MOLECULAR CHARACTERIZATION OF AEROMONAS HYDROPHILA AND ANTIMICROBIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS AGAINST PATHOGENIC ISOLATES FROM WATER AND STOOL SAMPLES IN THE ERA OF HIV/AIDS IN LIMPOPO PROVINCE, SOUTH AFRICA

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mm	Milimiter
µl	Microlitre
µg	Microgram
°C	Degree celcius
%	Percentage
AIDS	Acquired immune deficiency syndrome
AMK	Amikacin
AMP	Ampicillin
AFLP	Amplified fragment length polymorphism
AST	Antimicrobial susceptibility testing
ATCC	American type culture collection
AZT	Aztreonam
Bp	Based pair
BA	Blood agar
BHI	Brain heart infusion
BPW	Buffered peptone water
Bfp	Bundle-forming pili
CF	Cefazolin
CFP	Cefepime
CTX-M	Cefotaximase
CFT	Cefotaxime
CTZ	Ceftazidime
CTX	Ceftriaxone
CPM	Cefuroxime
CPN	Cephalothin
CphA	Cephalosporinase A
CIP	Ciprofloxacin

CA	Clavulanic Acid
CFU/mL	Colony forming units per millilier
CMT	Complex mutants of TEM
CS	Conserved segment
CLED	Cysteine Lactose Electrolytes Deficient
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ERT	Ertapenem
ESBLs	Extended-spectrum ß-lactamases
GCAT	Glycerophospholipid cholesterol acyltransferase
GES	Guiana extended spectrum
GM	Gentamicin
GNB	Gram-negative bacilli
HCL	Hydrochlroric acid
HGs	Hybridization groups
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
IMI	Imipenem
Kda	Kilodaltons
LCR	Ligase chain reaction
LTD	Limited
MBC	Minimum Bactericidal Concentrations
MDR	Multiple drug resistance
MEM	Meropenem
MIC	Minimum inhibitory contraction
MIN	Minutes

MLST	Multilocus sequence typing
Mm	Milimolar
N-CO	Nitrogen-carboxyl
NCCLS	National Committee for Clinical Laboratory Standards
N	Number
ND	Not Done
NI	Nitrofurantoin
O	Somatic antigen
Omps	Outer membrane proteins
OXA	Oxacillinase
PRL	Piperacillin
PIP	Pipemidic acid
PBP	Penicillin bending protein
PCR	Polymerase chain reaction
PER	Beta-lactamse named after (P.Nordmann, E .Ronco,R.Labia)
PFGE	Pulsed-filed gel electrophoresis
PG	Prostaglandin
PNA	Peptide nucleic acid
RFLP	Restriction fragment length polymorphism
RPM	Revolutions per minute
RAPD	Random amplified polymorphic DNA
RFLP	PCR-Restriction fragment length polymorphism
Rrna	Ribosomal ribonucleic acid
SAS	Statistical analysis system
SDS	Sodium dodecyl sulfate
Sesbl	Stool extended spectrum beta-lactames
SHV	Sulphydryl variabr
SSCP	Single–strand conformational polymorphism

TCBS	Thiosulfate citrate bile salts sucrose
TE	Tris-EDTA
TEM	Temoniera
TN	Tobramycin
VEB	Vietnamese extended spectrum betalactames
WHO	World health organisation
XDCA	Xylose deoxycholate citrate agar

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DECLARATION

I, Ramalivhana Naledzani Jeffrey, hereby declare that the work on which this dissertation is based, is original (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or shall be submitted for another degree at this or any other university or higher education or examining body.

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DEDICATION

I dedicate this study to my son Dakalo Ramalivhana, my mother Vho-Elisa Mufanadza Thenga and my late father, Vho-Ganyani Ramalivhana ndiri fola khelo kha vhadahe vhasielisane na vhothe vho makhulukuku wanga na vhane ndasa vhadivhe ndiri ndaa!!

Summary

Aeromonas hydrophila is distributed widely in nature and is responsible for an array of human infections. Several studies on the isolation and characterisation of the organism abound. Although there are reports on the antibiotic resistance profiles of the organism, these reports have not been updated in Limpopo province, South Africa despite the established fact that antibiograms vary with time and geographical area. Antibiotic resistance and pathogenesis of an organism are dependent on a host of factors such as the production of extended spectrum beta-lactamases and the genetic profiles such as the genes coding for resistance and possession of integrons and how these characteristics overach with the phylogenetic inter-relatedness of isolates from different sources. In spite of the aforementioned concerns on the efficacy of antibiotics due to the acquisition or endowment of microorganisms with intrinsic and extrinsic factors , which enhances resistance to antibiotics , medicinal plants are reportedly offering promise as alternative sources of efficacious management of infections. Medicinal plants are employed by traditional healers in the management of infections in developing countries especially in Africa. However, the antimicrobial activities of medicinal plants against *Aeromonas hydrophila* have received only a cursory attention.

In an endeavour to undertake a comprehensive study on the isolation, characterisation, antibiograms, activities of medicinal plants as well as the genetic profiles, including phylogenetics relatedness of *Aeromonas* isolates from different sources, stool and water samples were collected over a two year period from designated places in Limpopo Province and analysed using standard techniques applicable to the constituent research activity. The research findings are presented in six chapters as presented hereunder.

The first chapter focussed on the literature review of the organism and reflects areas such as the morphology, laboratory diagnosis, clinical manifestations, pathogenesis, antimicrobial susceptibility profiles, antibacterial activities of medicinal plants as well as the genetic aspects of *Aeromonas hydrophila*.

In the second chapter, a total of 300 isolates of *Aeromonas hydrophila* isolated from water and stool samples were tested using the Vitek 2 system, disk diffusion, MicroScan Walkaway and E test for antimicrobial susceptibility testing. For the total of 34 antimicrobial tested, the MICs agreement was 99.7% for isolates from all sources. The aim of this study was to determine antimicrobial susceptibility testing of *Aeromonas hydrophila* isolated from Limpopo Province, South Africa by different methodology. Almost 100% of isolates were resistant to ampicillin using both two methods with the MIC ranging from 1 µg/ml to 64 µg/ml. Overall; the agreement of the AST results among all four methods for the drugs tested was (100%) Aminoglycosides, (100%) Carbapenems, (100%) Monobactams, 93% Cephalosporins and 89.4% Beta-lactam/ Beta-lactam inhibitors. Overall agreement between the disk diffusion, MicroScan Walkaway and Vitek methods was 98%, respectively. This study indicated that AST methods correlated with one another when testing Aeromonas hydrophila isolates, with a few exceptions. In general, discrepancies among the methods were due to isolates being interpreted as intermediately susceptible or due to an increased number of resistances detected with disk diffusion and a lower number with Vitek and MicroScan Walkaway.

