ability to prevent the growth of other microorganisms. Antibiotics are generally used to treat infectious diseases. Depending on their mode of action, antibiotics can either kill the microorganism (bactericidal) or inhibit its growth (bacteriostatic). Not all antibiotics are active against all microorganisms. The age of antibiotics began in 1927, when the antibiotic penicillin was discovered. Penicillin and many other antibiotics interfere with bacterial cell wall synthesis. Because the human body has no structural component similar to the bacterial cell wall, treatment with the penicillins selectively destroys the bacteria without causing harm to the patient. Since the discovery of the first antibiotic, hundreds of antibiotics, have been developed. The key to antibiotic therapy is to find a target in the microbe, some metabolic structure or process that human cells do not have. In this way the antibiotic will selectively inhibit the disease-causing organism without harming the patient (Madigan *et al.*, 2003)

In the evolutionary struggle to survive bacteria acquire the ability to resist the action of the antibiotic to which they are normally susceptible. Antibiotic resistance is a predictable outcome of natural selection. Although antibiotic resistance can be intrinsic/species specific (some bacteria have an inherent resistance to certain specific antibiotics) studies have shown that acquired resistance is more predominant (Madigan *et al.*, 2003).

Although acquired resistance to antibiotics can only develop in the presence of selection pressure, antibiotic resistant bacteria can still persist long after removal of the antibiotic and susceptibility may take long to return. Some microbial pathogens are resistant to more than one class of antibiotics, exhibiting multiple-drug resistance (Colloquium report to the American Academy of Microbiology, 1999).

Given the ability of *Enterobacteriaceae* and clinically important bacteria to acquire antimicrobial resistance, consistent surveillance of the agents commonly prescribed to treat infections arising from these organisms is imperative (Sahm *et al.*, 2000).

Obtaining information on the prevalence of antibiotic resistance is the first step in surveillance studies. Nevertheless, the development of genotyping tools for detecting and tracking antibiotic resistance genes in commensal and pathogenic bacteria, as well as in the environment is needed for understanding the ecology of antibiotic resistance. Such information is indispensable if the problem of antibiotic resistance is to be tackled (Aminov *et al.*, 2001).

1.2. Literature review

1.2.1. Antibiotics

The production of antibiotic sis associated with soil microbes. Natural antibiotics are metabolic products from bacteria, fungi and actinomycetes, which kill or retards the growth of other microorganisms. In the natural environment, this is thought to provide a selective advantage for the antibiotic-producing microbes in their competition for space and nutrients. Most anti-fungal and antibacterial agents in clinical use today are derivatives of fermentation

products, some with improved pharmacological properties; while others are totally synthetic e.g. the quinolones (Madigan *et al.*, 2003; Mims *et al.*, 1993).

1.2.1.1. Classification of antibiotics

Antibiotics are classified based on: their effect, target site and by chemical structure.

1.2.1.1.1. By effect (bactericidal or bacteriostatic)

Antimicrobial agents can be classified based on the effect they have on the target microbe. Normally antimicrobials affect microbes in two ways; they either kill or inhibit microbial growth. There is no clear line of demarcation between bacteriostatic and bactericidal antimicrobials; in some cases the same antimicrobial can be bacteriostatic at low concentrations and bactericidal at high concentrations (Mims *et al.*, 1993).

Bactericidal

Within the cytoplasmic membrane osmotic pressure is high, creating a tendency for the bacterial cell to take up water from the environment and swell. If it were not for the rigid cell wall, water would be absorbed to such an extent that the bacterial cell would eventually burst. Bactericidal antibiotics weaken the cell wall causing bacteria to take up water and rupture for example polymyxin. This weakening of the cell wall results from two processes (a) Inhibition of transpeptidases, the enzymes essential for cell wall synthesis and (b) Activation of autolysins, the enzymes that cleave bonds in bacterial cell walls. In this way the antibiotic simultaneously inhibits cell wall synthesis and promotes its active destruction ((Madigan *et al.*, 2003; Mims *et al.*, 1993).

Bacteriostatic

The antibiotics suppress bacterial growth by inhibiting protein synthesis. They do this by inhibiting the binding of transport-RNA (tRNA) to the ribosomal RNA (rRNA)-ribosome complex thereby blocking the addition of amino acids to the growing peptide chain (Pelczar *et al.*, 1993; Madigan *et al.*, 2003)

1.2.1.1.2. By target site

Bacterial cells have four major sites that are sufficiently different from animal and human cells that antibacterial agents can target; these are the cell wall; ribosomes; cell membrane and the nucleic acid synthetic pathway (Mims *et al.*, 1993).

1.2.1.1.3 By chemical structure

Antibacterial agents are diverse chemical structures; classification on these bases alone is not useful. If combined with target sites it does provide a useful classification (Mims *et al.*, 1993).

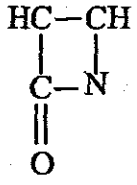
1.2.2. Classification and action mechanisms of some commonly used antibiotics

1.2.2.1.The inhibition of cell wall synthesis and cell membrane function

(a) Beta-lactams

This is a very large family of chemical compounds unrivalled in the treatment of infectious diseases (Franklin and Snow, 1981). All members of this group contain the beta-lactam ring. They are differentiated by the structure of the ring attached to the beta-lactam ring. Penicillins have a five-membered ring and cephalosporins have a six-membered ring. Beta-lactams inhibit cell wall synthesis by binding to enzymes known as penicillin- binding

proteins (PBPs), which are carboxypeptidases responsible for cross-linking in bacterial cell wall synthesis. Their inhibition causes the accumulation of precursor cell wall units and this causes the cell's autolytic pathway to be activated and cell lysis is the ultimate result. Most beta-lactams are active against Gram positive bacteria. There are more than forty beta-lactam antibiotics currently registered for clinical use e.g. penicillins; ampicillin; amoxycillin; cefuroxime; cefoxitin; cefaclor; carbenicillin; aziocillin; cloxacillin; imipenem etc. (Madigan *et al.*, 2003; Mims *et al*, 1993).



Beta lactam ring

Figure 1. Chemical structure of the beta-lactam ring (Franklin and Snow, 1981)

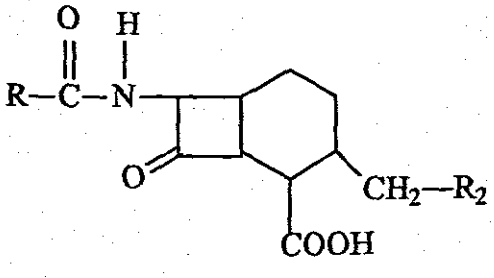


Figure 2. Chemical structure of a Cephalosporin antibiotic (Franklin and Snow, 1981)

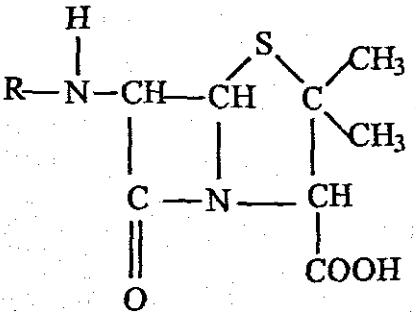


Figure 3. Basic structure of penicillin, R represent different chemical substituents (Franklin and Snow, 1981)

(b) Glycopeptides

These molecules have high molecular weights. Members of this group include vancomycin and teicoplanin. Glycopeptides interfere with cell wall synthesis by binding to terminal D-alanine-D-alanine at the end of the pentapeptide chains, which are part of the bacterial cell wall structure. This binding inhibits the transglycolysation reaction and addition of new sub-units into the cell wall is prevented. They normally affect the part of peptidoglycan synthesis that occurs while the intermediates are bound to the cytoplasmic membrane, though each affects different parts of the process. They are mainly used for infections by Gram positive cocci and Gram positive rods resistant to beta-lactams (Franklin and Snow, 1981; Mims *et al.*, 1993).

(c) Polymyxin and penta-N-benzylpolymyxin

Polypeptide antibiotics do not inhibit cell wall synthesis, they owe their antimicrobial action to their binding with the cytoplasmic membrane, and disturbing its function. Polymyxin is active against Gram negative bacteria and penta-N-benzylpolymixin is active against Gram positive bacteria (Franklin and Snow, 1981).

1.2.2.2. The inhibition of protein synthesis (suppression of gene function)

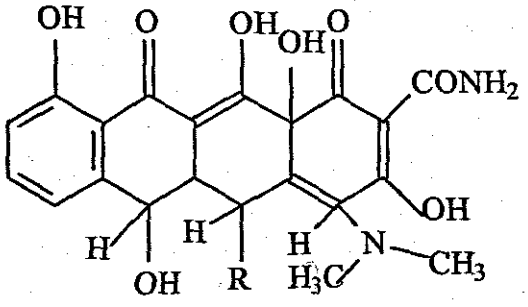
(a) Aminoglycosides

This is a group of related molecules with either streptidine (streptomycin) or 2-deoxystreptidine (gentamicin) that act by preventing the formation of initial complexes from which protein synthesis proceeds. They do this by interfering with the binding of formyl methionyl tRNA (fMet tRNA) to the ribosome. Streptomycin also causes misreading of the tRNA codons. Gentamicin and the newer aminoglycosides are used in the treatment of

serious Gram negative infections or as reserve antibiotics, when other antibiotics fail (Madigan *et al.*, 2003).

(b) Tetracyclines

This is a family of big cyclic structures with several sites for possible chemical substitutions. This group includes tetracycline; chlortetracycline; doxycycline and minocycline.



(a). R = - H TETRACYCLINE

(b). R = - OH DOXYCLINE

Figure 4. Skeletal structure of the tetracyclines (Franklin and Snow, 1981)

They inhibit the entry of aminoacyl-tRNA to acceptor sites on the 30S ribosomal sub-unit. Tetracyclines are mainly used to treat infections caused by chlamydiae, mycoplasmas and rickettsiae (Aminov *et al.*, 2001).

(c) Chloramphenicol

A simple molecule with a nitrobenzene nucleus, responsible for some of the toxic problems associated with the drug. It blocks peptidyl-transferase. It inhibits bacterial protein synthesis selectively because it has a higher affinity for the transferase in the 50S sub-unit of the

bacterial ribosome than it has for the 60S sub-unit of the mammalian ribosome. It is active against various species of both Gram positive aerobes and anaerobes (Mims *et al.*, 1993).

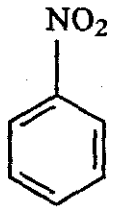


Figure 5. Nitrobenzene nucleus of chloramphenicol (Franklin and Snow, 1981)

(d) Macrolides; lincosamides and streptogranins

The three groups share overlapping binding sites on ribosomes, resistance to macrolides confer resistance to the other two. Examples are erythromycin (microlide) which is active against Gram positive *cocci*; *mycoplasmas*; chlamydiae and rickettisiae and clindamycin (lincosamide) which is active against Gram negative bacteriodes and Gram positive *Clostridium* spp. (Mims *et al* 1993).

1.2.2.3. The inhibition of nucleic acid synthesis

Nucleic acid synthesis is an essential function of growing and dividing cells; its inhibition affects cell division. Cessation of protein synthesis results if there is inhibition of RNA synthesis. Antimicrobials inhibiting nucleic acid synthesis fall into two groups based on their mode of action.

(I) The first group is made of compounds that affect the elaboration of nucleic acid building blocks, the purines and pyrimidine nucleotides. A lot of such drugs are used as anticancer and antiviral agents.

(II) The other group is made of compounds that interfere with the polymerization stage of nucleic acid biosynthesis. This is achieved through the interaction of inhibitor DNA with DNA or its template. The inhibitors of polymerization disrupts the function of the polymerases by interacting directly with enzymes or enzymes-template complexes (Mims *et al.*, 1993; Franklin and Snow, 1981).

(a) Sulphonamides

Members of this group are structural analogues of para-amino benzoic acid (PABA), produced by chemical synthesis. They act in competition with PABA for the active site of enzyme dihydropteroate synthetase, which catalyses an essential reaction in the pathway of tetrahydrofolic acid (THFA) synthesis. THFA is used in purine and pyrimidine synthesis. They are active against Gram negative organisms (Mims *et al.*, 1993).

(b) Trimethoprim (and co-trimoxazole)

It also prevents THFA synthesis at a later stage by inhibiting dihydrofolate reductase. It is active against Gram negative rods; cotrimoxazole is active against a range of urinary tract pathogens and against *Salmonella typhi*. Selective toxicity depends on the greater affinity of trimethoprim for bacterial dihydrofolate reductase (Madigan *et al.*, 2003).

(c) Quinolones

A family of potent, bactericidal synthetic antimicrobials, that act by inhibiting bacterial DNA gyrase activity. This prevents supercoiling of bacterial chromosomes. Nalidixic acid is active against Enterobacteria only. Newer quinolones have a greater degree of activity than nalidixic acid against Gram negative rods. Many high level quinolone–resistant clinical

isolates show multiple antibiotic resistance and increased tolerance to organic solvents (Kern *et al.*, 2000).

(d) Rifamycins

Rifampin is the most important member of this family in clinical use today. It binds to RNA polymerase (affinity is higher for bacterial RNA polymerase) thereby blocking mRNA synthesis. It is primarily used for the treatment of mycobacterial infections (Kern *et al.*, 2000)

1.2.3. Antibiotic resistance

Antibiotic resistance is the ability of microorganisms to resist antibiotic action. It can be an intrinsic character of the microorganism or an acquired resistance which is more predominant (Madigan *et al.*, 2003). There are two general categories of antibiotic resistance traits displayed by microorganisms:

(I) Those that allow the microorganism to withstand high levels of a specific antimicrobial agent, which are conferred by mutations in genes responsible for antibiotic uptake or binding sites.

(II) Those provided by genes conferring low-level resistance to multiple antibiotics such as the *mar* (multiple antibiotic resistance) locus (Mims *et al.*, 1993).

1.2.3.1. Intrinsic antibiotic resistance

Some bacteria possess an inherent resistance to certain specific classes of antimicrobials, either because they lack a susceptible target or they are impermeable to the antibacterial agent for example tetracycline resistance in *Proteus* spp. and *Providencia rettigeri*. Sulfamethoxazole resistance in *Morganella morganii*. However, within species that are

innately susceptible there are strains, which have developed/acquired resistance (Osterblad *et al.*, 2000).

1.2.3.2. Acquired antibiotic resistance

In addition to DNA in the chromosome, bacteria may have extra-chromosomal DNA in the form of mobile genetic elements such as plasmids and transposons. Plasmids are independent self-replicating units, which may be conjugative (Large, 60-120 kilobases) and carry several genes or non-conjugative (small, 1.5-15 kilobases). Plasmid genes control activities of the plasmid itself and of the parent cell, antibiotic resistance is one such activity and plasmid transfer is rapid within bacterial populations (Davison, 1999).

Many resistance plasmids (R factors/plasmids) can confer multiple-drug resistance, due to single R plasmids containing several different genes. Bacteria are also able to acquire resistance genes on transposons/jumping genes (Madigan *et al.*, 2003). Dissemination of antibiotic resistance genes by mobile genetic elements provides a rapid response to the antibiotic challenge.

1.2.3.3. Acquired antibiotic resistance and mobile genetic elements

The ability of bacteria to develop multiple drug resistance is due in part to their ability to acquire new antibiotic resistance genes on mobile genetic elements, especially integrons. Integrons are either located on plasmids or transposons. Members of the family *Enterobacteriaceae* often carry integrons, encoding resistance to various antibacterial compounds. Integrons are mobile DNA elements able to incorporate single or groups of antibiotic resistance genes by site-specific recombination within and between bacterial species (Bunny *et al.*, 1995; Darlsgaard *et al.*, 1999). A large number of antibiotic resistance

genes conferring resistance to aminoglycosides; chloramphenicol; beta-lactams and trimethoprim have been found in integrons (Bunny *et al.*, 1995; Darlsgaard *et al.*, 1999). These genes are contained in individual mobile units, called cassettes, that can be inserted into and excised from an intergron by site specific recombination (Bunny *et al.*, 1995).

Integrons most commonly associated with plasmids and transposons from clinical antibiotic resistant isolates are associated with the *sul*1 (sulfonamide resistance determinant) and the *qacE*1 (quartenary ammonium compounds resistance determinant) genes. Such integrons are designated as class 1 integrons (Bunny *et al.*, 1995). Class 1 integrons commonly possess two conserved regions, located on either side of the integrated gene cassette(s). The 5′-conserved segment (5′-CS) includes a gene, *intI*1, encoding the intergrase, *attI*1; the cassette integration site and the promoters that are responsible for expression of cassette genes. The 3′-conserved segment (3′-CS) includes an open reading frame of unknown function; the *qacE*1 and *sul*1 genes (Darlsgaard *et al.*, 1999; Naas *et al.*, 1999).

1.2.4. The relationship between antibiotic use and the development of resistance

Transferable plasmids with resistance (R) factors existed before the antibiotic era. The use of antibiotics has applied selective pressure in favor of the organism to be able to resist the antibiotic agents (Mims *et al.*, 1993). Given enough drug exposure and time, resistance will develop to all known antimicrobial drugs (Madigan *et al.*, 2003). The evolution of antibacterial resistance in human pathogenic and commensal organisms is the result of the interaction between antibiotic exposure and the transmission of resistance within individuals (Prats *et al.*, 2001). Introduction of antimicrobial agents into the environment via medical therapy and animal husbandry has resulted in new selective pressures on bacterial populations.

In microbial communities, there is cumulative evidence that exposure of populations to antibiotics promotes acquired antimicrobial resistance in the community of pathogens. Moreover, frequent use of antibiotics is associated with an increase in the frequency of antibiotic resistance in hospitals. This is most evident when resistance is due to mutations selected during therapy, resulting in clinical failure. Resistance tends to develop not only in the bacteria involved in the infections being treated, but also in the commensal bacterial flora. Resistance develops as a result of the ability of bacterial populations to adapt (Colloquium report to the American Academy of Microbiology, 1999). Selection of resistant organisms in nature may result from natural production of antibiotics by soil organisms; runoff from animal feed or crops or waste products from treated animals/humans (Ash *et al.*, 2002).