In Chapter 3, a total of 230 isolates of *Aeromonas hydrophila* were screened for production of ESBLs by the double disk diffusion test and for AmpC production by assessing resistance to cefoxitin. *Bla*SHV, *bla*TEM, *bla*CTX-M, and *bla*CMY-2 were sought from all ESBL-positive and cefoxitin-resistant isolates. Only 21 isolates were found to be ESBL producers. All 21 isolates were screened for production of SHV, TEM and CMY-2 ESBL and, they were all (100%) found to be producers of TEM 63 and CMY-2.

Chapter 4 investigated the intergron gene production by ESBL producers. Results indicated that 5% of ESBL producing isolates carries integron class 1 enzyme which is located on the plasmids. In chapter 5, a total of 32 *Aeromonas* strains were analyzed by random amplified polymorphic DNA PCR (RAPD). The RAPD fingerprints obtained proved reproducible when repeated on three different occasions using whole-cell DNA isolated from the *Aeromonas* strains. In total, 12 unique RAPD fingerprints were found.

The results revealed a tendency of the isolates to cluster according to their origin of isolation (best-cut test 0.80 and bootstrap values >50%). However, a certain degree of similarity was also observed between isolates of water sources and clinical sources which indicated genetic relatedness. There were also genetic similarities between the clinical and the environmental strains of *Aeromonas* spp. isolated from different geographical areas.

The study has demonstrated the genetic relatedness of *Aeromonas hydrophila* isolates from household drinking-water and clinical sources in South Africa, which may be due to cross-contamination from water to patients or vice-versa. This observation is of public health significance, particularly in the era of HIV/AIDS. This study points to the importance of monitoring and evaluating infection-control measures for improved hygiene and to prevent cross- contaminations.

Chapter 6 investigated the antibacterial activities of *Pyrenacantha grandiflora* Baill and *Ficus sycomorus* against *Aeromonas hydrophila* in the era of HIV/AIDS. The ground plant materials were extracted with different extractants and screened for anti-microbial activity using the disk diffusion and micro-dilution techniques. Inoculated strains were serially diluted and plated at time intervals of 0, 2, 4, 6, 8 and 24h.

Results obtained showed that *F. sycomorus* extracts killed all the bacterial cells of *A. hydrophila* at a concentration of 3 mg/ml in 8h. A higher concentration of 6mg/ml had the same effect after 6h. Acetone extracts were most active for both plants with the MIC of 1.5mg/ml and MBC of 1.5mg/ml.

This study has revealed the strong in vitro activity of *Pyrenacantha grandiflora* Baill and *Ficus sycomorus* extracts against *Aeromonas hydrophila*. This study may mark a reference investigation on the activities of *Pyrenacantha grandiflora* Baill and *Ficus sycomorus* in the Venda region of South Africa against *Aeromonas hydrophila* and justifies their use by local traditional healers to treat gastrointestinal related infections. Chapter 7 deals with the general conclusions and recommendations of the study in a concise manner.

Published articles

- **J.N. Ramalivhana**, C.L. Obi, A. Samie, C. Labuschagne, and G.F. Weldhagen. Random Amplified Polymorphic DNA Typing of Clinical and Environmental *Aeromonas hydrophila* Strains from Limpopo Province, South Africa, 2010 Feb;28(1) Journal of Health and population pp. 606-0997

- **J. N. Ramalivhana,** C. L. Obi and S. R. Moyo**.** Antimicrobial susceptibility testing of *Aeromonas hydrophila* isolated from Limpopo Province, South Africa using VITEK 2 system, Micro Scan WalkAway, disk diffusion and E-test method: African Journal of Microbiology Research Vol. 3(12) pp. 903-913, Dec, 2009

- **J.N. Ramalivhana**, C. Labuschagne, C.L. Obi, G.F. Weldhagen. The integron deposited in the Genbank under accession number: EU328347.1 GI:163963004: 2008

- **J. N. Ramalivhana,** C. L. Obi and S. R. Moyo (2010). Prevalence of Extended-Spectrum *β*-Lactamases producing *Aeromonas hydrophila* isolated from stool sample collected in the Limpopo Province, South Africa: African Journal of Microbiological Research

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J.N. Ramalivhana, C. L. Obi and S. R. Moyo (2010) Incidence of Class 1 integrons isolated from Aeromonas hydrophila in Limpopo Province, South Africa.

Presentations at National and International Conferences

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and HIV Community Care: Perspectives from HAPG in Bela-Bela ", Aventura Spar, Bele-Bela, Limpopo Province, South Africa, 24 February 2009.

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Ramalivhana JN, Obi CL, Moyo S and Samie A (2009). The use of medicinal plants against *Aeromonas hydrophila* isolated from stool samples of HIV/AIDS patients. A paper presented at the Limpopo Provincial Research Day held at 25 November 20009

Chapter One: General Introduction and Literature review

1.1 Introduction and literature review

Members of the genus *Aeromonas* are facultative anaerobic, oxidase positive, Gram-negative bacteria whose natural habitat is in the aquatic environment. Some species are pathogenic for animals and humans (Martin-Carnahan and Joseph, 2005). The taxonomy of the genus *Aeromonas* has been confusing because of lack of congruity between phenotypic and genotypic characteristics of species and multiple methods are required for accurate classification. Currently, 17 DNA-DNA hybridization groups (HG) have been described and 14 phenospecies are described in the 2005 edition of Bergey's Manual (Martin-Carnahan and Joseph, 2005). Several additional *Aeromonas* species have been described recently, including *A. simiae*, *A. molluscorum* and *A. culicicola*. Other species that may be synonyms of recognized species include *A. ichthiosmia* and *A. enteropelogenes.*

1.2 Description of the Genus

Aeromonas species are autochthonous to the aqueous environment where they may be pathogenic for poikilotherms. They are found in soil, sediments, and water in freshwater, estuarine, and marine environments. They are present in foods, sewage, and biofilms of drinking water distribution systems. Aeromonads are found in the intestinal tract of animals and humans, with and without evidence of gastrointestinal disease. Some aeromonads are pathogenic for humans, and most human clinical isolates belong to HG-1, HG-4, HG-8, HG-9, HG-10, HG-12 or HG-14 (Janda and Abbott, 1998). HG-2, HG-3, HG-5, HG-6, HG-7, HG-11, HG-15, HG-16 and HG-17 are isolated from the environment or diseased animals, and they are not considered human pathogens (Janda *et al*, 1996). Pathogenic speciess causing human diseases are associated with a variety of infections such as septicemia, wound infections, meningitis, peritonitis, and hepatobilary infections. Some strains of *Aeromonas* produce enterotoxins responsible for

causing gastroenteritis in humans; however, isolation of aeromonads from feces does not indicate pathogenicity, since these bacteria are widely distributed throughout the environment in water and foods, especially during summer months (Janda and Abbott, 1998).