1.2.5. Mechanisms of antibiotic resistance

Several mechanisms are responsible for the ability of microbes to tolerate antibiotics and the incidence of resistance to these antibiotics within bacterial species has increased since the commercial use of antibiotics became widespread. The three main mechanisms of antibiotic resistance in bacteria are alteration in target site, altered uptake and production of enzymes which modify or destroy the antibacterial agent.

1.2.5.1. Alteration in target site

The target enzyme may be altered, such that its affinity for the antimicrobial is lowered. This can be achieved without any disturbances in normal metabolism. Macrolide resistance is due to alteration in 23S rRNA target by methylation of two adenine nucleotides in the RNA, fusidic acid resistance results from alteration of elongation factor G (EF-G). Rifamycin

resistance is due to alteration in the RNA polymerase target which then has a lowered affinity for rifamycin (Franklin and Snow, 1981; Mims *et al.*, 1993).

1.2.5.2. Alteration in access to the target site (altered uptake)

Altering entry either by increasing cell wall impermeability or by pumping the drug out of the cell (efflux mechanism) decreases the amount of drug that reaches the target organ. Efflux of antibiotics out of cells is recognized as a major component of bacterial resistance to many classes of antibiotics. It occurs due to membrane transporter proteins, called efflux pumps. These pumps can selectively extrude specific antibiotics; others expel various structurally diverse antibiotics. These are called multi-drug resistance pumps (MDR). Antibiotic specific pumps are usually encoded on mobile genetic elements for example plasmids and transposons. Genes encoding most MDR pumps are constituents of the bacterial chromosome (Lee *et al.*, 2000). Efflux pumps occur as single component for example tetracycline and chloramphenicol- specific pumps TetA and ClmA, respectively and the MDR pump MdfA encoded in the chromosome of *E. coli* or multi-component systems (exclusive to Gram-negative bacteria). Multi-component pumps traverse both the inner periplasm and the outer membrane (Lee *et al.*, 2000).

1.2.5.3. Production of enzyme(s) which modify/destroy the antibacterial agent

Examples of enzymes capable of destroying/modifying antibacterial agents are:

(i) Beta-lactamases, cause hydrolysis of the beta-lactam ring to yield microbiologically inactive products. Penicillin resistance results from destruction of the beta-lactam ring by penicillinase to yield inactive penicillonic acid.

(ii) Aminoglycosides are inactivated by plasmid-borne modifying enzyme, three reactions 1) phosphorylation, 2) adenylation and 3) acetylation can achieve this.

(iii)Chloramphenicol resistant bacteria e.g. *S. aureus* strains carry the resistant marker on an extrachromosomal plasmid. *E. coli* carries the resistance determinant on an R factor. A gene for chloramphenicol resistance occurs on a Tn9 transposon. Bacterial isolates resistant to some classes of antibiotics may exhibit more than one mechanism of resistance (Table 1.) (Mims *et al.*, 1993).

Table 1: The three types of resistance mechanisms

ANTIBACTERIAL	ALTERED TARGET	ALTERED UPTAKE	DRUG INACTIVATION
Beta-lactams	-	+	++
Glycopeptides	-	-	-
Aminoglycosides	-	+	++
Tetracyclines	-	+	-
Chloramphenicol	-	-	+
Macrolides	++	-	-
Lincosamides	++	-	-
Fusidic acid	++	-	-
Sulphonamides	++	-	-
Trimethoprim	++	-	-
Quinolones	-	+	-
Rifampicin	++	-	-

Key: (-) mechanism has never been observed, (+) mechanism sometimes observed, (++) mechanism observed in most cases (Mims *et al.*, 1993).

1.2.6. Change between susceptibility and resistance

Given enough time and exposure bacteria have the ability to develop resistance to every available antibiotic. Resistance to antimicrobial agents occurs as a result of bacterial

exposure to antimicrobials (Colloquium report to the American Academy of Microbiology, 1999). Resistance may result from a single chromosomal mutation in one bacterial cell. In the presence of the antibiotic the mutants have a selective advantage and survive and outgrow the susceptible population. Bacteria also acquire resistance genes on transmissible plasmids, which often code for resistance determinants to several unrelated families of antimicrobials. Epidemiological studies suggest that bacteria exposed to low and prolonged concentrations of an antibiotic may have a role in the selection of antibiotic resistance genes (Madigan *et al.*, 2003).

The persistence of resistance determinants in bacterial genomes over hundreds of generations, even in the absence of antibiotics as selective agents has been reported. Resistance to certain antibiotics may continue to be prevalent among clinical isolates, even when they are no longer in us as therapeutic agents, because genetic mechanisms involved in acquired resistance may favor stability of resistance genes even in the absence of ongoing exposure. The presence of integrons among clinical bacterial isolates may account for multiple drug resistance and continued resistance to antibiotics that have been withdrawn from use in public health. Even with the rotation of antibiotics, antibiotic resistance may persist due to the linkage of some antibiotic and heavy metal resistance genes provided for their perpetuation as the selection pressures change (Bass *et al.*, 1999).

If the selective pressure applied by an antibiotic is removed a previously resistant microbial population often reverts to the drug sensitive phenotype. This depends on factors that may be controlled, such as rate and pattern of antibiotic use, and also on factors over which we have no control. These include the biological cost antibiotic resistance imposes on bacterial fitness and the rate and degree to which natural selection will ameliorate these costs. Sometimes the

resistant cells are at a selective disadvantage to the drug sensitive cells in the drug free environment and are therefore eventually, outgrown by the sensitive cells. In some cases the genetic elements conferring antimicrobial resistance are lost from the cells and the resistant cells are progressively wiped out of the population (Franklin and Snow, 1981).

1.2.7. The impact of aquatic systems on antibiotic resistance

Microorganisms do not occur in isolation, they are linked to lives of many other organisms, the ecosystems and eventually the larger environments they inhabit (Colloquium report to the American Academy of Microbiology, 1999). Antibiotics and antibiotic resistance genes are discharged in increasing amounts into the environment due to the high and often indiscriminate use of antibiotics in medical, veterinary and agricultural practices. Water bodies like rivers are major receptacles of these pollutants and since they are the major source of water for animal and human consumption (directly or indirectly), their pollution may contribute to the maintenance and spread of bacterial antimicrobial resistance (Goni-Urriza *et al.*, 2000).

Bacteria that possess intrinsic antibiotic resistance are found in nature. These bacteria may acquire additional antibiotic resistance genes from bacteria introduced into water or soil and the resident bacteria may become reservoirs / sources of antibiotic resistance genes found in many environments (Ash *et al.*, 2002). The occurrence of antibiotic resistant bacteria in freshwater sources has been documented all over the world (Ash *et al.*, 2002). The aquatic environment has a role not only as a reservoir of clinical resistance genes, but also as a medium for spreading and evolution of resistance genes and their vectors, such as mobile genetic elements (Yin and Stotzky, 1997).

The wide use of antibiotics does not only selectively promote drug-resistant pathogenic bacteria but also exerts selective pressure on the normal commensal biota (Aminov *et al.*, 2001). However, the environmental consequences of the widespread use of antimicrobial agents, and antimicrobial resistance are very little understood (Colloquium report to the American Academy of Microbiology, 1999). Commensal fecal flora and bacteria of animal origin have long been considered to be important reservoirs of resistance genes in human bacterial pathogens. In order to measure the environmental pool of resistance and to understand the ecology of antibiotic resistance it is therefore, important to track antibiotic resistance genes in a variety of commensal; pathogenic and environmental bacteria (Aminov *et al.*, 2001; Goni-Urriza *et al.*, 2000). Aquatic (river) ecosystems are ideal since they may support a wide array of microorganisms e.g. pollution indicator organisms (fecal and total coliforms); pathogenic bacteria; members of the normal commensal biota as well as environmental bacteria.

In a study don comparing antibiotic and heavy metal resistance percentages of *E.coli* isolated from hospitalized inpatients, bred animals and environmental isolates, resistance including multiple antibiotic resistance levels were highest for clinical and river isolates. The potential for aquatic organisms to function as reservoirs of clinically important resistance genes requires that these organisms should be capable of acquiring resistance genes from commensal or pathogenic organisms introduced into the environment and that they should be able to act as donors of genetic information for reintroduction to the human host. Evidence suggests that *in situ* gene transfer occurs in the aquatic environment. The three well-established mechanisms of gene transfer namely, that is transduction, transformation and conjugation are believed to occur in aquatic environments (Yin and Stotzky, 1997).

Studies have shown the presence of biologically significant concentrations of dissolved DNA in marine and freshwater environments. Analysis of isolated DNAs have shown that fragments large enough to encode entire or part of gene sequences are present, providing evidence for the potential presence of a gene pool within the dissolved DNA content of aquatic environments. Such DNA is suitable for natural transformation (Yin and Stotzky, 1997). Thus aquatic environments provide a source and reservoir of resistance genes.

1.2.8. Methodology

Antibiotic resistance pattern (ARP) data can be used as 'fingerprints' to determine the source of fecal pollution in natural waters (Harwood *et al.*, 2000). Antibiotic resistance profiles are obtained from bacterial isolates from suspected sources, using conventional culture methods. Agar – diffusion methods are used to characterize the antibiotic susceptibility and obtain any antibiotic resistance phenotype. Molecular methods have been effectively used in characterizing antibiotic resistance. Molecular techniques that assay the genotype rather than the phenotype have significant advantages over methods only studying the phenotype (Aminov *et al.*, 2001; Goni-Urriza *et al.*, 2000; Colloquium report to the American Academy of Microbiology, 1999). This is because many aquatic microbes are probably non-culturable. The bacteria that can not be cultivated may be part of the reservoir of resistance genes as well (Aminov *et al.*, 2001; Ash *et al.*, 2002). Methods such as restriction fragment length polymorphism analysis (RFLP), DNA sequencing and DNA fingerprinting are very useful in genetic studies of microbes in general, and antibiotic resistance in particular (Aminov *et al.*, 2001; Goni-Urriza *et al.*, 2000; Bezuidenhout *et al.*, 2001).

1.3.0. Aim

The overall aim of this study was to evaluate the usefulness of temporal and spatial antibiotic resistance pattern (ARP) data in determining the source of fecal pollution, and the effect of the pollution on antibiotic resistance in a natural water source.

1.3.1. Objectives

- To determine the distribution of microbial water quality indicators (fecal and total coliforms) as well as general bacterial community distribution along the eastern region of the Mhlathuze River.
- To determine the incidences of antibiotic resistance in the isolated microbes.
- To characterize antibiotic resistance in isolates by determining:

1. Whether the genes responsible are chromosomal or plasmid bound.
2. The presence and genetic variations of certain transposable elements like plasmids and integrons.
3. The presence and genetic variations of certain specific antibiotic resistance genes particularly intergron bound antibiotic resistance genes.
4. The conjugative nature of the extracted resistance plasmids.

CHAPTER TWO

MICROBIAL MONITORING OF THE MHLATHUZE RIVER (FEBRUARY 2001-SEPTEMBER 2002)

2.1. GENERAL OVERVIEW

2.1.1. Study area

In developing countries like South Africa, most rural communities do not have access to potable water and therefore, rely mainly on water from rivers, streams and wells for their domestic water supply. In most cases such water is fecally contaminated and there is no way it can be treated (Obi *et al.*, 2002). The Mhlathuze River is situated in a subtropical region of Southern Africa. This catchment area supports a fast growing agricultural and industrial community in the northern coast region of KwaZulu-Natal. Industries in this area include sugar milling, paper and pulp factories, aluminium smelters, fertilizer producing companies and mineral mines. All these are concentrated along the area close to the estuary (Bezuidenhout *et al.*, 2001).

The rural communities make up 78.5% of the total population of this catchment area and 63% of the rural households in the area depend on water from dams, rivers and streams, 34.8% of these people lack proper sanitary systems (Treasury Department, 2000). At present there is a gradual increase in both the demand for water from the river for agricultural and industrial development and pollution from industrial and domestic wastes. The lack of quality drinking water coupled with non-existent sanitary systems in

the area make communities of the rural areas along the Mhlathuze river more vulnerable to water-borne diseases such as diarrhea. A study by Pretorius (2000) illustrated how sanitation in a developing urban area can affect the quality of surface water. According to a report by the Institute for Water Quality Studies the Mhlathuze catchment area (W12-C-J) is high on the national microbial water quality monitoring programme's priority list, it is number 14 in a list of 120 areas in South Africa with a potentially high health risk due to fecally polluted surface waters (IWQS, 2000). Diarrhea outbreaks have been reported in the past. The 2000-2001 cholera epidemic that spread from this catchment to other areas was preceded by a diarrhea outbreak (May-August 2000) in the Mangeza area (a reserve of the Mhlathuze catchment area) which claimed at least 40 lives and affected most of the local households (Biyela, 2000). At present the water demand of the Mhlathuze River is high (Steyl *et al.*, 2000). The need for the collection of microbial and physico-chemical data of the water from the river to provide comprehensive baseline information for the river that can be utilized for future monitoring purposes is indispensable for this catchment.

In aquatic ecosystems there is an intricate relationship between external and internal factors. Temperature influences the amount of dissolved gases present in water, the cooler the water the more soluble the gases will be. Turbidity depends on factors like vegetation cover, geological formations, water-flow, total dissolved solutes etc. Very low dissolved oxygen is considered to pose a threat to aquatic life. Sudden fluctuations of these parameters may indicate changing conditions in the system (river) which affect internal factors between and within bacterial and planktonic populations in the water

body (Nubel *et al.*, 1999; Byamukama *et al.*, 2000; Goni-Urriza *e .al.*, 2000; Lobitz *et al.*, 2000)

Coliform bacteria have been accepted and used universally as indicators of microbial water quality, this is due to the following reasons: (a) Fecal coliforms have lifespans of 48hours in the environment. (b) Coliform bacteria are present in larger numbers than pathogens. (c) Coliforms react to treatment processes and environmental processes in a way reasonably similar to pathogens (USA Environmental Protection Agency, 1999). Total coliforms, include organisms that are generally found in soil and water, and they give an indication of the bacterial contamination of the water. The presence of fecal coliforms indicates recent fecal contamination, and therefore, the possibility of the presence of pathogenic microorganisms from human and/or animal waste. Increase in bacterial contamination in general and fecal contamination in particular is not only a problem of developing countries, but that of developed countries as well. Outbreaks of waterborne diseases have serious medical implications and they cause a tremendous economic burden to many countries worldwide (WHO, 2000; WHO, 1996).

A report of strategic environmental assessment for water use in the Mhlathuze catchment (Steyl *et al.*, 2000) by the Department of Water Affairs and Forestry provides comprehensive information on the physical and ecological environment as well as social and economical aspects in the catchment. However, very little has been done on the microbiological quality aspect of the water from this river .

Thus our primary aim was to determine the microbial quality and basic physical and chemical parameters of the Mhlathuze River and also determine whether there was any temporal or spatial distribution of bacterial communities in the study area.

2.2. METHODOLOGY

2.2.1. Study area and sampling sites

Samples for this study were collected from five sites along the length of the Eastern region of the Mhlathuze River. The sampling sites were as follows:

1. Site 1: Kwa-Dlangezwa, this site is downstream from a sewage treatment plant.
2. Site 2: Dlangubo, this site is the furthermost from industrial activities.
3. Site 3: Mhlathuze Estuary, Richards Bay, this site is next to mineral mining industries and a coal terminal.
4. Site 4: Mhlathuze pump station.
5. Site 5: situated at Felixton brigde, this site had the most human activities of all the five sampling sites. It is next to a sugar milling plant, a paper pulping factory, sugar cane plantations and informal agricultural gardens.

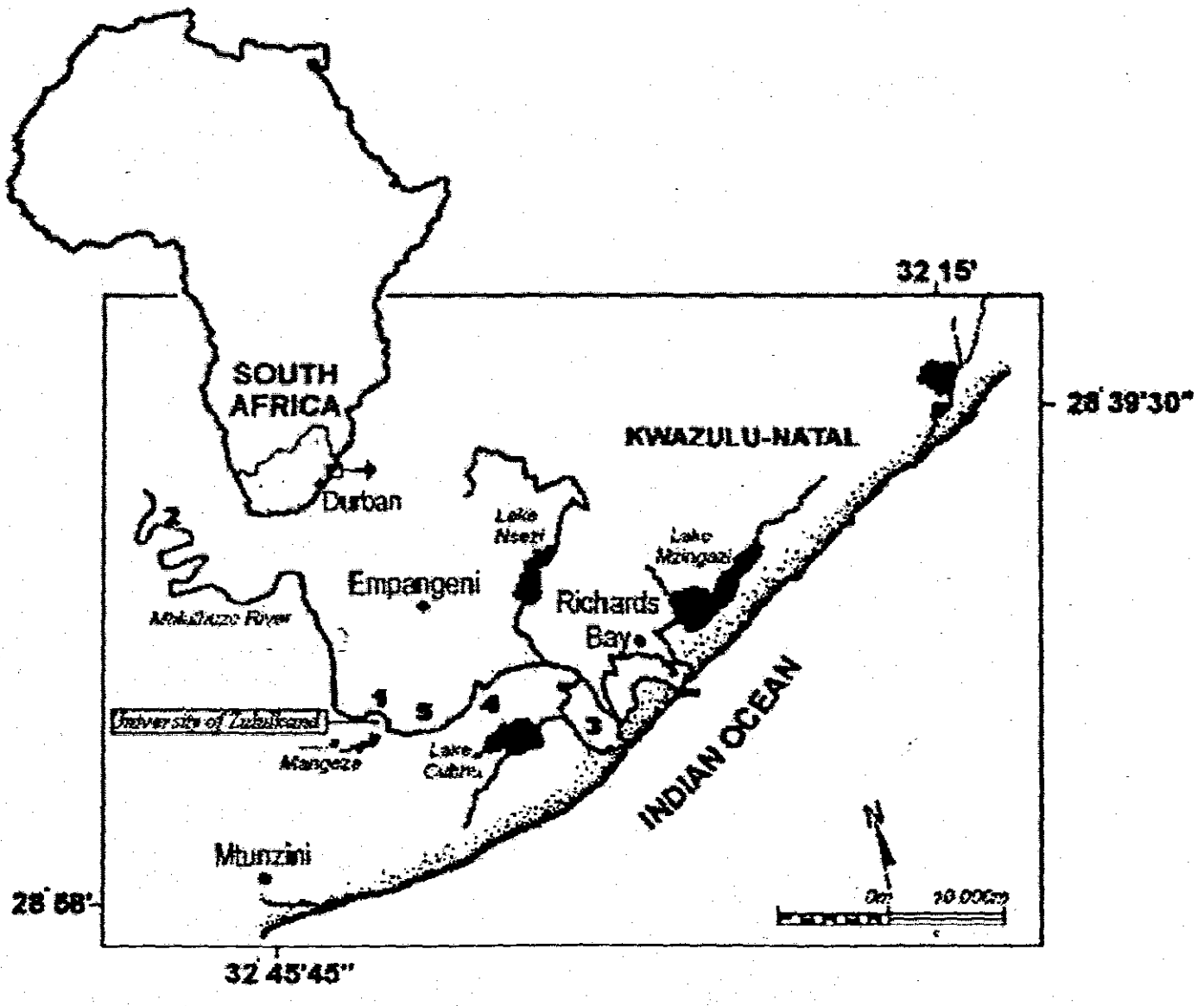


Figure 1. Map of the eastern region of the Mhlathuze catchment area indicating the sampling sites (site 1-KwaDlangezwa, site 2-KwaDlangubo, site 3-Mhlathuze estuary, site 4-Mhlathuze pump station and site 5-Felixton bridge).

2.2.2. Sampling

Water samples were collected biweekly (February 2001-September 2002) in sterile Schott bottles, at five different locations (Fig. 6). Once collected, the samples were immediately stored on ice in a dark cooler box and transported to the laboratory. The samples were stored at 4°C and analyzed within 6 hours of collection (Bezuidenhout *et al.*, 2001).

2.2.3. Physicochemical properties

Temperature, pH, total dissolved solutes/conductivity and percentage dissolved oxygen were measured *in situ* using CORNING checkmate II meter. Turbidity was also measured in situ using the AQUALYTIC turbidity meter.

2.2.4. Microbiological analysis

2.2.4.1. Culture conditions

Enumeration of bacteria was done using plate count and membrane filtration (Millipore, HANG 47 mm) methods. A series of ten-fold dilutions of each sample were used. Nutrient agar, m-Endo, m-FC, KF-Streptococcus, Vibrio Diagnostic (TCBS) and

Salmonella-Shigella agars (Merck) were used to culture heterotrophic bacteria, total coliform, fecal coliform, streptococci, *Vibrio* spp. and *Salmonella* and *Shigella* spp. respectively. Plates were incubated at 35°C for 24 hours except the m-FC plates, which were incubated at 44.5°C for 24 hours (Bezuidenhout *et al.*, 2001).

2.2.4.2. Identification of bacteria

Several single colonies from each plate were randomly isolated based on their morphology. The isolates were identified by Gram staining and biochemical tests, and confirmed by the API 20E (BioMerieux, France) system (Madigan *et al.*, 2003).

2.2.4.3. Resuscitation of VBNC bacteria

Resuscitation was carried out using the DVC method of Kogure (1979). Nine thousand seven hundred and thirty µl of sample was resuscitated using 20 µl (0.002%) nalidixic acid and 250 µl (0.025%) yeast extract. The sample was then incubated in the dark on a shaking platform (165 rpm) at room temperature for 6 hours. Bacterial numeration after resuscitation was obtained using membrane filtration method as described above and compared with the one without resuscitation.

2.2.4.4. Statistical Analysis

Geometric means of microbiological physical and chemical analysis data were used to present monthly values for these factors. Pearson's correlation coefficient analysis was added to determine linear relationships between bacterial counts and temperature as well as bacterial counts and rainfall figures.

2.3. RESULTS

Table 2 shows geometric means of the physical and chemical parameters and fecal coliform counts of the water samples collected from 5 different sites along the Mhlathuze River during the study period. Most of the values obtained were acceptable within South African standards. During the rainy period, the turbidity of Mhlathuze River increased to unacceptable levels, especially downstream. Water samples from sites 4 and 5 seemed to contain higher concentrations of total nitrogen and phosphates with averages of 3.33 and 2.78mg/l total nitrates and 2.9 and 3.6 mg/l total phosphates respectively. The two sites subsequently possessed higher fecal coliform contamination.

Resuscitation of water samples increased bacterial counts by up to 23-fold for fecal coliforms and up to 65 times for total coliforms (Table 2). Under stressful environmental conditions most bacteria revert to the viable but non-culturable stage, and therefore, can not be grown on laboratory media. Bacterial counts for site 3 samples were the lowest of all the sites. High metal concentrations (Cd^{+2}: 0.011mg/L; Pb^{+2}: 0.056 mg/L) were detected from water samples from Felixton and the estuary.

Figure 7 shows the mean values of the total coliform counts from all sites during the study period (2001-2002) as well as data from a study conducted between 1998-1999 in the same area. Surface water temperature presented a similar trend for the two study periods, however the average water temperatures during the summer months were higher than those detected in the previous study, with the exception of January 2002. Total coliform counts for this study (2001-2002) were significantly higher than those obtained

in the previous study (1998-1999). The rainfall figures were also higher in the same period than those in the previous study except for February. During 2001, the total coliform population in the Mhlathuze River was the most unpredictable of the 4 years, there were major fluctuations than all other years. In 2002, this was gradually changing. There was positive correlation between the fecal coliform counts and the water temperature (r=0.48) and fecal coliform counts and rainfall figures (r=0.31).

The fluctuation trend observed in the total coliform counts was also observed in the fecal coliform counts during the same period. Despite the increase in total coliform counts in the water system, the fecal coliform counts generally were of the same level as those observed in the period of 1998 (Figure 8) except for the fluctuations in 2001. There was significant increase in fecal coliform counts in November and December of 2001. This sudden increase of fecal coliform counts was mainly due to the significant increase at Site 4 (Figure 9). Felixton (Site 5) continues to be the main source of fecal contamination as observed during the 1998 and 1999 period. Although Mhlathuze Pumping Station also emerged as a main source of fecal contamination during 2001, the situation was recovered in 2002.

Table 2: The geometric means of fecal coliform counts, physical and chemical characters measured in each site of the Mhlathuze River between February 2001 and September 2002.

Site	Site 1	Site 2	Site 3	Site 4	Site 5
T(^{0}C)	15.0-31.6 (23.3)	17.0-30.85 (25.2)	16.5-31.2 (25.9)	18.0-29.4 (24.2)	15.0-30.7 23.2)
PH	5.8-8.3 (7.6)	5.93-8.47 (7.74)	5.94-8.19 (7.51)	6.9-8.58 (7.73)	6.83-8.38 (7.70)
Turbidity (NTU)	0.8 – 9.0 (5.63)	2.5-7.6 (6.1)	2.1-20.2 (7.56)	2.3-17.8 (13.4)	1.4-33.0 (7.80)
Conductivity (µS/cm)	350-696 (480.9)	218-524 (391.9)	1970-51800 (25700)	263-705 (491.5)	221-717 (462.6)
DO (mg/L)	36.2-51.6	33.3-51.0	32.0-43.3	32.0-41.9	31.7-43
Total N (mg/L)	1.6-5.6 (2.41)	0.5-5.9 (1.43)	1.4-2.4 (1.67)	1.5-8.5 (3.33)	1.3-7.7 (2.78)
NO_2^-(mg/L)	0.06-1.9 (0.20)	0.06-0.8 (0.15)	0.03-0.8 (0.17)	0.02-4.8 (0.22)	0.02-0.8 (0.15)
NO_3^-(mg/L)	0.3-1.0 (0.59)	0.5-0.7 (0.59)	0.2-0.6 (0.33)	0.3-0.9 (0.62)	0.2-1.1 (0.48)
NH_4^+(mg/L)	0-0.23 (0.06)	0.01-0.06 (0.03)	0.03-0.24 (0.07)	0.02-0.34 (0.08)	0-0.13 (0.04)
Total P(mg/L)	1.3-2.8 (1.9)	1.8-4.8 (2.9)	3.6-32.0 (10.7)	1.0-8.3 (2.9)	1.3-10 (3.6)
Ortho-P (mg/L)	0.2-0.5 (0.28)	0.19-0.87 (0.53)	0.20-0.94 (0.44)	0.46-0.80 (0.67)	0.20-0.68 (0.34)
Cd^{+2} (mg/L)	nd	0-0.001	0-0.006	nd	0-0.011
Cu^{+2} (mg/L)	nd	0.002-0.022	0.039-0.11	nd	0.004-0.047
Al^{+3} (mg/L)	nd	10.9-12.7	27.9-44.7	nd	13.1-32.2
Hg^{+2} (mg/L)	nd	0-1.19	0.52-3.29	nd	0-0.08
Pb^{+2} (mg/L)	nd	0 - 0.001	0.003-0.056	nd	0.002-0.004
BOD	0-1.35 (0.21)	0-0.90 (0.23)	0-1.00 (0.41)	0-1.2 (0.48)	0-0.80 (0.24)
COD(mg/L)	9-72.5 (22.3)	2.5-21.5 (11.9)	18.0-126.0 (33.6)	4.0-25.0 (12.5)	1.3-34.5 (19.7)
Fecal Coliform Count/100ml (mean)	10-900 (292)	20-2600 (642)	0-200 (54)	40-26800 (1937)	20-6120 (1118)
Ratio after resuscitation*	1.5-23.3	1.5-10	1.25-4.0	1.25-7.5	1.3-2.5

Key: DO: dissolved oxygen; COD: chemical oxygen demand: BOD, biological oxygen demand. Values in brackets are the averages for the different parameters. Nd., analysis not done

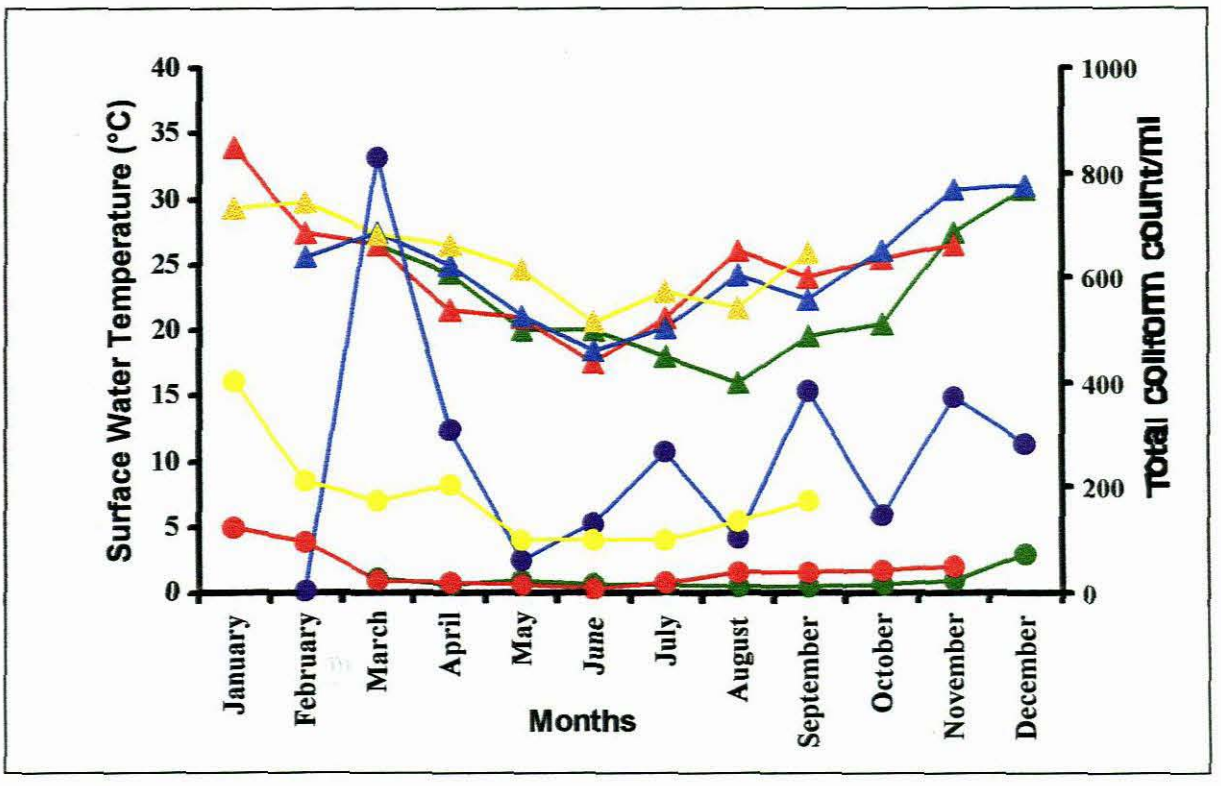


Figure 2: The monthly mean of total coliform counts and temperature of all sites along Mhlathuze River during the study period (February 2001 – September 2002) and from the pervious study (March 1998-November 1999). (● Total coliform count for 2001; ● Total coliform count for 2002; ● Total coliform count for 1999; ● Total coliform count for 1999; ▲ Temperature for 2001; ▲ Temperature for 2002; ▲ Temperature for 1999; ▲ Temperature for 1998)

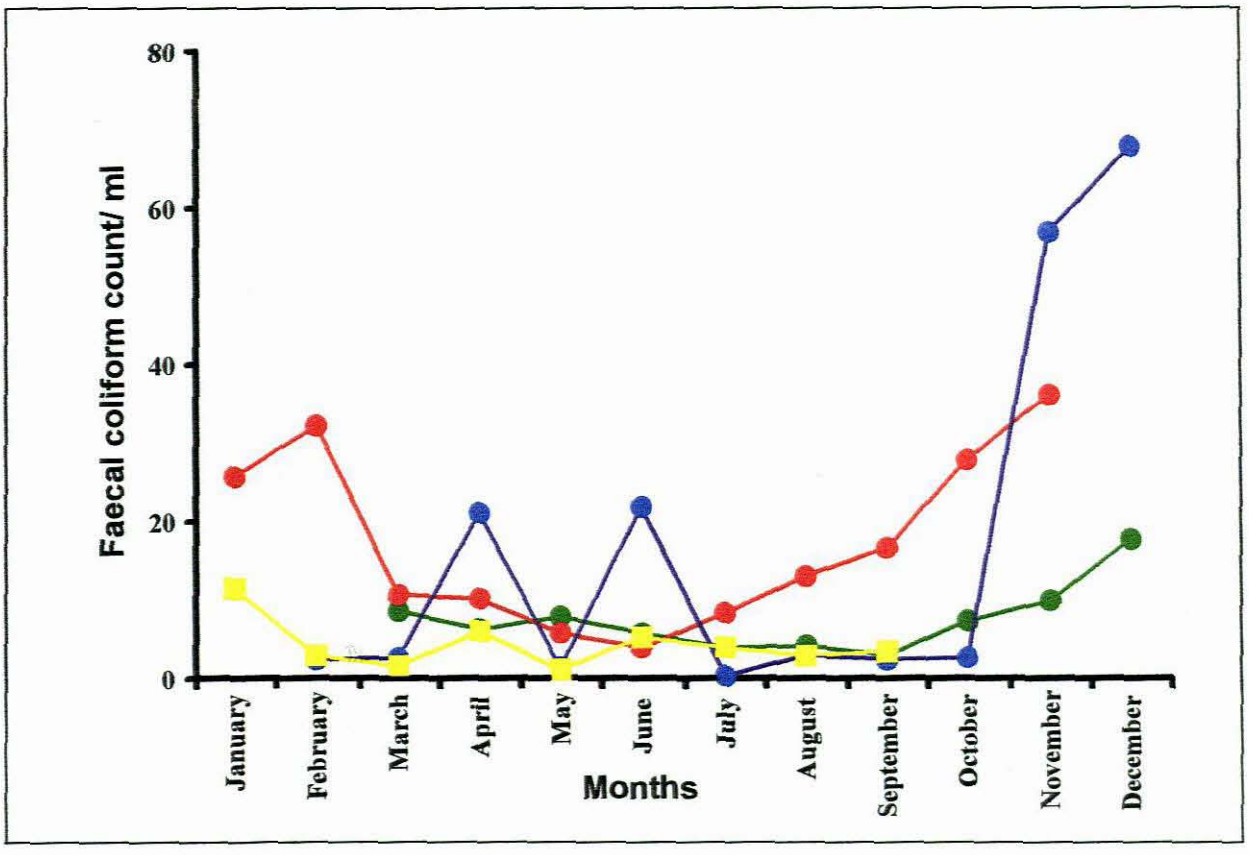


Figure 3: The monthly mean fecal coliform counts of all the study sites along Mhlathuze River during the study period (February 2001 – September 2002) and from the pervious study (March 1998-November 1999). (● Fecal coliform count for 2001; ● Fecal coliform count for 2002; ● Fecal coliform count for 1999; ● Fecal coliform count for 1998).