The genus *Aeromonas* consists of straight, coccobacillary to bacillary gram-negative bacteria with rounded ends measuring 0.3-1.0 x 1.0-3.5 Fm (Martin-Carnahan and Joseph, 2005). They occur singly, in pairs, and rarely as short chains. Motile strains produce a single polar flagellum, though peritrichous or lateral flagella may be formed on solid media by some species (Martin-Carnahan and Joseph, 2005). *Aeromonas* spp. is facultative anaerobic, catalase positive, oxidase positive, chemoorganotrophic bacteria that exhibit both oxidative and fermentative metabolism on carbohydrates (Obi *et al*., 2007). *Aeromonas* spp. produces a wide variety of extracellular hydrolytic enzymes such as arylamidases, amylase, deoxyribonuclease, esterases, peptidases, elastase, chitinase, and lipase (Carnahan *et al*., 1988). They grow optimally within a temperature range between 22 and 35° C, but growth occurs in a temperature range from 0 to 45° for some species (Mateos *et al*., 1993). Some species, including most nonmotile *A. salmonicida* strains, do not grow at 35° C (Martin-Carnahan and Joseph, 2005). They tolerate a pH range from 4.5 to 9.0, but the optimum pH range is from 5.5 to 9.0 (Isonhood and Drake, 2002), and optimum sodium chloride concentration range is from 0 to 4%.

Serotyping is based upon somatic (O) antigen determinants as described by Sakazaki and Shimada (1984). Several typing schemes have been proposed (Fricker 1987; Cheasty *et al*., 1988; Thomas *et al*., 1990), but only one comparison study of two of these schemes has been published (Shimada and Kosako, 1991). The scheme of Sakazaki and Shimada recognizes 44 serogroups, with an additional 52 provisional serogroups proposed by Albert *et al*. (1995). *Aeromonas* spp. are found to be serologically heterogeneous, with individual serogroups found in more than one species (Janda *et al*., 1996). . Three serotypes predominate in clinical specimens, O: 11 (24%), O: 16 (14%), and O: 34 (10%). Korbsrisate *et al*. (2002) characterized the distribution of *A. hydrophila* serogroups in

clinical specimens and developed polyclonal antibodies for rapid identification of clinical isolates by direct agglutination. Only 50% of strains fell into the common serogroups O: 11, O: 16, O: 18, O: 34, or O: 83. Rough strains (15.2%) and untypable strains (2.3%) reduced the effectiveness of serotyping for identification of clinical strains. A polyvalent antiserum was produced that resulted in positive agglutination of 102 or 105 strains, for a calculated sensitivity of 97.1% and specificity of 90.7%. This test could be useful in rapid identification of aeromonads to genus level where they are isolated from samples that may also contain vibrios.

Motile aeromonads are generally resistant to penicillin, ampicillin, carbenicillin and ticarcillin. They are typically susceptible to second and third generation cephalosporins, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, trimethoprim-sulfamethoxazole, and quinolones (Koehler and Ashdown, 1993; Janda and Abbott, 1998). Most aeromonads produce an inducible chromosomal β-lactamase (Walsh *et al.*, 1997). *A. trota* and up to 30% of *A. caviae* are susceptible to ampicillin. Antibiotic resistance to streptomycin, chloramphenicol, tetracycline, cephalexin, cefoxitin, erythromycin, furazolidone, and sulfathiazole is mediated by plasmids (Chaudhury *et al.*, 1996).

The genus *Aeromonas* is differentiated from *Plesiomonas* and *Vibrio* by its resistance to O/129 (150 Fg) and variable presence of ornithine decarboxylase (Martin-Carnahan and Joseph, 2005). Other key differential characteristics include its inability to grow in the presence of 6.5% sodium chloride, gelatin liquefaction, inability to ferment inositol, and a negative String Test. Additional useful but variable phenotypic characteristics include an inability to grow on thiosulfate citrate bile salts sucrose agar (TCBS), and ability of most but not all *Aeromonas* species to ferment D-mannitol and sucrose.

1.3 Occurrence

Aeromonads are found in foods, including fresh grocery produce, seafood, raw meats, packaged ready-to-eat meats, cheese, and milk (Palumbo, 1996). While *Aeromonas* spp. are not considered fecal bacteria, they are present in the feces of healthy animals and humans, presumably as the result of ingestion of food and water containing these organisms (Holmes *et al*., 1996; Demarta *et al*., 2000). They are present in high numbers in sewage before and after treatment (Monfort and Baleux, 1991), thus they have been proposed as an indicator of sewage-contaminated surface water. *Aeromonas* spp. may colonize drinking water distribution systems and produce biofilms that resist disinfection (Holmes *et al*., 1996; Bomo *et al*., 2004). Despite the presence of aeromonads in drinking water distribution systems, no point source outbreaks attributable to *Aeromonas* spp. have been reported from ingestion, inhalation, or dermal contact with drinking water.