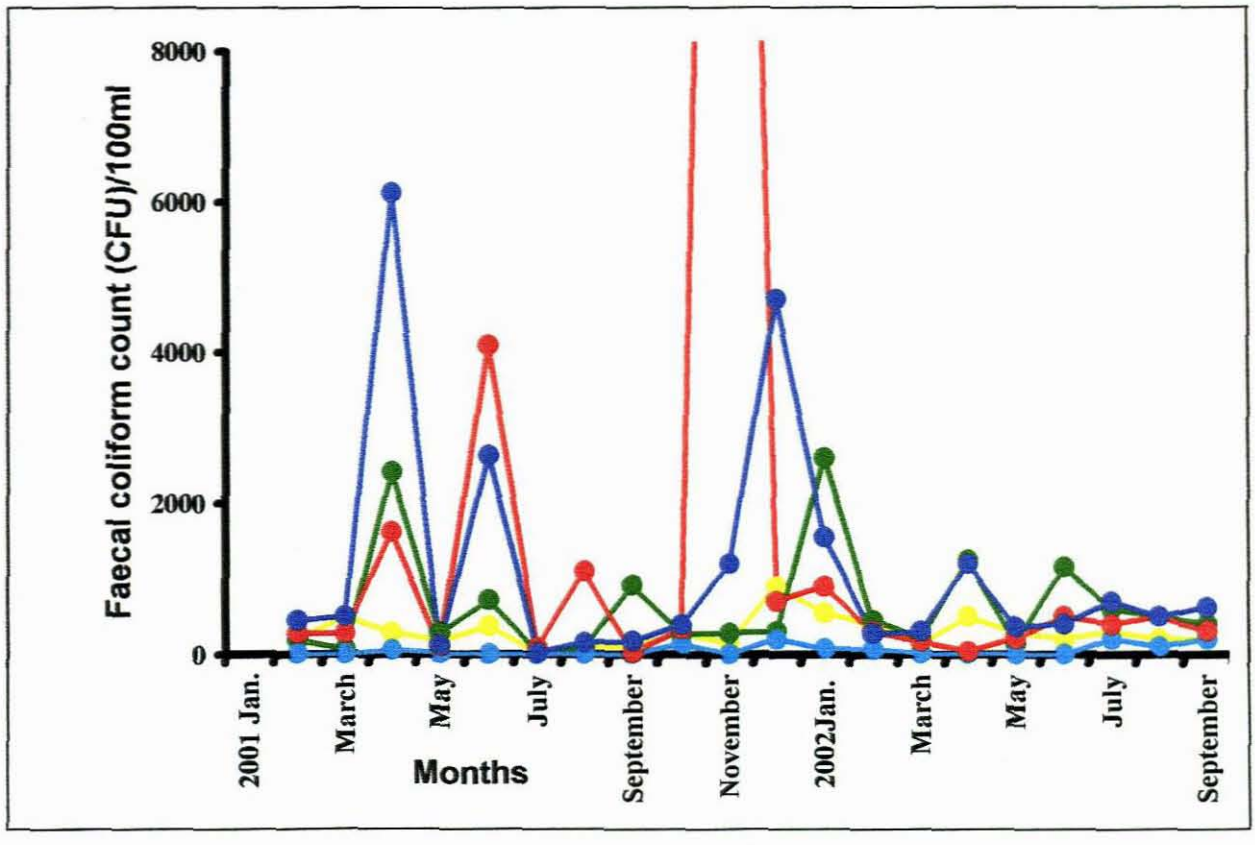


Figure 4: *The monthly mean fecal coliform counts of each site along Mhlathuze River during the study period (February 2001 – September 2002).* (● Felixton Site 5; ● Mhlathuze Pumping Station Site 4; ● Richards bay Estuary Site 3; ● Dlangubo Site 2; ● Kwa Dlangezwa Site 1).

When the trends of the fecal coliform contamination of each site along the Mhlathuze River during the two study periods were compared, the seasonal changes were not so obvious as suggested in the previous study (1998-1999) due to the fluctuation pattern in 2001. With the exception of sudden increase in fecal coliform on Sites 4 and 5 in 2001, the fecal contamination in the river was generally lower in this study period.

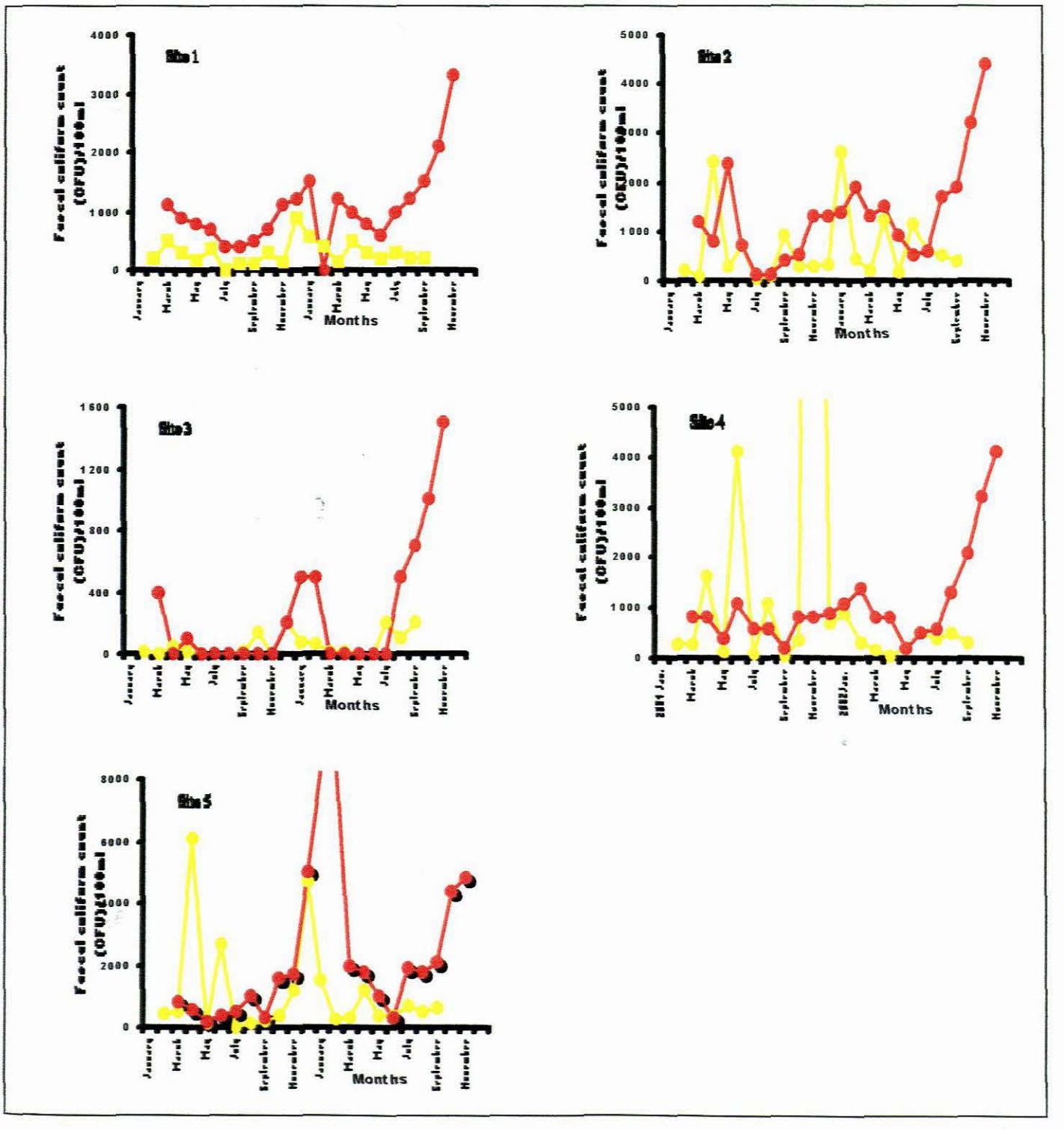


Figure 5: Comparison of monthly mean fecal coliform counts of each site along Mhlathuze River between study period (● February 2001 – September 2002) and the previous one (● March 1998 – November 1999).

2.4. DISCUSSION

The distribution of bacterial communities observed in the study showed a seasonal trend in which bacterial counts were higher during the summer period than in winter. When the monthly means were analyzed statistically using the Pearson correlation method, the following correlation co-efficient values were obtained: fecal coliform counts and surface water temperature (r=0.48), and fecal coliform counts and rainfall figures (r=0.31). Total coliforms and temperature (r=0.35) and total coliforms and rainfall figures (r=0.28). The seasonal pattern of microbial growth that was observed in this study (Figures 2-5) is strongly supported by other studies (Nubel *et al.*, 1999; Byamukama *et al.*, 2000; Lobitz *et al.*, 2000; Nishiguchi, 2000; Solo-Gabrielle, 2000). The mean surface water temperature of Mhlathuze River was constantly between 20-34°C during the summer period. This represents an ideal temperature range for the prolonged survival of coliform bacteria as well as human and animal enteric pathogens in aquatic environments, which explains the increases in bacterial counts during the warmer months (Bezuidenhout *et al.*, 2001).

There was a significant increase in total coliform counts in this study (Figure 7) compared to the 1998-1999 study, and fluctuation patterns for the total coliform and fecal coliform counts were also observed in 2001 (Figures 7 and 8). Despite the substantial increase in the total coliform count, the levels of fecal contamination were similar to those observed in the 1998-1999 study. The Mhlathuze pumping station (site 4) and Felixton (site 5) were the main contributors to the sudden increases in total coliform counts. The observed fluctuating pattern, which occurred at site 4 (Figures 9 and 10),

coincided with the construction of the Mhlathuze pumping station in order to supply water for the new mining project in the region. A drop in bacterial counts was observed when the construction was complete. The increase in total coliform numbers, specifically at site 4, could be attributed to environmental degradation and destruction of the soil profile due to the construction of another pumping station. This premise is supported by the fact that there was no significant increase in fecal coliform numbers, which could have been the case if there was additional contamination.

Felixton (site 5) continued to have the highest levels of fecal contamination as was observed in 1998-1999 (Figure 9). This site is adjacent to a paper-pulping factory, a sugar mill and is downstream from a sewage treatment plant outlet. There is also a lot of human activities in this area, for example: the development of large informal gardens, swimming and the use of a bridge adjacent to the sampling site as a thoroughfare around this site. Both the sugar mill and the informal agricultural activities are in full operation during the summer months. These factors could have contributed, independently or in combination, to the very high levels in bacterial counts that were observed at this sampling site most especially in summer (Bezuidenhout *et al.*, 2001; Bezuidenhout *et al.*, 2002).

Lower fecal coliform counts were obtained in sites 1 and 3 (Figure 10). Low levels of bacterial contamination and the complete absence of fecal coliforms in marine waters have been reported in other studies. Another factor could be that it is a remote area, which is far from domestic activities and therefore, it does not receive direct fecal contamination from domestic waste. Mean fecal and total coliform counts were generally

very high, but lower than those observed in other studies (Pretorius, 2000; Byamukama *et al.*, 2000; Goni-Urriza *et al.*, 2000). According to the DWAF guidelines, (1996) the bacteriological quality of the water of the Mhlathuze River posed an increased risk of transmission of water-borne infectious pathogens to communities that depend on the river for domestic use.

Studies on heterotrophic, autotrophic and other bacterial types showed that other factors such as light, salinity, predation, available nutrients and environmental pollutants are some of the key factors that influence bacterial growth and abundance in water bodies (Pernthaler *et al.*, 1998; Lobitz *et al.*, 2000; Solo-Gabriele *et al.*, 2000). Our results (Table 2) show that most of the physical and chemical parameters are within the acceptable levels of water quality for domestic supply. The turbidity of the Mhlathuze River was generally higher during the summer period and with levels of up to 33.0 NTU, which might have serious health hazard for drinking purposes

The "viable but non-culturable" state, an important survival strategy, of several pathogenic microorganisms such as *Salmonella, Shigella* and *Vibrio* spp. has caused increasing concerns for the public health sector. After resuscitation, fecal coliform counts of the water samples increased up to 23 times in this study (Table 2). The results indicate that fecal contamination in the Mhlathuze catchment area is severe. Fecal contamination by human and/or animal waste is the major health risk associated with drinking water directly from natural sources (Obi *et al.*, 2002). The use of other detection techniques, for example molecular methods, could be of help in future studies, since this study clearly

demonstrated that viable bacteria only represent a small fraction of the total microbial community of the system. It is estimated that in most ecosystems, less than 1% of bacteria are culturable using traditional culture methods. An added advantage to the incorporation of molecular methods in bacterial detection is their high sensitivity and specificity.

2.5. CONCLUSION

The abundance of fecal and total coliform bacteria showed seasonal correlation with surface water temperature and rainfall figures in the Mhlathuze River. Of all the environmental parameters measured in this study, temperature seemed to have the strongest impact on the fecal and total coliform communities. The results of this study show that, although bacterial contamination in the river is generally constant over time, there are periods when huge increases in bacterial counts are observed. This information could be vital in medical management in Northern KwaZulu-Natal, commensal organisms could be used to indicators the environmental pool of antibiotic resistance, and this might lesson the burden of over prescription of antibiotics which may not be effective against the infective agents. The following could be some of the factors that influenced the observed elevated levels of fecal and total coliform bacteria:

1. Human activities (domestic, recreational, agricultural, industrial) along the river increase during the summer period. At Felixton, for example, a sugar-mill and paper factory is operating at full capacity alongside informal agricultural activities.

2. The construction at the Mhlathuze pumping station caused severe fluctuation of bacterial growth observed in 2001 particularly at site 4.
3. The catchment area is situated in an area with high summer rainfall and increased run-off is therefore expected in the summer annually.
4. Surface water temperatures of between 25°C and 30°C were regularly detected during the summer period. This temperature range is ideal for longer survival of fecal coliforms and enteric pathogens (Bezuidenhout *et al.*, 2001).

CHAPTER THREE

ANTIBIOTIC RESISTANCE PATTERNS OF THE ISOLATED MICROBES

3.1. GENERAL OVERVIEW

The extensive use of antibiotics in humans, animals and plants has resulted in the establishment of a pool of antibiotic resistance in the environment. Non-pathogenic and pathogenic microorganisms that become resistant to antibiotics may serve as reservoirs of resistance genes. This may provide the opportunity for exposure of naïve populations of bacteria, animals and humans to antibiotic resistance genes and antibiotic resistant bacteria and the opportunity for the transfer of these genes. This problem is further compounded by the increasing incidence of pathogens with antibiotic resistance (Ogan and Nwiika 1993; DePaola *et al.*, 1995).

Exposure to environmental contaminants and changes in nutrient composition may lead to selection pressures favoring certain organisms or organisms with certain genotypes. Recent studies demonstrated positive correlation between industrial (Goñi-Urriza *et al.*, 2000; McArthur and Tuckfield, 2000) pollution and the spatial distribution of antibiotic resistance. This is because of the association of antibiotic and heavy metal resistance genes. Many plasmids and integrons encoding antibiotic resistance genes also encode resistance to heavy metals or quartenary ammonium compounds that could be the selective factor (Colloquium report to the American Academy of Microbiology, 1999). This results in the selection of antibiotic resistance genes even in the absence of the

antibiotics, as a selective pressure. The wide and often indiscriminate use of antibiotics in medical and veterinary practices (Balagué and García Véscovi,2001; White *et al.*, 2000) as well as domestic and agricultural use of pesticides and related compounds (Balagué and García Véscovi, 2001) cause significant antibiotic contamination of the natural environment and consequent development of resistance in communities of non-disease organisms. These organisms could create a widespread antibiotic resistance gene pool that could be transferred back into human and animal pathogens. The transmission of antibiotic resistant bacteria and antibiotic resistance genes flow in both directions, between animals and humans either by direct contact or via indirect mechanisms (Colloquium report to the American Academy of Microbiology, 1999). A recent study by Ash (2002) showed that several rivers in the United States have become major reservoirs of antibiotics and antibiotic resistant microbes. With the world gradually becoming a global village, drug and antibiotic resistance can be expected to spread steadily all over the world. In medicine, these generate significant health and economic impacts.

Like antibiotic contamination, heavy metal pollution is also a serious environmental problem as a result of the increase in human and industrial activities. The accumulation of toxic heavy metals in the environment consequently leads to their accumulation in bacteria. The mechanisms of heavy metal resistance have been suggested to enhance the antibiotic resistance ability of microorganisms (Davidson, 1999; Edlund *et al.*, 1996). This leads to the co-selection of the linked markers. The results from monitoring physical, chemical and microbiological quality of the Mhlathuze River (Chapter 2) indicates that there are high levels of bacterial as well as heavy metal contamination in

this water system. A previous study of the causes of diarrhoea in this area, showed that most of the isolated microorganisms were resistant to at least two antibiotics. When water samples were collected from one of the reserves that was struck by a diarrhoea outbreak, *Salmonella* (which was found by that study to be the cause of the outbreak) isolates from water samples had antibiotic resistance patterns similar to those of isolates from infected patients (Biyela, 2000). These findings are supported by other studies, which have also reported that the environment has a role in the spread and maintenance of antibiotic resistance in both commensal and pathogenic microbes (Colloquium report to the American Academy of Microbiology, 1999). The industrial and intensive farming activities in this region might also contribute to the selection of antibiotic resistance genes in bacteria that colonize humans and animals. However, the role of environmental pollution with antibiotics and other selective agents remains unknown and needs to be factored into any equation of selective influences for antibiotic resistance.

This part of the study aimed to determine the antibiotic susceptibility/resistance patterns of the *Enterobacteriaceae* as well as *Pseudomonas* and *Aeromonas* isolates from the Mhlathuze River and to determine if there was any spatial distribution of antibiotic resistance in the study area.

3.2. MATERIALS AND METHODS

3.2.1. Isolation and Identification of bacteria

For the isolation, the membrane filtration technique was used (see section 2.2.2.1.). Several single colonies from each plate were randomly isolated according to their

morphology. Pure cultures of the isolates were obtained by sub-culturing on nutrient agar plates. The isolates were identified by Gram staining and biochemical tests, and confirmed by the API 20E (BioMerieux, France) system (Madigan *et al.*, 2003).

3.2.2. Antibiotic sensitivity test

The disc diffusion method was used to determine antibiotic sensitivity of the isolates. Overnight broth cultures were spread on Mueller-Hinton agar plates. The plates were dried at room temperature for 2 hours. Antibiotic discs were placed at equi-distances. The plates were incubated for 24 h at 37°C and organisms were classified as sensitive, intermediate or resistant based on the NCCLS standards. The antibiotic discs used corresponded to those commonly used in treatment of human infections. A total of 15 antibiotics used in this study can be classed into 10 different groups (Table 3).