1.4 Virulence associated and health effects in humans

Von Graevenitz and Mensch (1968) reviewed 30 cases of infection and established *Aeromonas* spp. as significant human pathogens causing a variety of extra-intestinal infections. Extra-intestinal and gastrointestinal infections are now known to occur in previously healthy hosts as well as immunocompromised or otherwise susceptible populations (Janda *et al*, 1996; Joseph, 1996; Janda and Abbott, 1998). Besides diarrheal illness, *Aeromonas* spp. cause wound infections, septicemia, meningitis, ophthalmitis and endocarditis. Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesins, agglutinins, and various hydrolytic enzymes (Janda *et al*, 1996). Virulence factors are present in two forms, cell-associated structures, and extracellular products. Among the cell-associated structures are pili, flagella, outer membrane proteins, lipopolysaccharide, and capsules. The major extracellular products include cytotoxic, cytolytic, hemolytic, and enterotoxic proteins. Aeromonads produce an array of filamentous structures, including short rigid, and long wavy pili, and polar

and lateral flagella. Short rigid pili are similar to those of *E. coli* type I and Pap pili (Ho *et al*., 1992), while long wavy pili belong to a class of type IV bundle-forming pili (Bfp) (Kirov and Sanderson, 1996). Removal of pili or neutralization of attachment sites by homologous antibody treatment impedes adherence to cell culture systems (Iwanaga and Hokama, 1992; Kirov *et al*., 1999). The type IV pili are controlled by the tapABCD gene cluster (Pepe *et al*., 1996). TapD encodes a Type IV peptidase/methyltransferase which is responsible for extracellular secretion of aerolysin and other enzymes via a Type II secretion system (Strom and Pepe, 1999), in addition to its tap pilin-related function. Barnett *et al*. (1999) cloned the tap cluster and demonstrated conclusively that Bfp and Tap pili were distinct families. Tap pili have a molecular mass of 17 kilodaltons (kDA), while Bfp pili have a molecular mass of 19-23 kDA. Tap pili are thought to facilitate attachment to enterocytes and enhance colonization. Bfp pili facilitate adherence to erythrocytes (Kirov *et al*., 2004). Polar flagella and lateral flagella were described by Rabaan *et al*. (2001) and Kirov *et al*. (2002). Polar flagellins function as adhesions, while lateral flagellins are thought to serve as colonization factors (Kirov *et al*., 2004).

Capsule production has been reported for *A. salmonicida* and *A. hydrophila* serogroups (Martinez *et al*., 1995), but the function of capsule material is vague. It is presumed to resist complement activity and perhaps enhance adherence (Kirov *et al*., 2004). S-layers (originally termed A-layer in *A. salmonicida*) are paracrystalline structures made up of identical protein subunits that are translocated across the cell membrane and assembled on the cell wall surface via an interaction with O-polysaccharide side chains of lipopolysaccharide. Stains producing S-layers are more pathogenic for fish, but the role of S-layer in human infection is not clear. Studies suggest that strains containing S-layers autoagglutinate (Kokka *et al*., 1991).

The hemolysin produced by some *Aeromonas* species is termed 'aerolysin', and it possesses both hemolytic and enterotoxic activity. This hemolytic enterotoxin (aerolysin), has been shown to share significant homology with the cytotoxic

enterotoxin (Act), and two cytotonic toxins (Alt and Ast) as reviewed by Xu *et al*. (1998). Early investigation of *Aeromonas* toxins was responsible for recognition of 'aerolysin' in several species of aeromonads; however, it appears that 'aerolysin' is really a family of toxins whose mechanisms of action are gradually being understood. The toxins reported by various investigators have fundamental differences in properties, making comparisons difficult (Buckley *et al*., 1981; Chakraborty *et al*., 1987). Act increased levels of tumor necrosis factor " (TNF-") and interleukin (IL-1) in macrophage cell lines and other inflammatory cytokines that result in tissue damage. Prostaglandin activity (PG1 or PG2) is increased. Aerolysin is released from cells as proaerolysin that is activated by proteolytic cleavage of a C-terminal peptide fragment of approximately 40 amino acids. Another 'aerolysin' has been described that is released by a type II secretion pathway under control of exeAB and exeC-N genes. The gene products produce a pore in the outer membrane protein for toxin secretion (Howard *et al*., 1996). This 'aerolysin' is a dimer with hemolytic activity for mouse erythrocytes. This toxin is involved in channel formation in target cells in a similar manner to the way it forms pores in bacterial outer membrane protein, by producing a heptamer that inserts into the cell membrane and forms a 1-2 nanometer (nm) channel which results in loss of cell permeability, cell leakage, and eventual cell destruction (Howard *et al*., 1996).

Aeromonas spp. produce a lipase, glycerophospholipid cholesterol acyltransferase (GCAT), that results in production of cholesteryl esters and phospholipase activity that digests plasma membranes of host cells (Buckley, 1983). Another class of enzymes, the metallo- and serine proteases are involved in toxin activation and have a protective role in inimical environments (Rodriguez *et al*., 1992). Other enzymes that contribute to virulence include amylase, chitinase, elastase, lecithinase, and nucleases (Gosling, 1996a).

Aeromonas salmonicida strains produce siderophores that facilitate iron acquisition (Chart and Trust, 1983). Some species of mesophilic aeromonads produce an enterobactin-like siderophore under iron-limiting conditions.

1.5 Health Effects in Animals

Aeromonads have been reported as pathogens of fish, amphibians, and reptiles (Gosling, 1996b). *Aeromonas* spp. cause hemorrhagic disease, ulcerative disease, furunculosis, and septicemia in fish (Austin and Adams, 1996). Aeromonads cause pneumonia, peritonitis, abortion and other diseases in birds and domestic animals (Gray, 1984).

1.6 Risk Assessment

Exposure to *Aeromonas* spp. through ingestion of food and water is continuous, and case reports suggest that susceptible individuals may acquire gastrointestinal illness from chronic exposure to high numbers of aeromonads in untreated water, and foodborne disease has been reported (Morgan *et al.*, 1985). The infectious dose by ingestion is remarkably high, based upon bacterial counts in foods implicated in disease. A human feeding study failed to induce illness in a significant percentage of volunteers (Morgan *et al.*, 1985), however, this study is considered inconclusive since the strain used was selected for the presence of only one virulence factor (enterotoxin) and the subjects were healthy adults. In the only risk assessment study for exposure via drinking water, Rusin *et al*. (1997) reported that the oral infectious dose exceeded $10log^{10}$ colony-forming units per milliliter (CFU/mL) and the risk of infections attributable to drinking water was 7.3 per billion exposures.

The use of water purification devices installed at the tap has become popular, yet devices employing activated carbon filtration may actually degrade microbial quality of drinking water. Chaidez and Gerba (2004) demonstrated that counts of *Pseudomonas* and *Aeromonas* were higher in water samples taken after filtration though point-of-use devices. Despite these increases in total bacterial load in drinking water, the levels remained substantially below the number required to cause gastrointestinal disease. Risk assessment studies have demonstrated an increased rate of transmission in daycare

centers in Ecuador (Sempertegui *et al.*, 1995), nursing homes in the U.S. (Bloom and Bottone, 1990; Sims *et al.*, 1995), and intensive care units in Italy (Torre *et al.*, 1996). Poor personal hygiene and reuse of water have been shown to increase the risk of transmission. The risk of *Aeromonas* infections is significant for animals in aquaculture, where crowding promotes transmission (Austin and Adams, 1996).

1.7 History of *Aeromonas* species

Bacteria resembling motile *Aeromonas* species were first isolated from water and diseased animals over 100 years ago. The history of their isolation and taxonomy was reviewed by Carnahan and Altwegg (1996) and Martin-Carnahan and Joseph (2005). Associations of aeromonads with human disease were reported by von Graevenitz and Mensch (1968) in a review of 30 cases of *Aeromonas* infection or colonization, providing evidence for their recognition as human pathogens and suggesting that some aeromonads may be associated with gastrointestinal disease. *Aeromonas* spp. are isolated most frequently from fecal specimens from children under five years of age, while isolation of aeromonads from other body sites typically occurred in adult populations. Aeromonads cause acute diarrheal disease of short duration or chronic loose stools in children, the elderly, or the immunocompromised, and they have been implicated as a cause of travelers' diarrhea. *Aeromonas* spp. cause cellulitis or wound infections following traumatic injury in an aqueous environment. They also cause septicemia associated with underlying disease such as cirrhosis, leukemia, cancer, and various infections associated with hospitalization such as rare urinary tract infections, surgical wound infections, meningitis, peritonitis, endocarditis, or other serious infections. Predisposing conditions for *Aeromonas* infection include cirrhosis or other hepatic disease, hematologic malignancies, hepatobiliary disease, diabetes, and renal disease. Use of medicinal leaches has resulted in infections in patients undergoing reconstructive breast surgery. The species distribution of *Aeromonas* infections is *A. hydrophila* (48%), with *Aeromonas sobria* and *Aeromonas caviae* equally distributed (25-27%).

An environmental source of *Aeromonas* implicated in gastrointestinal infection was first proposed by Holmberg *et al* 1986, who presented epidemiological evidence to support untreated well water as the source of infection in patients with diarrheal disease. *Aeromonas caviae* was present in 18 of 34 cases. The role of *A. caviae* as an agent of gastrointestinal disease remained controversial for the next decade until an overwhelming number of case reports overcame the last objections. Today, seven species of *Aeromonas* are recognized to cause a variety of intestinal and extra-intestinal infections in humans (Janda and Abbott, 1998). *Aeromonas* spp. have been identified as the cause of community acquired infection, nosocomial infection, and travelers' diarrhea (Rautelin *et al*., 1995). The intense interest in *Aeromonas* spp. in the 1980s lead to the search for improved culture methods for their recovery from water, food, clinical specimens, and especially feces, since the role of aeromonads as agents of gastrointestinal disease was controversial (Joseph, 1996). Isolation and enumeration methods for all sample types were reviewed by Moyer (1996), and phenotypic identification methods were reviewed by Millership (1996). Current identification methods for the recognized phenospecies are contained in the Second Edition of Bergey's Manual of Systematic Bacteriology (Martin-Carnahan and Joseph, 2005).

An understanding of the mechanisms by which aeromonads cause gastrointestinal disease has been frustrated by an inconclusive human feeding study (Morgan *et al*., 1985) and the lack of suitable animal model systems for demonstrating the Koch-Henle postulates (Kelleher and Kirov, 2000). Numerous virulence factors and toxins (Gosling 1996a; Howard *et al*., 1996; Chopra and Houston, 1999) have been proposed and examined, but a complete understanding of the multifunctional mechanisms of pathogenesis is elusive. The first evidence supporting a mouse model for *Aeromonas* virulence studies was published by Sha *et al*. (2002). Other models include the medicinal leech model (Graf, 1999) and the blue gourami model (Yu *et al*., 2005). None of these models reproduce gastrointestinal disease in humans. Aeromonads are ubiquitous in

the aquatic environment (Holmes *et al*., 1996). *Aeromonas* spp. causes disease in poikilothermic animals, and occasionally in mammals.

Diseases caused by aeromonads represent a significant source of loss to the aquaculture industry. *Aeromonas* spp. have been found in foods (Palumbo, 1996), and they have been shown to cause foodborne outbreaks of gastrointestinal disease. They have been isolated from treated drinking water (Burke *et al*., 1984a; van der Kooij, 1988; Fernandez *et al*., 2000). Drinking water standards for *Aeromonas* are in effect in The Netherlands (van der Kooij, 1988), a bottled water standard is in effect in Canada and *A. hydrophila i*s listed by EPA on the CCL in the U.S. (USEPA, 2001). No disease outbreaks associated with treated drinking water have been reported, and the role of drinking water as a source of aeromonads causing gastrointestinal disease in not clearly and convincingly established. Burke *et al*. (1984) demonstrated a seasonal association between the presence of aeromonads in a public drinking water supply and the presence of aeromonads in human fecal specimens collected from patients with gastroenteritis; however, the causal relationship of aeromonads to gastroenteritis was not shown.

The taxonomy of aeromonads between 1980 and 1990 was complicated by the fact that few phenotypic markers were available to reliably differentiate species for the newly recognized hybridization groups. Further complication arose from reports of genetically identical strains published under different names by different investigators. DNA hybridization groups (HGs) were recognized for which there were no reliable phenotypic characteristics, resulting in confusion among microbiologists and physicians. Some of the newly recognized aeromonads were described from a handful of strains, making it difficult to describe the species phenotypically. For example, *A. allosacchrophila* was described from three isolates (Martinez-Murcia *et al*., 1992), *A. encheleia* was described from four isolates (Esteve *et al*., 1995), and *A. popoffii* was described from eight isolates (Huys *et al*., 1997).

The current taxonomy of the genus *Aeromonas* is based upon DNA-DNA hybridization and 16S ribosomal DNA relatedness studies. The genera of the family *Aeromonadaceae* now include *Aeromonas*, *Oceanimonas*, *Oceanisphaera,* and *Tolumonas* (incertae sedis) (Martin-Carnahan and Joseph, 2005). *A. hydrophila* ssp. dhakensis (subsp. nov.) (Huys *et al.*, 2002), *A. hydrophila* ssp. ranae (subsp. nov.) (Huys *et al.*, 2003), *A. culicicola* (sp. nov.) (Pidiyar *et al.*, 2002), *A. simiae* (sp. nov.) (Harf-Monteil *et al.*, 2004), and *A. molluscorum* (sp. nov.) (Minana-Galbis *et al*, 2004) have been proposed as new species and subspecies of *Aeromonas*, and more will undoubtedly be described.