3.2.3. Statistical Analysis

The percentage data were arcsine transformed before analysis. Paired t-tests (Wilkinson, 1988) were used to examine the statistical significance between different species. Spatial variability in antibiotic resistance was analyzed with the Chi-square test. Pearson's correlation coefficient (r) was used to represent the relationship between the clinical isolates and the *Enterobacteriaceae* environmental isolates.

Table 3: The classes and quantity of antibiotics used in the study

Class	Antibiotic used	Quantity
Fluoroquinolones	Ciprofloxazine	5 µg
Phenicols	Chloramphenicol	30 µg
Aminocoumarin	Novobiocin	30 µg
Folate Inhibitors	Co-trimoxazole	25 µg
Ansamycins	Rifampicin	5 µg
Quinolones	Nalidixic acid	30 µg
Cephalosporin	Cefuroxime; Cephalothin; Cefotaxime; Cefoxitin	30 µg
Aminoglycosides	Streptomycin; gentamicin	10 µg
Tetracyclines	Tetracycline	30 µg
β-lactamase	Ampicillin; Penicillin	10 µg, 10 U

3.3. RESULTS

3.3.1. Antibiotic Resistance Levels

From February 2001 to January 2002, a total of 114 enteric bacteria were isolated and identified as follows: *E. coli*, (42), *Citrobacter freundii* (27), *Klebsiella* spp. (23), *Enterobacter* spp. (12), *Serratia marcesens* (7) and *Proteus* spp. (3). In addition several *Pseudomonas* and *Aeromonas* spp. were also identified.

All isolates were sensitive to gentamicin and to ciprofloxacine except one *Pseudomonas* isolate which was resistant to ciprofloxacine. This isolate had the highest level (12) of resistance to the tested antibiotics (15). Cefotaxime inhibited the growth of all enteric bacteria isolated except one *Klebsiella* and one *Enterobacter* isolate. All *S. marcesens* isolates were resistant to rifampicin. 94.7% of enteric bacterial isolates were resistant to at least one antibiotic and 80.8% to two or more antibiotics (Table 4). Four enteric bacterial isolates, including one *E. coli* and three *S. marcesens*, were resistant to 8 out of 15 different antibiotics tested. Among the non- *E.coli* isolates, only two *Enterobacter* isolates were sensitive to all antibiotics. Other genera (*Klebsiella* spp, *S. marcesens, Serratia* spp. *C. freundii and Proteus* spp.) were resistant to at least one antibiotic. Resistance to three and more classes of antibiotics was common in the tested microbes (Table 4).

Table 5 shows antibiotic resistance patterns (ARP) of all *Enterobacteriaceae* isolates from the five sites. ARP of *E. coli* and non-*E.coli* isolates is also shown in Table 4.

Table 4: Level of antibiotic resistance of different genera of *Enterobacteriaceae* isolated from the Mhlathuze River

No. of Antibiotic Determinant(s)	0 (%)	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)
Total *Enteric bacteria* isolates	5.3	13.9	18.4	19.3	22.8	11.4	3.5	0.9	3.5
E. coli	9.5	16.7	14.3	21.4	23.8	4.8	7.1	0.0	2.4
Others	2.8	13.9	20.8	18.1	22.2	15.3	1.4	1.4	4.2

Highest levels of resistance among all isolates were shown to the following antibiotics: penicillin, 72.6%; rifampicin, 69.2%; novobiocin, 52.1%; ampicillin, 43.6% and cephalothin, 28.2 %. The ARPs were very similar between *E. coli* and non-*E. coli* groups. The non *E. coli* groups have higher resistance against nalidixic acid, novobiocin, cefuroxime and cefotaxime than *E. coli* isolates.

3.3.2. Comparison of Antibiotic Resistance Patterns (ARP) of different sources

ARPs for the tested antibiotics were generally similar among different sites. However, there were some differences. The isolates from Site 1 were more resistant to penicillin. Except for cefotaxime and nalidixic acid, the enteric bacteria isolated from Richards Bay Estuary (Site 3) had higher percentage of resistance towards other types of antibiotics. And the enteric bacteria isolated from Dlangubo (Site 2), which is about 40 km upstream generally showed mild resistance. This was expected, site 2 is the furthermost from industrial and agricultural activities. Microbes resistant to chloramphenicol and rifampicin were mainly isolated downstream (Site 3). Microbes resistant to cefotaxime

were mainly isolated upstream (Sites 1 and 2). When comparing the ARP of *E. coli* isolates from the different sites, it was observed that Site 4 had a different pattern.

Table 4: Antibiotic resistance patterns of enteric bacteria isolated from the five sampling sites between February 2001 and February 2002.

Antibiotic	A	P	T	S	CXM	NA	RIP	KF	TS	C	CTX	FOX	NO
Total	43.6	72.6	9.4	16.2	5.1	6.8	67.0	26.6	10.3	4.3	1.7	6.0	52.1
E. coil	46.5	69.8	11.6	18.6	2.3	2.3	67.4	27.9	11.6	4.7	0.0	7.0	39.5
Others	40.6	75.4	7.2	15.9	7.2	10.1	66.7	26.1	8.7	4.4	2.9	4.3	58.0
Site I	38.1	85.7	14.3	14.3	9.5	4.7	66.7	28.6	4.8	0.0	4.8	4.8	52.4
E. coli	55.6	88.9	11.1	22.2	0.0	0.0	55.6	33.3	0.0	0.0	0.0	11.0	44.4
Others	25.0	83.3	16.7	8.3	16.7	8.3	75.0	25.0	8.3	0.0	8.3	0.0	58.3
Site 2	42.3	65.4	11.5	11.5	3.9	3.9	61.5	26.9	11.5	3.9	3.9	3.9	34.6
E. coli	54.5	63.6	18.2	9.1	0.0	0.0	63.6	0.0	18.2	0.0	0.0	0.0	27.3
Others	33.3	66.7	6.7	13.5	6.7	6.7	60.0	46.7	6.7	6.7	6.7	6.7	40.0
Site 3	62.5	81.3	12.5	31.3	6.3	0.0	81.3	37.5	16.7	6.3	0.0	6.3	76.5
E. coli	60.0	100	0.0	60.0	0.0	0.0	80.0	80.0	0.0	0.0	0.0	20.0	80.0
Others	66.7	75.0	16.7	25.0	8.3	0.0	83.3	33.3	25.0	8.3	0.0	0.0	75.0
Site 4	40.9	63.6	4.5	18.2	4.5	9.1	77.3	13.6	9.1	4.5	0.0	9.1	63.3
E. coli	42.9	57.1	14.3	28.6	14.3	0.0	85.7	28.6	28.6	14.3	0.0	0.0	42.9
Others	40.0	66.7	0.0	13.3	0.0	13.3	73.3	6.7	0.0	0.0	0.0	13.3	73.3
Site 5	42.1	73.7	0.0	15.8	0.0	10.5	78.9	36.8	10.5	10.5	0.0	5.3	68.4
E. coli	20.0	40.0	0.0	0.0	0.0	0.0	80.0	40.0	0.0	20.0	0.0	0.0	40.0
Others	50.0	85.7	0.0	21.4	0.0	14.3	78.6	35.7	14.3	7.1	0.0	7.1	78.6

Key: A, ampicillin; P, penicillin; T, tetracycline; S, streptomycin; CXM, cefuroxime; NA, nalidixic acid; RIP; rifampicin; KF, cephalothin; TS, co-trimoxazole; NO, novobiocin; C, chloramphenicol; CTX, cefotaxime; FOX, cefoxitin.

3.3.3. A comparison of the resistance pattern of the *E.coli* isolates and members of other genera of enteric bacteria

The antibiotic resistance patterns of different genera of *Enterobacteriaceae* isolated from the Mhlathuze River are shown in Table 6. *S. marcesens* isolates showed significantly higher resistance against tetracycline, streptomycin, cefuroxime, cephalothin, novobiocin, chloramphenicol and 100% resistance against rifampicin. Isolates of *Enterobacter* spp. were mainly resistant to nalidixic acid and cefotaxime, but usually showed resistance against most of the other antibiotics tested.

Table 6: Antibiotic resistance patterns of each *Enterobacteriaceae* genus isolated from the Mhlathuze River from February 2001 to January 2002.

Antibiotic	A	P	T	S	CXM	NA	RIP	KF	TS	C	CTX	FOX	NO
E.coli	46.5	69.8	11.6	18.6	2.3	2.3	67.4	27.9	11.6	4.7	0.0	7.0	39.5
Klebsiella spp	47.8	70.0	0.0	8.7	4.3	8.7	73.9	21.7	4.3	0.0	4.3	0.0	60.9
C. freundii	33.3	88.9	3.7	18.5	0.0	11.1	70.4	25.9	11.1	3.7	0.0	11.1	66.7
Enterobacter spp	41.7	58.3	16.7	0.0	16.7	16.7	25.0	0.0	8.3	8.3	8.3	0.0	25.0
S.marcesens	42.9	71.4	28.6	57.1	28.6	0.0	100	85.7	14.3	14.3	0.0	0.0	71.4

Key: A, ampicillin; P, penicillin; T, tetracycline; S, streptomycin; CXM, cefuroxime; NA, nalidixic acid; RIP; rifampicin; KF, cephalothin; TS, co-trimoxazole; NO, novobiocin; C, chloramphenicol; CTX, cefotaxime; FOX, cefoxitin.

3.3.4. Comparison of Antibiotic Resistance patterns of *Enterobacteriaceae* isolates from the Mhlathuze River and those of clinical isolates from diarrhea patients in this area.

When antibiotic resistance patterns of the isolates from this study were compared to those obtained from clinical isolates of diarrhea patients from a previous study (Biyela, 2000), there was a strong correlation in resistance patterns from the two studies (r=0.97) Table 7. Although the clinical isolates showed high level of resistance as was expected, the environmental isolates in this study had higher percentages of resistance when compared with results from similar studies (Goni-Urriza *et al.*, 2000; Harwood *et al.*, 2000).

Table 7: Comparison of Antibiotic Resistance patterns of environmental *Enterobacteriaceae* isolates from the Mhlathuze River and of clinical isolates from the diarrhoea patients in this area

Antibiotic R	A	P	T	S	CXM	NA	C	CTX	CIP	GTA
Environmental isolates (total)	43.6	72.6	9.4	16.2	5.1	6.8	4.3	1.7	0.0	0.0
E. coli (enviro)	46.5	69.8	11.6	18.6	2.3	2.3	4.7	0.0	0.0	0.0
Clinical isolates*	75.0	80.0	17.0	16.9	16.9	18.3	4.2	1.4	2.8	1.4

Key: A, ampicillin; P, penicillin; T, tetracycline; S, streptomycin; CXM, cefuroxime; NA, nalidixic acid; GTA, gentamicin; C, chloramphenicol; CTX, cefotaxime, CIP, ciprofloxacine.

3.4. Discussion

The emergence of antibiotic resistance has become a significant public health problem, since eradication of resistance is not as simple as its development. The assumption is that the use of any antibiotic whether for therapeutic or non-therapeutic purposes will create selective pressure. Whenever antibiotics end up in the environment, for example in water bodies, it gradually leads to the development of organisms with resistance genes and consequently, the extinction of the sensitive species. This then leads to clinical failure when the antibiotics are used for therapeutic purposes.

From the point of view of a researcher interested in pure drug resistance, *in vitro* tests avoid many of the confounding factors, which influence other tests by removing bacteria and placing them into a controlled experimental environment. However, the test has certain significant disadvantages. The correlation between *in vitro* response and clinical response in patients is neither clear nor consistent, and the correlation appears to depend on the level of acquired immunity within the population being tested. The removal of antibiotics from therapy might seem to be the easy way out, a pool of antibiotic resistance genes and antibiotic resistant bacteria already exists.

Antibiotic resistance genes do not only spread among bacteria involved in the infection being treated. Commensal bacteria have a role in the continuance of antibiotic resistance as reservoirs, results from this study show that there is a similarity in ARPs of commensal and pathogenic bacteria. This indicates the possibility of the exchange and/or sharing of resistance genes most probably in the environment.

The Mhlathuze catchment sustains major human, agricultural and industrial activities in the coastal region (Steyl *et al.*, 2000). Without proper infrastructure in the form of quality potable water and sanitary systems, the rural communities that depend on the river for domestic purposes are thus vulnerable to water-borne diseases. Diarrhea outbreaks, which are strongly correlated with the water quality and sanitary systems, continue to pose a public health risk in the communities of Mhlathuze's many reserves. This problem is further compounded by the increasing incidence of pathogens with antibiotic resistance (Ogan and Nwiika 1993; DePaola *et al.*, 1995). Heavy use of antibiotics for medical and veterinary purposes (Balagué and García Véscovi, 2001, White *et al.*, 2000) as well as industrial effluents, pesticides and related compounds (Balagué and García Véscovi, 2001) and heavy metal pollution, accelerate the level of antibiotic contamination of the natural environment and consequent development of resistance in communities of non-disease organisms. These organisms could create a widespread antibiotic resistance pool, from which resistance could be transferred back into human and animal pathogenic organisms (Kruse and Sorum 1994).

The results from our water quality monitoring (Chapter 2) indicate that there is a high level of fecal contamination in this water system especially at Felixton (Site 5), which embraces higher human and industrial activities. Several heavy metals have been detected in this water system, antibiotic resistance was also a common occurrence among the enteric bacteria isolated from the studied area (Lin, 2003). Widespread antibiotic resistance across all the sites, for both *E.coli* and other genera indicates that there is indeed a pool of resistance in the environment. The strong correlation (r=0.97) between

ARPs of the clinical and the environmental isolates in this region suggest a strong link between the diarrhea incidences and the water quality in the region. With poor sanitary facilities and the lack of portable water, the high levels of multiple antibiotic resistance amongst the isolated enteric bacteria is a major cause for concern which expectedly will lead to extremely high economic burden on disease management and local economy (Lo Presti *et al.*, 2000; White *et al.*, 2000).

Several mechanisms render antibiotics inactive. One of these is the beta-lactamase hydrolysis of the beta-lactam antibiotics. Clinical studies demonstrate that resistance predicted by beta-lactamase is a critical issue. Microbiological surveillance data provided by the Nosocomial Infections Surveillance System (N.I.S.S) in the United States, showed that resistance in many Gram negative bacteria is caused by the beta-lactamase production (Thornsberry, 1995). Also many *Enterobacteriaceae* can be induced to produce beta-lactamase by exposure to broad spectrum of cephalosporins. High levels of resistance shown by the isolates to penicillin and ampicillin indicate that the beta-lactamase gene is widely available in the environment.

Several reports have demonstrated that the presence of heavy metals and industrial effluents is associated with increased antibiotic resistance among bacterial communities (McArthur and Tuckfield, 2000; Nies, 1999). The presence of several heavy metals has been detected in sites 3 and 5. Richard Bay Estuary (site 3) is located near aluminium smelters and fertilizer manufacturing factories. The enteric bacteria isolated from Richard Bay Estuary (site 3) showed high resistance against several antibiotics compared to those

from other Sites. And site 5 (Felixton) contains a sugar mill and paper pulping factories as well as other human activities. Microbes isolated from this site showed high resistance to chloramphenicol. Dlangubo (site 2), which is away from the industrial region and mainly for agricultural developments, showed the lowest level of antibiotic resistance. These observations suggest that industrial and/or human activities play a prominent role and have an impact on the level of antibiotic resistance in the environment. This finding is supported by other studies that have reported the presence of high numbers of antibiotic resistant microbes in industrially polluted waters (Nies, 1999). A study by Davidson (1999) reported that water and soil ecosystems which are not in direct proximity of the rhizosphere have thecharacteristics of being oligotrophic, such that bacteria are in a state of semipermanent starvation. *In situ* conjugation frequencies increase upon addition of a carbon source, e.g., in activated sludge water treatment plants. Gene transfer frequencies have also been reported to have a linear relationship with water temperature in rivers (Davidson, 1999).

3.5. Conclusion

Levels of antibiotic resistance observed in this study were high. Multiple resistance was also found to be very common among the isolates. These results indicate that industrial and human activities might contribute to this trend. The antibiotic resistance gene pool especially β-lactamase should be widely available in the environment. Microbes with heavy metal resistance have also been isolated. These isolates also showed high levels of antibiotic resistance. It is thus imperative that the determination of antibiotic susceptibility/resistance patterns of isolated microbes be part of the microbial monitoring

process. The observations should be closely correlated with disease management practices and other possible factors that may contribute to antibiotic resistance (Harwood *et al.*, 2000).

CHAPTER FOUR.

THE MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE GENES

4.1. GENERAL OVERVIEW

The wide and often indiscriminate use of antibiotics as well as the use of pesticides in agriculture and related compounds has resulted in the establishment of a pool of antibiotic resistance in the environment. Water bodies are major receptacles of these pollutants. This may provide the opportunity for exposure of naïve populations of bacteria to antibiotic resistance genes and the opportunity for the transfer of these genes back into human and animal pathogens, which subsequently generates significant health and economic impacts (Colloquium Report to the American Academy of Microbiology, 1999).

Aquatic ecosystems harbor a large pool of antibiotic resistance genes. In some instances environmental bacteria have been reported to harbor resistance genes that are of clinical importance. This is partly due to the fact that bacteria are able to exchange antibiotic resistance genes and their vectors, that is plasmids and transposons within aquatic habitats. The fact that antibiotic resistance genes can be transferred and therefore, spread among microorganisms in the aquatic environment and even from pathogenic bacteria to environmental bacteria within aquatic systems necessitates the need to include antibiotic resistance assaying in water quality monitoring studies. Ash *et al.*, (2002) showed that several rivers in the United States have become major reservoirs of antibiotics and

antibiotic resistant microbes. The ability of bacteria to develop multiple drug resistance is due in part to their ability to acquire new antibiotic resistance genes on mobile genetic elements, especially integrons. Integrons are either located on plasmids or transposons. *Enterobacteriaceae* often carry integrons, encoding resistance to various antibacterial compounds. Integrons are mobile DNA elements able to incorporate single or groups of antibiotic resistance genes by site-specific recombination within and between bacterial species (Bunny *et al.*, 1995; Darlsgaard *et al.*, 1999). A large number of antibiotic resistance genes conferring resistance to aminoglycosides; chloramphenicol; beta-lactams and trimethoprim have been found in integrons (Bunny *et al.*, 1995; Darlsgaard *et al.*, 1999). These genes are contained in individual mobile units, called cassettes, that can be inserted into and excised from an integron by site specific recombination (Bunny *et al.*, 1995).