The first attempts to identify aeromonads to genotype relied upon differences in 16S ribosomal DNA sequences (Martinez-Murcia *et al.*, 1992), and several investigators developed probes for detection of various *Aeromonas* spp. (Demarta *et al.*, 1999; Khan *et al.*, 1999). *Aeromonas* taxonomy based upon 16S ribosomal DNA is complex and investigators have examined alternative means to sort out the genotypic maze. Borrell *et al.* (1997) were able to identify ten species using endonucleases AluI and MboI, but needed to add NarI and HaeIII to differentiate *A. salmonicida* from *A. encheleia,* and their system did not distinguish between *A. salmonicida* and *A. bestiarum,* nor identify *A. popoffii*. Figueras *et al.* (2000) added two additional endonucleases NlwNI and PstI to this restriction fragment length polymorphism (RFLP) method to differentiate *A. salmonicida* and *A. bestiarum* and for recognition of *A. popoffii*. Genetic crossover or recombination may obscure phylogenetic relationships among aeromonads (Morandi *et al.*, 2005).

Various other genetic methods are under investigation for species identification. Pidiyar *et al.* (2002) sequenced the 16S-23S intergenic spacer regions and found that the resulting phylogeny did not agree with the results of 16S ribosomal DNA and DNA-DNA hybridization studies. The significance of intergenic spacer region analysis is under investigation. The sequence of the gyrB gene was used to construct a phylogenetic tree of all 17 hybridization groups (Pidiyar *et al.*, 2002). *A. culicicola* grouped with *A. veronii*, based upon gyrB, but it grouped with *A. jandaei* based upon

16S ribosomal gene sequence. From sequence analysis of the polymerase chain reaction (PCR) amplicon of gyrB, the gyrB gene sequence was viewed as a better phylogenetic chronometer than the 16S ribosomal gene. Yanez *et al*. (2003) reported that the gyrB gene agree with the 16S ribosomal data which lead to placement of the genus *Aeromonas* in the family *Aeromonadaceae*, and gyrB gene sequences were useful in resolving discrepancies between 16S ribosomal gene sequences and DNA-DNA hybridization results.

Several other methods have been used to characterize *Aeromonas* species. Minana-Galbis *et al*. (2004) examined the genetic diversity between *A. hydrophila*, *A. bestiarum, A. salmonicida* and *A. popoffii* by multilocus enzyme electrophoresis (MLEE). By MLEE, *A. popoffii* and *A. bestiarum* were closely related. MLEE has been used in genomospecies determination since 1991 (Altwegg *et al*., 1991; Figueras *et al*., 2000). While some authors are enthusiastic about MLEE, few studies have applied the method and none of them have looked at the entire population structure of the genus *Aeromonas*. Multilocus sequence typing (MLST) using the four gene loci of 16S rDNA, recA, chiA and gyrB has revealed the taxonomic limitations of 16S rDNA alone (Carnahan, 2001).

The current classification of some species is questionable (Martin-Carnahan and Joseph, 2005). *A. ichthiosmia* is a later synomym of *A. veronii*, and the taxonomic status of *A. allosaccharophila* is unclear and deserves further investigation, though a later study supports the status of *A. allosaccharophila* as a separate species based upon gyrB gene sequences (Yanez *et al*., 2003). *A. allosccharophila* and *A. ichthiosmia* belong to *A. veronii* complex. The taxonomic status of *A. enteropelogenes* and *A. trota* is questionable as they share 81-99% relatedness and identical API E20 and API 50CHE biochemical profiles. While *A. enteropelogenes* has taxonomic status, *A. trota* has been cited more frequently and it should be used preferentially in future publication to avoid ambiguity among ampicillin-susceptible aeromonads (Carnahan *et al*. 1991; Huys *et al*. 2002).

Aeromonas salmonicida contains four subspecies: *salmonicida*, *achromogenes*, *masoucida*, and *smithia*. However, the lack of congruence between phenotypic and genetic data results in great difficulty in accurately assigning isolates to the correct subspecies (Martin-Carnahan and Joseph, 2005). A. salmonicida has not been shown to cause disease in humans, however *A. hydrophila* like HG-3 strains that are motile and grow at 37° C have been isolated from clinical specimens on rare occasions (Carnahan *et al* , 2001), and its importance relates to animal diseases in the marine environment and in aquaculture (Austin and Adams, 1996).

Other species of *Aeromonas* have been described, but they have not yet received taxonomic recognition. These species include *A. arequipensis*, *A. dechromatica*, *A. guangheii*, and *A. pastoria* (Martin-Carnahan and Joseph, 2005).

1.8 Treatment of Gastrointestinal *Aeromonas* infections

The role of antibiotics in the treatment of gastrointestinal *Aeromonas* infections is controversial, since most patients regain health without treatment. Antimicrobials are indicated for only severe and unresponsive cases of *Aeromonas* gastroenteritis (Phavichitr and Catto-Smith 2003). Antimicrobials should be considered for chronic gastrointestinal disease or extra-intestinal infection (Ghenghesh *et al*., 1999). For adult patients with severe gastrointestinal illness such as colitis, quinolones, chloramphenical, trimethoprim-sulfamethoxazole, and tetracyclines are effective (Farraye *et al*., 1989). However, Ko *et al*., (1996) reported resistance in strains from Taiwan to tetracycline, trimethoprim-sulfamethoxazole, ceftriaxone, cefotaxime, cefixime and tobramycin. Prophylactic antimicrobial therapy is recommended for patients undergoing leech therapy (Snower *et al*., 1989). In addition to selection of antibiotic therapy in the clinical setting, antibiotic sensitivity patterns are sometimes useful as phenotypic characteristics for species identification, especially for clinical isolates (Overman and Janda, 1999). *Aeromonas* spp. are characteristically resistant to ampicillin (94.9%), with variable resistance to cephalexin (76.3%), trimethoprim (37.3%), tetracycline (11.9%), cefuroxime

(5.1%), and ceftazidime (1.7%). Strains may be susceptible to gentamicin, chloramphenicol, and ciprofloxacin. In another study, antibiotic susceptibility tests were performed on 164 strains, and resistance to ciprofloxacin (12-22%), nalidixic acid (54-62%), and norfloxacin (14-19%) were recorded (Overman and Janda, 1999). Wide annual fluctuations in resistance during two consecutive years occurred with furazolidone (7% vs. 74%) and streptomycin (45% vs. 72%). These data support antibiotic susceptibility testing of isolates from extra-intestinal source.

Disc diffusion and broth dilution tests for amoxicillin-clavulanic acid, sulfonamide, trimethoprim, and trimethoprim-sulfamethoxazole are unreliable (Koehler and Ashdown, 1993). Overman and Janda (1999) published antimicrobial susceptibility patterns for *A. jandaei*, *A. trota*, *A. schubertii*, and *A. veronii biovar veronii*, using the Microscan system and found that the antibiograms were useful in establishing the identification of these four species when isolates originated from clinical specimens. In the U.S., more than 90% of strains were reportedlysusceptible to third-generation celphalosporins (cefotaxime, ceftriaxone, ceftazidime and cefoperazone) and aminoglycosides (gentamicin, tobramycin, amikacin, sisomicin, netilmicin, kanamycin, and neomycin and almost all aeromonads were susceptible to quinolones (ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, sparfloxacin, moxifloxacin and gatifloxacin (Zong *et al*., 2002). Most U.S. strains were noted to be susceptible to chloramphenicol, tetracycline, minocycline, doxycycline, and nitrofurantoine, but resistant to clindamycin, vancomycin, and erythromycin whereas imipenem was observed to be effective for treatment of *Aeromonas* infection (Lupiola-Gomez *et al*., 2003).

Aeromonas spp. are typically sensitive to tetracycline, aminoglycosides, trimethoprim-sulfamethoxazole, third-generation cephalosporins, and quinolones (Koehler and Ashdown, 1993; Janda et al, 1996). Ko *et al*. (1996) reported increasing resistance to tetracyclines, trimethoprim-sulfamethoxazole, some extended-spectrum cephalosporins (ceftriaxone, cefotaxime, and cefixime), and tobramycin in strains from Taiwan. Huys et

al. (2001) found oxytetracycline resistant strains in water from fish farms and in hospital sewage.

Aeromonas species exhibit differences in their susceptibility to antibiotics. Clinical isolates of *A. caviae* were more susceptible to ticarcillin than *A. veronii* and *A. hydrophila* (Vila *et al*., 2002). Use of clavulanic acid with amoxicillin enhanced antibacterial activity but tazobactam did not enhance the effect of piperacillin. *A. veronii* (79%) were more susceptible to cefazolin than *A. caviae* (53%) or *A. hydrophila* (40%). Gentamicin and amikacin were more active than tobramycin. Tobramycin resistance and gentamicin susceptibility has been observed in Australia, Taiwan and the U.S. Fluoroquinolones have been the first choice treatment for *Aeromonas* infections. Strain resistant to nalidixic acid and susceptibility to ciprofloxacin are known to have a mutation in the gyrA gene; thus, naladixic acid resistant strains should not be treated with fluoroquinolones. Resistance to nalidixic acid is a function of a mutation of the gyrA, gyrB, genes which make up the quinolone resistance-determining regions (Goni-Urriza *et al*., 2002). Gyrase and topoisomerase IV are the primary and secondary targets for quinolones, respectively. Ko *et al*. (2001) demonstrated that minocycline and cefotaxime administered together produced a synergistic effect against *A. hydrophila* using a murine model. Ciprofloxacin was as effective as cefotaxime-minocycline in vitro and in a murine model, suggesting that clinical studies are warranted (Ko *et al*., 2003).

Two metal ion binding sites are conserved in the metallo-β-lactamase produced by *A. hydrophila* (Valladares *et al*., 2000). Class B metalloproteins require bivalent transition-metal ions for activity, and Zn^{2+} is required for activation. Quiroga *et al*. (2000) found that cefotetan was efficacious against *Aeromonas* spp. because it behaved as a transitory inactivator of metallo-β-lactamase activity. Walsh *et al*. (2002) used E - test strips for the detection of metallo-β-lactamases in clinical isolates. The test is based upon reduction of imipenem or ceftazidime in the presence of EDTA or 2-mercaptopropionic acid. Fosse *et al*. (2003) demonstrated the β-lactamase classes produced by *Aeromonas* spp., and proposed they had taxonomic value as an adjunct in species identification.

Edwards *et al.* (2001) reported antibiotic resistance in *Aeromonas* spp. isolated from a eutrophic lake in England. Goni-Urriza *et al.* (2002) reported antibiotic resistance to ampicillin (99%), ticarcillin (87%), cephalothin (93%), cefoxitin (56%), streptomycin (65%), sulfamethoxazole (90%), trimethoprim (42%), pipemidic acid (67%), oxolinic acid (67%), naladixic acid (59%), and tetracycline (14%) in *Aeromonas* spp. isolated from European rivers. Susceptibility to fluoroquinolones varied from 54 to 98%. Most strains were susceptible to ciprofloxacin, colistin, fosfomycin, cotrimoxazole, chloramphenicol, gentamicin, tobramycin, cefotaxime, and imipenem (Goni-Urriza *et al*., 2002). Urban wastewater effluents are thought to contribute to the increasing rate of antibiotic resistance in environmental aeromonads (Goni-Urriza *et al*., 2002).

Oxytetracycline is used in aquaculture and the development of resistant strains of *Aeromonas* spp. has both commercial and public health implications (Adams *et al*., 1998). Because resistance is plasmid mediated, it is easily transferred among strains, and has been demonstrated in isolates from hospital sewage and a German hospital patient, in addition to fish farming environments. Dissemination of resistant strains has occurred in Norway, Scotland, England, and Germany (Rhodes *et al*., 2000). Bruun *et al*. (2003) demonstrated transfer of large plasmids conferring oxytetracycline resistance among environmental aeromonas and between *Aeromonas* spp. and *E. coli*.

Aeromonas popoffii has been isolated from freshwater and seawater, but not from humans, though they are known to produce virulence factors and exhibit antibiotic resistance (Soler *et al*., 2002). Third-generation cephalosporins and quinolones are most active against *A. popoffii* in laboratory studies.