Enterobacteriaceae often carry integrons that are grouped into classes 1, 2 and 3. Integrons most commonly associated with plasmids and transposons from clinical antibiotic resistant isolates are associated with the *sul*1 and the *qac*E genes which code for resistance to sulfonamides and quartenary ammonium compounds, respectively. Such integrons are known as class1 integrons (Bunny *et al.*, 1995). Class1 integrons encode resistance to several antibacterial compounds and have been extensively studied in *Enterobacteriaceae* (Dalsgaard *et al.*, 1999). Class 1 integrons commonly possess two conserved regions, located on either side of the integrated gene cassette(s). The 5′-conserved segment (5′-CS) includes a gene, *intI*1, encoding the intergrase, *attI*1; the cassette intergration site and the promoters that are responsible for expression of cassette

genes (Figure 11). The 3′-conserved segment (3′-CS) includes an open reading frame of unknown function; the *qacE*1 and *sul*1 genes (Darlsgaard *et al.*, 1999; Naas *et al.*, 1999). The presence of integrons among clinical bacterial isolates may account for multiple drug resistance and continued resistance to antibiotics that have been withdrawn from use in medicine. Even with the rotation of antibiotics, antibiotic resistance may persist due to the linkage of some antibiotic and heavy metal resistance genes provided for their perpetuation as the selection pressures change (Bass *et al.*, 1999).

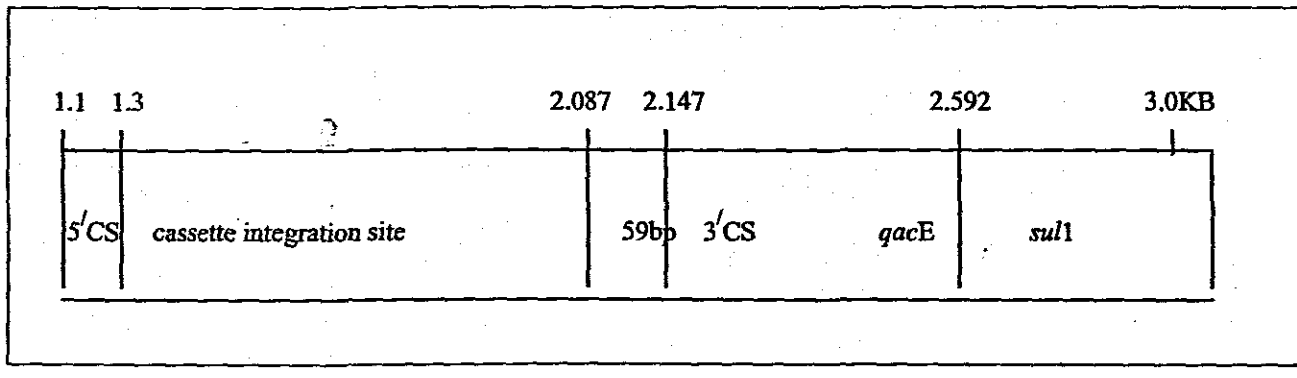


Figure 11: A schematic representation of a class1 integron (modified from Dalsgaard *et al.*, 1999). 5′CS and 3′CS represent the 5′ and the 3′ conserved segments of the integron; *qac*E and *sul*1 encode resistance to disinfectants and sulfonamides respectively (Dalsgaard *et al.*, 1999).

A different type of integron has been reported by Rechia and Hall (1995). It is a putative but defective integron1. The integrase gene (*intI2*) is interrupted by a termination codon, whose product is 40% identical to that of *intI1*. This gene was found to be located 5′ to the first cassette at the left end of the Tn7 and Tn2 transposons. This integron and its relatives form the second class of integrons. Class3 integrons are those in which the *intI*1 gene is located 5′ to the bla_{imp} cassette which was first found in a class1 integron. The

putative integron integrase (*intI3*) is 61% identical to the *intI*1 integrase. However, a study by Rechia and Hall (1995) reported that only one protein, the integron encoded integrase intl1 is required for the movement of gene cassettes (Bass *et al.*, 1999).

Given the ability of *Enterobacteriaceae* and clinically important bacteria to acquire antimicrobial resistance, consistent surveillance of the agents commonly prescribed to treat infections arising from these organisms is imperative (Sahm *et al.*, 2000). Obtaining information on the incidences of antibiotic resistance is the first step in surveillance studies. Nevertheless the development of genotyping tools for detecting and tracking antibiotic resistance genes in commensal and pathogenic bacteria, as well as in the environment is needed for understanding the ecology of antibiotic resistance. Such information is indispensable if the problem of antibiotic resistance is to be tackled (Aminov *et al.*, 2001).

While culturing studies are necessary to assess microbial physiology, they are poor indicators of microbial genetic diversity (Edwards *et al.*, 1999). One technique that is renowned for allowing insight into phylogenetic relationships of organisms at strain and isolate level is Enterobacterial Repetitive Integenic Consensus (ERIC) profiling. ERIC is used to yield strain specific genomic fingerprints, and had been used in studies to determine the relationship between antibiotic resistance patterns and genetic diversity of the isolates (Navarro *et al.*, 2001). The purpose of this part of the study was to characterize antibiotic resistance in *Enterobacteriaceae* isolates on a molecular basis and also to determine the relationship between antibiotic resistance patterns and molecular

types of isolates belonging to the same species. Molecular tests use polymerase chain reaction (PCR) to indicate the presence of genes encoding resistance to antibiotics. Theoretically, the frequency of occurrence of specific gene mutations within a sample of bacteria obtained from patients from a given area could provide an indication of the frequency of antibiotic resistance in that area analogous to information derived from *in vitro* methods. Advantages include the need for only small amounts of genetic material as opposed to live bacteria, independence from host and environmental factors, and the ability to conduct large numbers of tests in a relatively short period of time. Even though the confirmation of the association between given mutations and actual drug resistance is difficult, especially when resistance involved more than one gene loci and multiple mutations. If these complexities can be resolved, molecular characterization can become an extremely valuable surveillance tool for monitoring the occurrence, spread or intensification of antibiotic resistance

4.2. METHODS

4.2.1. DNA Extraction

4.2.1.1. Total DNA extraction

Total DNA extraction of all the isolated microorganisms was carried out using the thermolysis method (Sandvang *et al.,* 1997). One bacterial colony was suspended in 1.0 ml of phosphate buffered saline and centrifuged at 9000g. The pellet was resuspended in 100µl of TE buffer and boiled at 100°C for 10 min. The boiled suspension was stored at -20°C. For each PCR reaction, 2 µl of the lysed suspension was used.

4.2.1.2. Plasmid DNA extraction

Plasmid DNA extractions were carried out using the GFX™ Micro plasmid Preparation Kit (Roche). The purified plasmid was re-suspended in 50 µl TE buffer and incubated overnight at – 4°C before use. The extracted plasmid DNAs were analyzed on a 0.7 % agarose gel run in 1X TAE buffer for 40 minutes at 70 volts. The gel was stained with ethidium bromide and visualized under UV light.

4.2.2. Enterobacterial Repetitive Intergenic Consensus (ERIC) profiles of the *Enterobacteriaceae* isolates.

Total DNA templates were used for the ERIC PCR's. The reaction mixture was as follows: PCR master mix, 12.5µl; *Taq* polymerase, 1µl; ERIC 2B primer (100pmol), 1µl; DNA, 2µl and PCR water, 8.5µl. The mixture was spinned down and covered with mineral oil, then cycled in a Gene tech, thermocycler SPCR1 MK II (Stuart Scientific, UK). The PCR reaction conditions were as follows: 94 °C for 240s, 30° C for 60s and 74 °C for 60s, followed by 35 cycles of 94°C for 60s, 30°C for 60s, 74 °C for 180s and finally 94°C for 60s, 30°C for 60s and 74°C for 480s (Navarro *et al.*, 2000). The PCR products were electrophoresized on 1% agarose gel, run in 1XTAE buffer for three hours at 80 volts (Navarro *et al.*, 2001).

4.2.3. Detection of the class1 integron Gene Cassette using the polymerase chain reaction (PCR)

A total of 114 bacterial isolates belonging to the *Enterobacteriaceae* family and several *Psedomonas/Aeromonas* spp were tested for their resistance/susceptibility patterns to a

battery of 15 antibiotics, which are used as first line drugs in state hospitals and clinics. Total DNA templates of the multiple resistant isolates were used for integron detection. First a combination of primers, int1F and *sul*1B was used for the detection of the entire integron i.e. including the *sul*1 and *qac*E genes. All isolates that yielded a product with this set of primers were amplified with int1F and int1B, to amplify the variable region of the integron. The PCR conditions for all integron experiments were as follows: 94 °C for 5 min, followed by 30 cycles of 94°C for 1 min, 65°C for 60 s and 72°C for 2 min and finally for 5 min at 72°C. The reactions were briefly held at 4°C. The PCR conditions were optimized to detect amplicons of between 0.7 and 2.0 kilobases in size.

Table 8: The DNA sequences of the primers used in this study

Primer	Sequence	Reference
INT1 F[a] (5CS)	5′-GGC ATC CAA GCA GCA AGC –3′	Dalsgaard *et al.*, 1999
INT1 B[a] (3CS)	5′-AAG CAG ACT TGA CCT GAT -3′	Dalsgaard *et al.*, 1999
QAC E1 F	5′-ATC GCA ATA GTT GGC GAA GT-3′	Dalsgaard *et al.*, 1999
QACE1 B	5′-CAA GCT TTT GCC CAT GAA GC-3′	Dalsgaard *et al.*, 1999
SUL1 B	5′-GCA AGG CGG AAA CCC GCG CC-3′	Dalsgaard *et al.*, 1999
SUL1 F	5′-CCT CGA TGA GAG CCG GCG GC-3′	Dalsgaard *et al.*, 1999
ANT(3′) B	5′-ATT GCC CAG TCG GCA GCG-3′	Dalsgaard *et al.*, 1999
ANT(3′) F	5′-GTG GAT GGC GGC CTG AAG CC-3′	Dalsgaard *et al.*, 1999
PSE1 F	5′-CGC TTC CCG TTA ACA AGT AC-3′	Naas *et al.*, 1999
PSE1 B	5′-CTG GTT CAT TTC AGA TAG GC-3′	Naas *et al.*, 1999
ERIC 2B	5′ AAG TAA GTG ACT GGG GTG AGC G-3′	Navarro *et al.*, 2001

4.2.3.1. Detection of antibiotic resistance genes

Isolates that yielded a PCR product with the general class1 primers (*int*1) were purified using High Pure PCR Product Purification Kit (Roche Diagnostics, Germany). The purified products were amplified with primers for specific antibiotic resistance genes (*pse*1, *ant3'*, *sul*1& *qacE*). All PCR products were analyzed on 1% agarose gels. The gels were stained with ethidium bromide and visualized under UV light. A molecular weight marker (PCR product molecular weight marker, Sigma) was also loaded on the gel for band size determination. Products of interest were cut for sequencing.

4.2.4. Conjugation Experiments

Conjugation was carried out for 16 isolates representative of the different antibiotic resistance patterns (10 *E. coli*; 3 *Klebsiella* spp.; 2 *C. freundii* and 1 *S. marcesens*). Depending on the resistance pattern of the isolates, two recipient strains were used, *E. coli* HB101, containing the streptomycin resistance gene and the *E.coli* CSH56, containing the nalidixic acid resistance gene in their chromosome.

Broth cultures of the recipient and of the isolates used for mating experiments were grown for 16hours at 37°C, and mixed in a 1:5 (Donor: Recipient) ratio. The cultures were precipitated by centrifugation and the pellet was re-suspended in 50μl of nutrient broth. The suspensions were spotted onto non-selective nutrient agar plates and incubated overnight. The mating aggregates were scraped from the agar plates and suspended in test tubes with 5ml normal saline. The tubes were vortexed for one minute to disrupt the mating pairs, and 1ml of the suspension was precipitated again and washed 3 times in normal saline. The resultant cells were plated onto media with the antibiotics to select for

the donors and transconjugants. Antibiotic sensitivity or resistance patterns of the transconjugants were determined and compared to those of the donors (environmental isolates) prior to conjugation. Plasmid DNA extractions were carried out for the transconjugants (see 4.2.1.2. for procedure).

Table 9: Antibiotics and concentrations used for conjugation experiments

Antibiotic	**Stock concentration**	**Final concentration**
Ampicillin	100mg/ml	100μg/ml
Streptomycin	50mg/ml	50μg/ml
Nalidixic acid	25mg/ml	25μg/ml

4.3. RESULTS

4.3.1. ERIC profiles of some of the MAR isolates

The ERIC profiles of some of the *E. coli* and *C. freundii* with multiple antibiotic resistance are shown in Figure 6. The results obtained showed a degree of similarity between the resistance phenotypes and molecular types of the strains used for ERIC analysis especially for the *C.freundii* isolates (Figure 12B). Isolates, 4/031201; 5/031201; 3/210102 and 7/141101 (lanes, 1; 2; 6 and 12) each yielded two bands when amplified with the ERIC 2B primer. 4/120301, and 5/031201 were both resistant to penicillin, streptomycin, nalidixic acid, rifampicin and novobiocin, on top of the shared resistance 4/120301, was resistant to ampicillin and 5/031201, to cefotaxime and cephalothin. 3/ 210201 and 4/031201, shared 3 resistance determinants, ampicillin;

penicillin and rifampicin, 3/ 210201, was also resistant to tetracycline. 3/ 210201, and 7/141101 both were resistant to ampicillin, penicillin and co-trimoxazole, 7/141101, was also resistant to cephalothin.

Isolates 8/141101 and 2/101001 (lanes 7 & 8) yielded two bands of equal size and they were both resistant to ampicillin, penicillin, rifampicin and novobiocin. Isolates 10/260301 and 6/031201 (lanes, 9 &10) each yielded three bands two of which were common among these isolates, and both of them were resistant to penicillin, rifampicin and novobiocin. 6/031201, was also resistant to streptomycin and 10/260301, was resistant to cephalothin.

The relationship between molecular types and ARPs was also observed with the *E.coli* isolates (Figure. 12A). Isolates, 15/141101 and 15/141101 (lanes, 2 and 5) had similar ARPs and yielded band patterns that had 50 % similarity. Isolates, 9/031201 and 12/031201 (lanes 4 and 5) had ARPs that were 66% similar and their band patterns were also 66% similar.

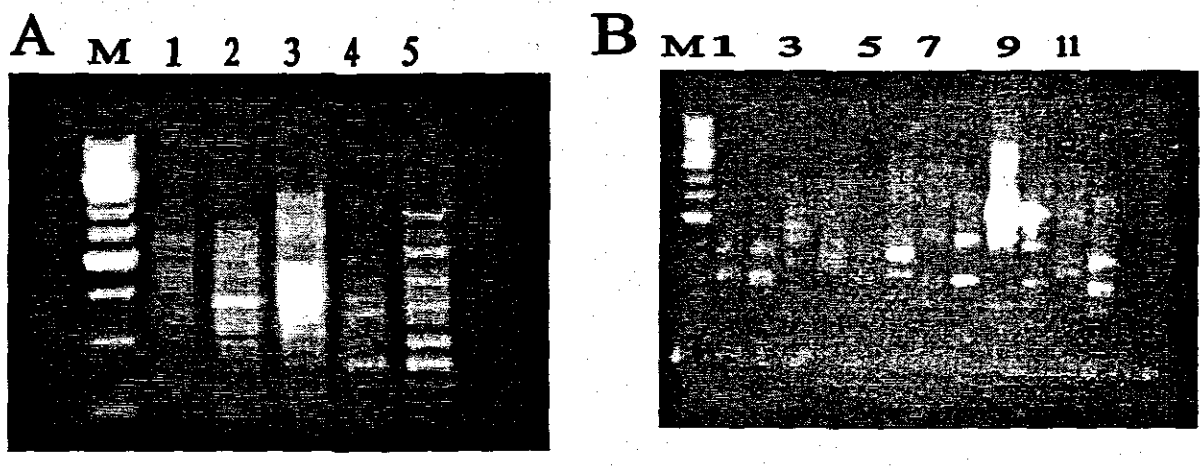


Figure 12: Examples of ERIC profiles of (A) *E. coli* (1: isolate 6/181601; 2: 15/141101; 3: 9/101001; 4: 9/031201 and 5: 12/031201) and (B) *C. freundii* (1: 4/031201; 2: 5/031201; 3: 3/101001; 4: 8/241001; 5: 2/180601; 6: 3/210102; 7: 8/141101; 8: 2/101001; 9: 10/260301; 10: 6/031201; 11: 3/010801; 12: 7/141001) with multiple antibiotic resistance isolated from the Mhlathuze catchment. M: molecular weight marker XVI (Roche molecular Biochemicals).

4.3.2. Detection of Integron bound antibiotic resistance genes

Of the 43 *Enterobacteriaceae* isolates that were used for integron detection experiments, 25 yielded PCR products with the general integron 1 primer set (Table 10). When amplified with primers for the entire integron genome i.e. including the associated genes (primers: int1F & sul1B), PCR products ranged between 0.70 and 2.0 KB (Figures 8A-8C). Some isolates possessed more than one copy of the integrons, of different sizes (Figure 13A & 13C). When primers specific for the variable region of the integrons (int1F & int1B) were used, products obtained ranged between 0.7 and 1.0kb (Figures 13D & 13E).

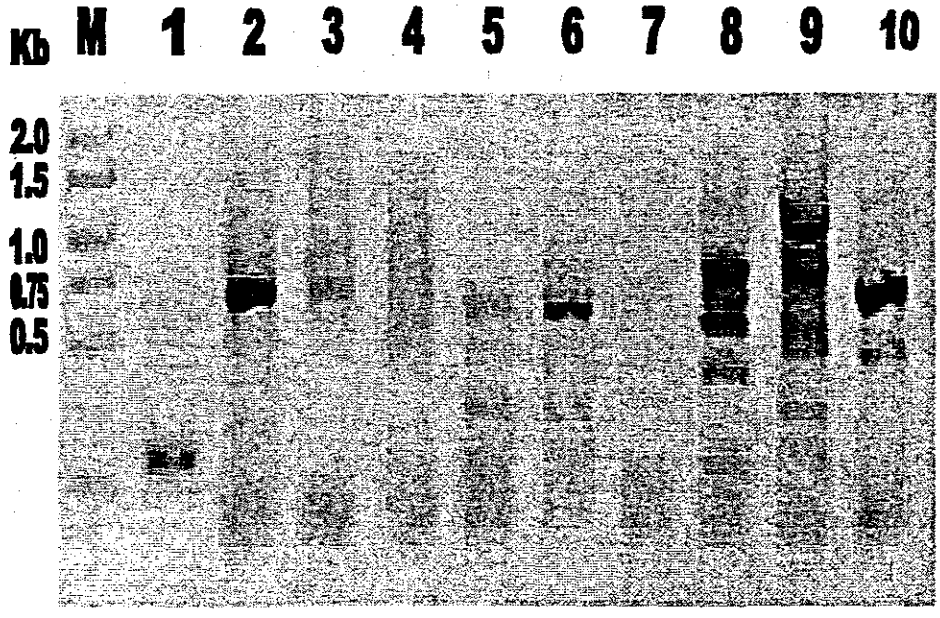


Figure 13A: Amplification of the entire integron. M, molecular weight marker, 1,9/210102; 2, 3/010801; 3, 11/031201; 4, 6/010801; 5,10/210102; 6, 8/241001; 7, 12/031201; 8, 2/101001; 9, 3/101001; 10, 4/110901.

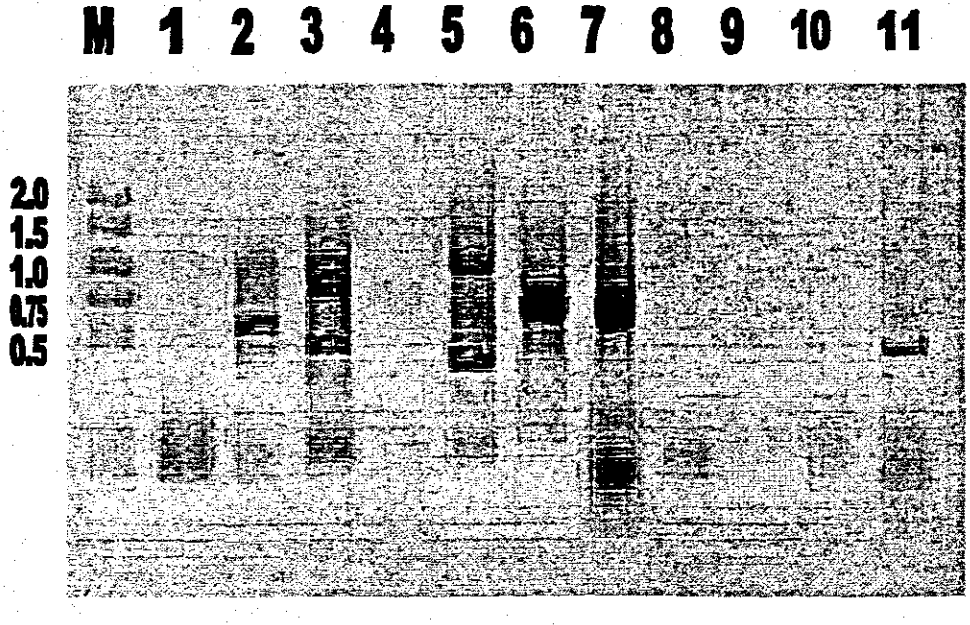


Figure 13B: Amplification of the entire integron. M, molecular weight marker; 1, 6/180601; 2,4/050601; 3, 180601; 4; 1/010801; 5, 3/210102; 6, 3/180601; 7, 14/031201; 8, 2/260301; 9, 6/090401; 10, 1/241001; 11, 8/141101.

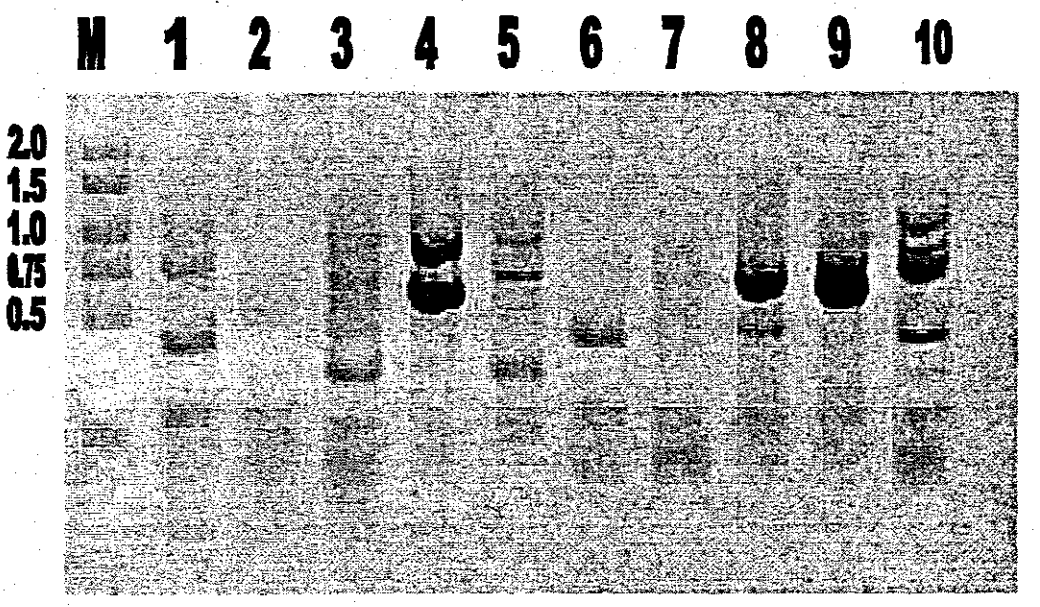


Figure 13C: Amplification of the entire integron. M, molecular weight marker; 1,7/141101; 2, 1/090401; 3, 1/260301; 4, 15/031201; 5,15/141101; 6,6/031201; 7, 8/260301; 8, 9/101001; 9, 6/230401; 10, 5/031201.

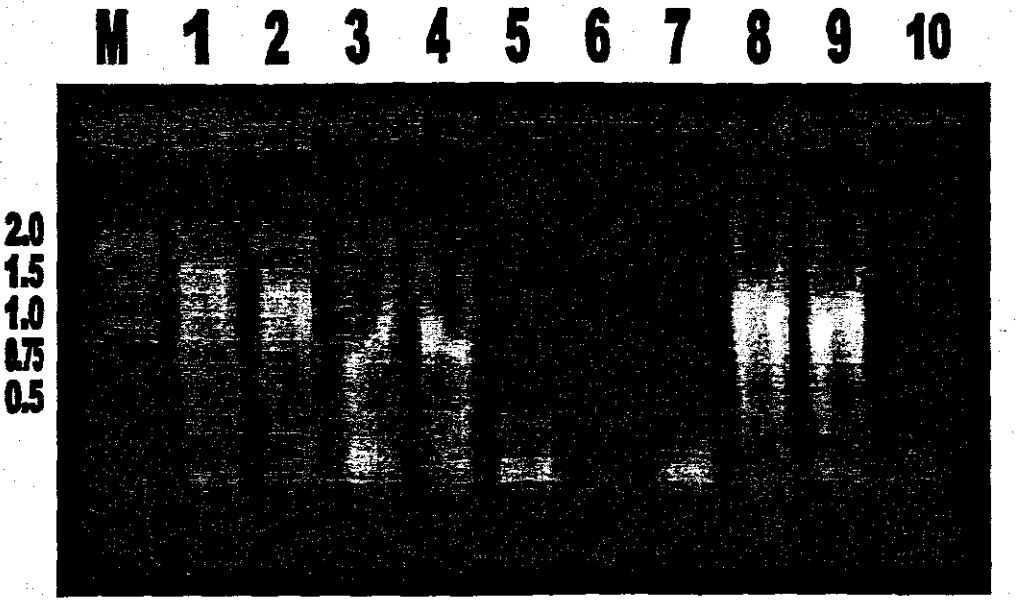
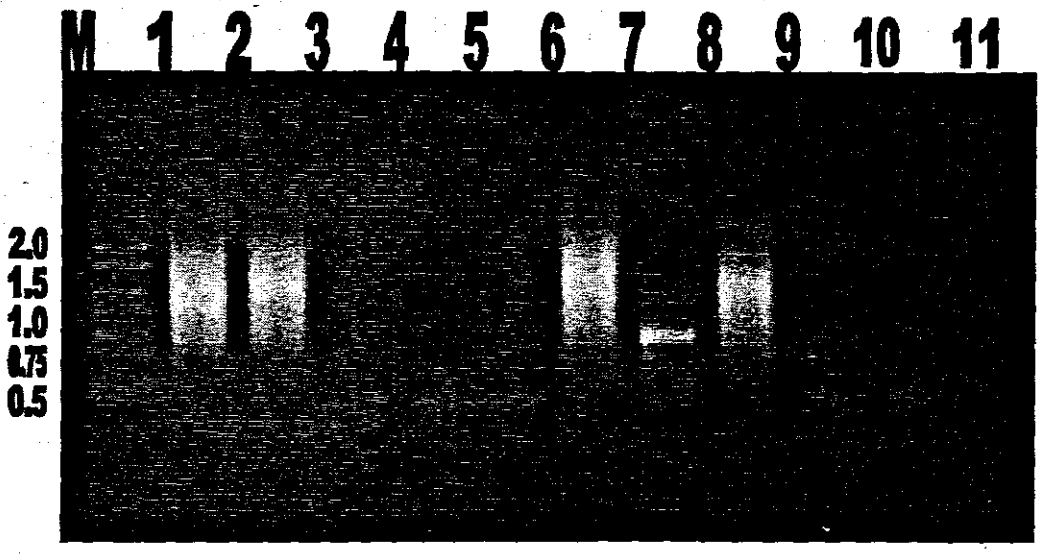


Figure 13D: Amplification of the variable region of the integron. M, PCR product molecular weight marker, 1, 6/230401; 2, 3/180601; 3, 140301; 4, 15/031201; 5, 8/241001; 6, 6/031201; 7, 8/260301; 8, 10/210102; 9, 9/101001.



Figures 13E: Amplification of the variable region of the integron. M, molecular weight marker (Sigma); 1, 4/050601; 2, 8/141101; 3, 2/101001; 4, 3/210102; 5, 7141101; 6, 5/031201; 7, 2/180601; 8, 15/14101; 9, 11/031201; 10, 4/110901.

These PCR products were purified and used to quantify the integrons by amplifying with different sets of diagnostic primers. The beta-lactamase gene (*pse*) was the most widespread, among the isolates studied, it was present in 44% of the twenty five integrons. The aminoglycoside resistance gene (*ant*3′) was present in 16%. The two genes that are associated with class 1 integrons *sul*1, coding for sulfonamide resistance and the *qacE*1, coding for resistance to quartenary ammonium compounds were detected in 24% of the integrons (Table 10).

Table10: Results of the detection of intergron1 and associated antibiotic resistance genes.

Isolate (# of R determinants)	Int1f & sul1b	Int1f & Int1B	Int1f & pseb	Int1f & ant'3	Sul1f & Qace1b
E.coli					
6/1806 (8)	+	+	+	-	-
9/0312(6)	+	+	+	-	-
10/210102(4)	+	+	-	-	-
12/0312(4)	+	+	-	-	-
9/1010(4)	+	+	-	-	-
11/0312(4)	+	+	-	+	+
15/1411(4)	+	+	-	+	+
Klebsiella spp.					
15/0312(5)	+	+	-	-	-
4/1109(4)	+	+	-	-	-
3/1806(4)	+	+	+	-	-
14/0312(4)	+	+	-	-	-
6/2304(3)	+	+	+	-	-
S.marcesens					
9/2603(8)	+	+	+	-	-
Enterobacter spp.					
1/2603(4)	+	+	-	-	+
8/2603(3)	+	+	-	-	-
C.freundii					
4/0312 (7)	+	+	+	-	-
8/2410(6)	+	+	-	-	-
3/210102(5)	+	+	+	-	-
5/0312(5)	+	+	+	+	-
2/1806(5)	+	+	+	+	+
3/1010(5)	+	+	+	-	+
3/0108(4)	+	+	-	-	-
2/1010(4)	+	+	-	-	-
8/1411(4)	+	+	-	-	+
6/0312(4)	+	+	+	-	-

Key: Int1f & sul1b, amplify the complete integron together with the associated genes; int1f &int1l, amplify the variable region of the integron; int1f &pseb amplify the gene that codes for resistance to beta-lactam antibiotics;int1f &ant3', amplify the gene that codes for aminoglycoside resistance; *qacEf* &sul1b, amplify the two genes sequences that are associated with class 1 integrons i.e. the quartenary ammonium compound that code for resistance to disinfectants and the sulfonamide resistance gene; +, PCR product; -, no PCR product.

4.3.3. Bacterial conjugation

The conjugation experiments were successful in 9 isolates, *E.coli* (6), Klebsiella spp. (2) and *C. freundii* (1) (Table 11). Antibiotic resistance patterns of the transconjugants were similar to those of the donors (environmental isolates) except for *E. coli* isolate 12/031201. This *E. coli* isolate developed resistance to nalidixic acid after the conjugation process. The sizes of plasmids, were about 10 KB.

Table 11: The horizontal transfer of plasmids from the environmental isolates to the recipient cells through the conjugation processes.

Recipient: CSH56	Selection for donors (NA+Nal)	Selection of transconjugants (Na+Amp+Nal)
E. coli 6/180601	+	+
E. coli 5/260301	+	-
E. coli 9/031201	+	-
E. coli 4/120301	+	-
E. coli 9/210102	+	+
S. marcesens 1/241001	+	-
Klebsiella spp 15/031201	+	-
Recipient: HB101	Selection for donors (NA+Sm)	Selection for transconjugants (NA+Amp+Sm)
E. coli 4/031202	+	-
*Klebsiella spp.*4/110901	+	+
*Klebsiella spp.*1/010801	+	+
E. coli 1/260201	+	-
E. coli 9/260201	+	+
E. coli 12/031201	+	+
15/141101	+	-
C. freundii 4/230401	+	+
E. coli 11/031201	+	+
E. coli 8/210102	+	+
C. freundii 5/031201	+	-

Key: NA, nutrient agar; Nal, nalidixic acid; Amp, ampicillin; Sm, streptomycin; -, no growth observed; +, growth observed.

4.4. DISCUSSION

Most of the microorganisms isolated from the Mhlathuze River belonged to the genera of coliform bacteria commonly found in water and soil (Lin, 2003). These microorganisms are mostly involved in the recycling of carbon, nitrogen and sulfur compounds. *Enterobacteriaceae*, *Pseudomonas* spp. and *Aeromonas hydrophila* are commonly isolated from water samples (Goni-Urriza *et al.*, 2000). *Aeromonas hydrophila*, is associated with gastroenteritis and *Pseudomonas aeroginosa,* on the other hand, affects patients that are immuno-compromised and ones with metabolic and hematological disorders (Pearson *et al.*, 2000). The presence of the opportunistic pathogens such as those mentioned above and *Klebsiella* spp. may have serious health hazard for the people that drink the water, especially young children, the elderly and those infected with HIV/AIDS.

The results of the Enterobacterial Repetitive Intergenic Consensus (ERIC) profiling of some *E. coli* (Figure 12A), *C.freundii* (Figure 12B) and *Klebsiella* spp. isolates showed different profiles among isolates of the same species, an indication of the genetic diversity of enteric bacterial species in the river. Although there was a relationship between antibiotic resistant patterns and genetic diversity of some of the isolates studied, one cannot overlook the differences in some of these cases. This then suggests that there is both vertical and horizontal transfer of the antibiotic resistance genes.

Among the 25 isolates with positive detection of class1 integrons, the beta-lactamase gene (*pse*) was the most widespread, it was present in 44% of these integrons. All the integrons in which this gene was detected were from isolates that had shown phenotypic

resistance to beta-lactam antibiotics. However, these isolates did not represent all the isolates that were resistant to the beta-lactam antibiotics. The aminoglycoside resistance gene (*ant3′*) was detected in 16% of the integrons. However, the detection of the aminoglcoside resistance gene (ant3′), growth observed.

did not correspond with the phenotypic detection of resistance to the aminoglcoside antibiotics used in this study (gentamicin and streptomycin). Although six of the twenty-five isolates that possesed the integrons were from isolates that had phenotypic resistance to streptomycin. This could mean either that the gene(s) coding for streptomycin resistance in these cases were not *ant*3-associated or that the genes were not part of the integrons. The two genes that are associated with class 1 integrons *sul*1, coding for sulfonamide resistance and the *qacE*1, coding for resistance to quartenary ammonium compounds were detected in 24% of the integrons. Cases of class1 integrons that lack the 3′CS have also been reported by Naas and co-workers (1999). The lack of the 3′ conserved segment in the integron results in the absence of the *qacE* and the *sul1* genes. In none of the cases studied did the integron bound antibiotic resistance genes account for the total phenotypic resistance of the isolates. Park *et al.* (2003) suggested that in the absence of sustained antibiotic pressures, such as in the natural environment, coliform bacteria might carry empty or non-functional class1 integrons.

In the science of antibiotic resistance, the mutational rate is frequently defined as *in vitro* frequency at which defective mutants arise in a bacterial population in the presence of a given antibiotic concentration. Therefore, phenotypic data only reports the selectively favorable mutations. The problem is complicated by the fact that the phenotype does not

always reflect the same genotype in all "selected" mutants, because mutations in different genes can produce similar antibiotic resistance phenotypes. The level of expression of antibiotic resistance genes in integron associated cassettes is affected by a number of factors, for example cassette position. A study by Collis and Hall (1995) reported that the expression of the genes was greatest when the cassette was promoter proximal and was reduced by the presence of preceding cassettes. This is because the efficiency of translation could be affected by the presence of upstream cassettes, loss of upstream cassettes led to an increase in the resistance conferred by the gene. Another factor that might influence the level of expression of antibiotic resistance genes is plasmid copy number. On top of transcription and translation initiation signals in genes associated with integrons, all these factors play a major role, not only in the maintenance but also in the spread of antibiotic resistance between environmental and clinical bacteria.

The results of this study showed that there exists a pool of antibiotic resistance genes within this water body. The difference in integron sizes, random distribution of the integrons across different genera and the degree of genetic diversity among same species indicates the possibility of convergent acquisition of resistance determinants by genetically unrelated strains of bacteria (Navarro *et al.*, 2001).

With the capability of spreading among different bacterial species mobile genetic elements like integrons and plasmids have the capacity to disseminate antibiotic resistance within bacteria in the aquatic environment. In this study conjugation was

successful in 56% of the cases studied. The antibiotic resistance profiles of transconjugants were similar to the ones of the donors. Interestingly, one of the transconjugants induced the nalidixic acid resistance after conjugation. Evidence suggests that the three main mechanisms of gene transfer, namely transformation, transduction and conjugation occur naturally in aquatic environments. (Madigan *et al.*, 2003) Aquatic environments are reservoirs of a large pool of bacterial DNA. Extracellular DNA can be derived from dead and viable cells. Many viable cells release DNA without cell lysis, suggesting that release may be a normal function of bacteria. In the aquatic environment percolation of water, flows of air, water or dust may move free or bound DNA. Half-lives of free DNA are longer in aquatic environments (0.017-235 hours) than in terrestrial environments (9.1-28.2 hours) (Yin and Stotzky, 1997). In the natural environment there might be a lot of stress for example the presence of antibiotics and or heavy metals, decreased nutrient levels and the struggle for existence among microbes. All these factors are capable of inducing a physiological state of competence in bacterial cells 'enabling' them to actively take up DNA from the environment.

4.5. CONCLUSION

From the results of this study one can conclude that there exists a large pool of antibiotic resistance genes within the Mhlathuze River. More worrisome than the phenotypic measurements of antibiotic resistance is the ability of commensal organisms to carry resistance genes of clinical importance and their ability to transfer such genes to other bacteria, as evidenced by results of this study. This suggests the possibility of transmission of resistance genes between bacteria in the environment, which might mean

gene exchange between commensal and pathogenic bacteria and also between animal and human pathogens. The difference in ERIC II patterns among isolates of the same species indicates the possibility of the horizontal transfer of genes in the environment rather than vertical dissemination of single clone(s) of antibiotic resistance gene(s) (Navarro *et al.*, 2001).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

More than half of surface freshwater is in the four largest lakes. Though these huge lacustrine sources dwarf the innumerable smaller lakes, springs and rivers of the earth, human habitation depends more on these widely distributed smaller sources. Which in total still far exceed the needs of man and the animal and plant kingdoms. Despite this, severe local problems can arise due to prolonged drought, the pollution of surface waters or the extension of settlements into more arid regions. Indeed, droughts have been endemic since ancient times, and even pollution of local sources has been a cause for concern and the subject of legislation since at least 1847 in the United Kingdom (Hyues, 1973). In developed countries, the quality of water supplies and amenities is rising steadily in most communities. On the other hand, the provision of portable drinking water for everyone continues to be one of the major challenges facing developing countries.

In South Africa the major problem to supplying high quality water is fecal contamination together with the associated human and animal enteric pathogens. A lot of water quality studies done in the past concentrated on the detection of pollution indicator microorganisms. With the advancement in molecular biology, molecular techniques can be used to determine microbial contamination. This ensures that even organisms present in the viable but non-culturable state in the environment are detected. However, for effective disease management, the prediction of potential sources of high risk bacterial contamination could be more useful than the detection of general bacterial communities.

Some studies have looked at the determination of antibiotic resistance profiles of the isolated indicator organisms.

Although the microbial contamination of the Mhlathuze River have been studied in the past (Bezuidenhout *et al.*, 2001; Bezuidenhout *et al.*, 2002), none of these studies determined the antibiotic resistance profiles of the isolated organisms. This is necessary not only to safeguard the health of people that depend on water from this river, but also to monitor it so that it will not become a reservoir of antibiotic resistant bacteria and antibiotic resistance. For example *E.coli*, an enteric organism routinely used as an indicator organism, was shown in the results of this study (Chapter 2) to be widely distributed in the environment. For this reason its significance in water quality monitoring becomes questionable . Nevertheless, multiple antibiotic indexing of *E.coli* has been used to differentiate *E.coli* of high-risk origin (human, poultry, swine) from those of other sources. The ability to identify high-risk sources of contamination in water could be useful in establishing standards to facilitate detection of sources of contamination (Kasper *et al.*, 1990).

The results of this study showed that commensal organisms are reservoirs of antibiotic resistance genes, most of which are of clinical importance. This problem is aggravated by the presence of some of these resistance genes on mobile genetic elements in particular integrons. There is no limit to the number of resistance gene cassettes that can integrate to an integron's gene cassette. The development of multiple drug resistance creates a serious problem in the therapy of infectious diseases. Multiple drug resistance is a form of

infective heredity. With the ability to spread among different bacteria and to integrate several resistance gene cassettes, integrons have the capacity to disseminate multiple drug resistance within bacteria in the environment.

But even more of a concern than the ability of environmental microbes to acquire resistance genes is their ability to exchange such genes within the environment. This shows that there is the possibility for the exchange of resistance genes between commensal and pathogenic organisms in the Mhlathuze River. Some of the isolated organisms harbored integrons, capable of incorporating multiple antibiotic resistance genes to their cassette integration sites. And since integrons are carried on mobile genetic elements, these resistance genes can be easily exchanged among bacteria in the environment. Such elements promote not only the spread of resistance genes but also their mantainence even with the discontinuation of antibiotic use in therapy.

From the results of this study one can conclude that water from the Mhlathuze River does not only pose a health risk due to its high level of fecal and total coliform contamination. This River also serves as a reservoir for antibiotic resistant bacteria and antibiotic resistance genes capable of transmitting among bacterial cells in the environment when the opportunity presents itself. The Mhlathuze River does not only pose a risk of infection by pathogenic organisms, but that of the transmission of antibiotic resistance and in particular multiple drug resistance elements. This calls for studies (not only in this river) that would determine the extent to which transmission of antibiotic resistance

bacteria from humans to animals occurs and the extent to which such transfer impacts the efficacy of antibiotic use in human medicine.

Future studies should include the following:

1. Looking at the use of ARP in the determination and if possible the tracking of the high-risk sources of bacterial contamination.
2. The mechanisms of gene transfer among bacteria within the aquatic environment.
3. Determination of where gene reservoirs are developed, maintained and amplified.
4. Role of commensal organisms as reservoirs of resistance genes of clinical importance.
5. Role of the environment in the spread of antibiotic resistance.

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Appendix A

A1. Reagents

Hydrogen sulfide production test

Materials

Peptone – iron agar tubes

Procedure

Inoculate a colony of the test culture into a tube of peptone – iron agar by making stab inoculation using a transfer needle. Incubate at 35 ^{O}C for 7 days.

Results- blackening along the line of inoculation is positive.

Indole production test

Materials

Tubes of tryptone broth

Kovac's reagent

Procedure

Inoculate a tube of tryptone broth with a test culture. Incubate at 35 ^{O}C for 48 hours. Add l ml kovac's reagent and allow to stand, for the reagent to rise to the top.

Results

Positive-A bright red color which develops in the reagent layer.

Methyl- red and the Vokes- Paskeur tests (MRVP)

Materials

Medium- 7.0g peptone; 5g each of glucose and pottassium in a liter of distilled water. Dispense 10ml into test tubes and autoclave.

Procedure

Inoculate 10ml of the MRVP medium with 2 loopfuls of a 4-6 hour old, peptone water culture of the test organism. Incubate for 3 days at 30 ^{O}C. Divide into 2 equal parts.

MR- to one part add 5 drops of 0,04% methyl red solution.

VP- to the other part add a trace of creatinine and 5ml of 40% pottassium hydroxide and shake well.

Results

MR- positive: magenta

Negative: yellow

VP- positive: pink

Negative: no color change

A2. Buffers and solutions

10x TAE buffer (total volume=1L)

Tris base	48.4g
Glacial acetic acid	11.42ml
0.5M EDTA	20ml (pH 8.0)

Distilled water 968.58ml

To prepare 1X, dilute in distilled water in a ratio 1: 9

TE buffer (500ml)

10mM tris pH 8.0 (0.605g)

1mM EDTA pH 8.0 (0.186g)

Dissolve in distilled water to make a final volume of 500ml. Sterilize and keep refrigerated until use.

Glucose Buffer (1L)

25mM Tris pH 8.0

50mM glucose

10mM EDTA

02M NaOH with 1% SDS (fresh every time)

10%SDS 5ml

10N NaOH 1ml

Water 44ml

Chloroform isoamyl 24:1 solution

Chloroform 24ml

Amylalcohol 1ml

Close with 10ml TE buffer

Keep refrigerated

Proteinase K

10mg/ml TE buffer

Lysozyme

10*ug*/ml TE buffer

PCR master mix (Roche)

Taq DNA polymerase 25U in 20mM Tris-HCl

100mM KCl

3mM $MgCl_2$

35, 0.01 % (v/v), dNTP mix (dATP, dCTP, dGTP, dTTP each 0.4 mM)

Final pH 8.3 (Kept at -20^0C)

Primers

All primers resuspended according to manufactures instructions, and used in 100pmol concentration.

Appendix B

B1. Antibiotic resistance patterns of the isolated microbes

	Isolate number	AP	PG	TET	CHL	SM	CXM	CTX	CIP	GM	NAL	FOX	RIP	KF	TS	NO	Site	IDENTIFICATIC
9	/12/03	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	*Enterobacter spp.*
1	/26/03	0	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	*Enterobacter spp.*
11	/26/03	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	*E.coli*
7	/23/04	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	*E.coli*
8	/07/05	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	*Klebsiella spp.*
7	/21/05	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	*Enterobacter spp.*
5	/05/06	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	*Klebsiella spp.*
4	/18/06	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	*Enterobacter spp.*
5	/09/07	1	1	0	0	1	0	0	0	0	0	0	1	1	0	1	1	*E.coli*
8	/14/08	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	*E.coli*
13	/10/10	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	*E.coli*
1	/11/09	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	*C.freundii*
9	/09/07	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	*S.marcesens*
1	/24/10	1	1	1	0	1	1	0	0	0	0	0	1	1	0	1	1	*S.marcesens*
8	/1109	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	*Pseudo/Aeromon*
4	/14/11	0	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	*Pseudo/Aeromon*
5	/14/11	1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	*Pseudo/Aeromon*
8	/14/11	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	*c.freundii*
12	/14/11	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	*Pseudo/Aeromon*
14	/14/11	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	*E.coli*
8	/o3/12	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	*S.marcesens*
10	/o3/12	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	*E.coli*
17	/o3/12	1	0	1	0	1	1	0	0	0	1	1	1	1	1	1	1	*P. auriginosa*
9	/21/1/02	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	*E.coli*
10	/21/1/02	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	*E.coli*
3	/12/03	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2	*S.marcesens*
6	/26/03	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	2	*Pseudomonas sp*
8	/26/03	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	2	*Enterobacter spp.*
3	/09/04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	*E.coli*
4	/09/04	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	2	*C.freundii*
7	/09/04	1	1	0	0	0	1	1	0	0	0	0	0	1	0	0	2	*Klebsiella spp.*
5	/23/04	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	2	*E.coli*
5	/21/05	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	2	*E.coli*
4	/05/06	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	2	*C.freundii*
8	/05/06	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	2	*E.coli*
2	/18/06	0	1	0	0	0	0	0	0	0	0	1	1	1	0	1	2	*C.freundii*
8	/18/06	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	*E.coli*
4	/01/08	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	2	*E.coli*
14	/14/08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	*E.coli*
3	/11/09	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	*E.coil*
6	/09/07	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	*Klebsiella spp.*
	Isolate number	AP	PG	TET	CHL	SM	CXM	CTX	CIP	GM	NAL	FOX	RIP	KF	TS	NO	Site	IDENTIFICATIC

11	/14/08	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	2	*Klebsiella spp.*
10	/11/09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	*Klebsiella spp.*
11	/24/10	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	*Enterobacter sp.*
2	/11/09	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	2	*C.freundii*
2	/09/07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	*Aeromonas spp*
2	/14/08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	*/Aeromonas spp*
5	/14/08	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	2	*Pseudo.monas sp*
11	/11/09	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	2	*Pseudo/Aero*
3	/14/11	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	2	*Pseudo/Aero*
6	/14/11	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	*Enterobactersp*
15	/14/11	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	2	*E.coli*
5	/03/12	0	1	0	0	1	0	0	0	0	1	0	1	1	0	1	2	*C.freundii*
12	/03/12	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	2	*E.coli*
18	/03/12	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	2	*P. auriginosa*
5	/21/1/02	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	2	*C.freundii*
8	/21/1/02	1	1	0	0	0	0	0	0	0	0	0	1	0	1	0	2	*E.coli*
11	/21/1/02	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2	*Klebsiella spp.*
2	/12/03	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3	*C.freundii*
8	/12/03	1	0	0	0	1	1	0	0	0	1	0	1	0	0	0	3	*Proteus spp.*
2	/07/05	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	*E.coli*
4	/21/05	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	3	*Klebsiella spp.*
7	/05/06	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	3	*Proteus spp.*
7	/18/06	0	1	0	0	0	0	0	0	0	0	0	0	0	0		3	*Proteus spp.*
4	/09/07	0	1	0	0	1	0	0	0	0	0	0	1	1	0	1	3	*E.coli*
8	/28/08	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3	*E.coli*
7	/11/o9	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	3	*Klebsiella spp.*
18	/14/08	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	3	*Enterobacter sp*
8	/24/10	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	3	*C.freundii*
2	/24/10	1	1	1	0	1	1	0	0	0	0	0	1	1	0	1	3	*S.marcesens*
7	/09/07	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	3	*Pseudo./ Aero.*
13	/14/11	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	3	*Klebsiella spp.*
6	/03/12	0	1	0	0	1	0	0	0	0	0	0	1	0	0	1	3	*C.freundii*
9	/03/12	1	1	0	0	0	0	0	0	0	0	1	1	1	0	1	3	*E.coli*
13	/03/12	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	3	*Kklebsiella spp.*
3	/21/1/02	1	0	1	1	0	0	0	0	0	0	0	1	0	1	0	3	*C.freundii*
7	/12/03	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	4	*E.coli*
6	/09/04	0	1	0	0	1	1	0	0	0	0	0	1	1	0	0	4	*E.coli*
4	/23/04	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	4	*C.freundii*
6	/23/04	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	4	*Klebsiella spp.*
3	/07/05	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	4	*Klebsiella spp.*
6	/21/05	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	4	*E.coli*
3	/05/06	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	4	*Klebsiella spp.*
6	/18/06	1	1	1	1	1	0	0	0	0	0	0	1	0	1	1	4	*E.coli*
13	/14/08				0	0	0	0	0	0	0	0	1	0	0	0	4	*E.coli*
16	/14/08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	*E.coli*
9	/10/l0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	4	*E.coli*
	Isolate number	AP	PG	TET	CHL	SM	CXM	CTX	CIP	GM	NAL	FOX	RIP	KF	TS	NO	Site	IDENTIFICATIC

1	/01/08	1	0	0	0	0	0	0	0	0	1		1	0	0	1	4	*Klebsiella spp.*
6	/14/08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	*Klebsiella spp.*
10	/14/08	0	0	0	0	0	0	0	0		0	0	0	0	0	0	4	*Enterobacter sp*
3	/09/07	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	4	*C.freundii*
9	/14/08	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	4	*C.freundii*
1	/10/l0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	4	*C.freundii*
2	/10/l0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	4	*c.freundii*
3	/10/l0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	4	*C.freundii*
1	/09/07	0	1	0	0	1	0	0	0	0	0	0	1	1	0	1	4	*S.marcesens*
5	/01/08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	*Pseudo./Aeromor.*
17	/14/08				0	0	0	0	0	0	0	0	0	0	0	1	4	*Psudo/Aero*
11	/14/11	1	1	0	0	0	1	0	0	0	0	1	0	0	0	1	4	*Pseudo/Aero*
7	/o3/12	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	4	*C.freundii*
14	/o3/12	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	4	*Kklebsiella spp.*
5	/12/03	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	5	*Aeromonas spp.*
6	/12/03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	*Enterobacter spp.*
2	/26/03	1	1	0	1	0	0	0	0	0	0	0	1	1	0	1	5	*E.coli*
4	/26/03	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	5	*E.coli*
7	/26/03	1	1	0	0	0	0	1	0	0	0	0	1	0	1	0	5	*Aeromonas spp.*
9	/26/03	1	1	0	1	1	0	0	0	0	0	0	1	1	1	1	5	*Serratia spp.*
10	/26/03	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	5	*C.freundii*
5	/09/04	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	5	*Klebsiella spp.*
1	/07/05	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	5	*C.freundii*
3	/18/06	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	5	*Klebsiella spp.*
2	/01/08	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	5	*E.coli*
15	/14/08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	*E.coli*
6	/11/o9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	5	*E.coli*
6	/01/08	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	5	*Klebsiella spp.*
4	/11/o9	0	1	0	0	0	0	0	0	0	1	0	1	0	0	1	5	*klebsiella spp.*
3	/01/08	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	5	*C.freundii*
7	/24/10	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	5	*C.freundii*
3	/14/08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	5	*Pseudo./Aero.*