1.8.1 Use of Antibiotics

Rehydration therapy is sufficient intervention in most pediatric cases of gastroenteritis caused by *Aeromonas* spp. (San Joaquin and Pickett, 1988). Draining obstructions and antibiotic therapy cleared infection in patients with acute suppurative cholangitis (Chan

et al., 2000). Debridment or more intensive surgical intervention may be necessary to arrest progression of soft tissue infection

1.8.2 Antibiotic resistance

1.8.2.1 Development and acquisition of resistance

The microbes responsible for these infections are often antibiotic resistant pathogens. The ability of the pathogens to grow despite the presence of antibiotics, through the development of antibiotic resistance, has rendered victims vulnerable. Modern uses of antibiotics have caused a huge increase in the number of resistance bacteria. In fact within eight to twelve years, after wide spread use, strains resistant to multiple drugs become widespread.

Several mechanisms have been developed by bacteria in order to deal with antibiotics but all require either the modification of existing genetic material or the acquisition of new genetic material.

Originally it was believed that all resistance was acquired through spontaneous mutation. Development of resistance through this method is called primary resistance. Errors in DNA synthesis during replication and occasional failures in the DNA repair systems results in a spontaneous mutation frequency for an individual base pair of about 10^{-7}-10^{-8}. In *E. coli*, it has been estimated that primary streptomycin resistance is acquired at a rate of approximately 10^{-9} when exposed to high concentrations of streptomycin. While this is an extremely rare event, the very fast growth rate of bacteria means that it doesn't take long before resistance develops in a population. Once the resistance genes are acquired, the genes can be transferred directly to all the bacteria's progeny. This is known as vertical gene transfer.

The widespread development of multi-drug resistance in many species of bacteria simultaneously led scientists to believe that another mechanism beyond spontaneous

mutation was responsible for the acquisition of antibiotic resistance. Lateral or horizontal gene transfer (HGT) is a process whereby genetic material contained in small packets of DNA can be transferred between individual bacteria. There are three possible mechanisms of HGT. These are transduction, transformation or conjugation. Transduction occurs when bacteria-specific viruses or bacteriophages transfer DNA between two closely related bacteria. Transformation is a process where parts of DNA are taken up by the bacteria from the external environment. This DNA is normally present in the external environment due to the death of another bacterium. Conjugation occurs when there is direct cell-cell contact between two bacteria and transfer of small pieces of DNA called plasmids takes place. This is thought to be the main mechanism of antibiotic resistant gene transfer. Figure 2.1 shows the three mechanisms of HGT.

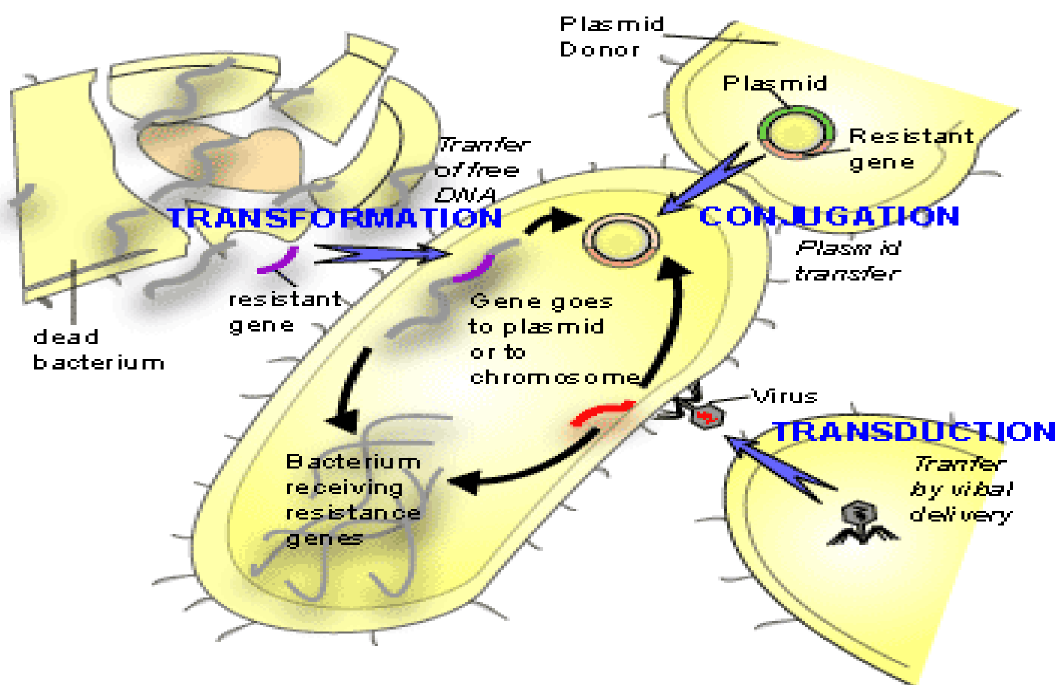

Figure 1.1: Illustration of the mechanisms involved in genetic exchange among bacteria showing transformation, conjugation and transduction (Yim, 2007)

1.8.2.2 Mechanisms of antibiotic resistance

Several mechanisms have evolved in bacteria which confer them with antibiotic resistance. These resistances to antibiotics have evolved due to misuse in clinical treatment (Chaibi *et al*., 1999; Essack *et al*., 2001). Resistance may be defined as the ability of a micro-organism to resist the action of antimicrobial agents at concentrations achievable in the body after normal dosage (Mims *et al*, 1993). Resistance to β-lactam antibiotics in Gram-negative bacteria can arise using three difference mechanisms. These mechanisms can either chemically modify the antibiotic or alternatively render it inactive through physical removal from the cell, or through modification of its target site (Dever and Dermody, 1991; Baker, 1999; Menashe *et al*., 2001). Figure 2.2 show several different mechanisms of antibiotic resistance. The most common mode is enzymatic inactivation of the antibiotic (Silva *et al*., 1999; Wright, 1999). An existing enzyme is modified to process the antibiotic and modifies it so that it no longer affects the microorganism (Baker, 1999; Silva *et al*., 1999). An alternative strategy utilized by bacteria is the alteration of the antibiotic target site (Hindler, Howard and Keiser, 1994; Sougakoff and Jarlier, 2000) and reduced permeability (loss of certain porin proteins) through alterations in the bacterial wall pores that may prevent the attainment of effective periplasmic β-lactam antibiotic concentration (Hindler, Howard and Keiser, 1994).

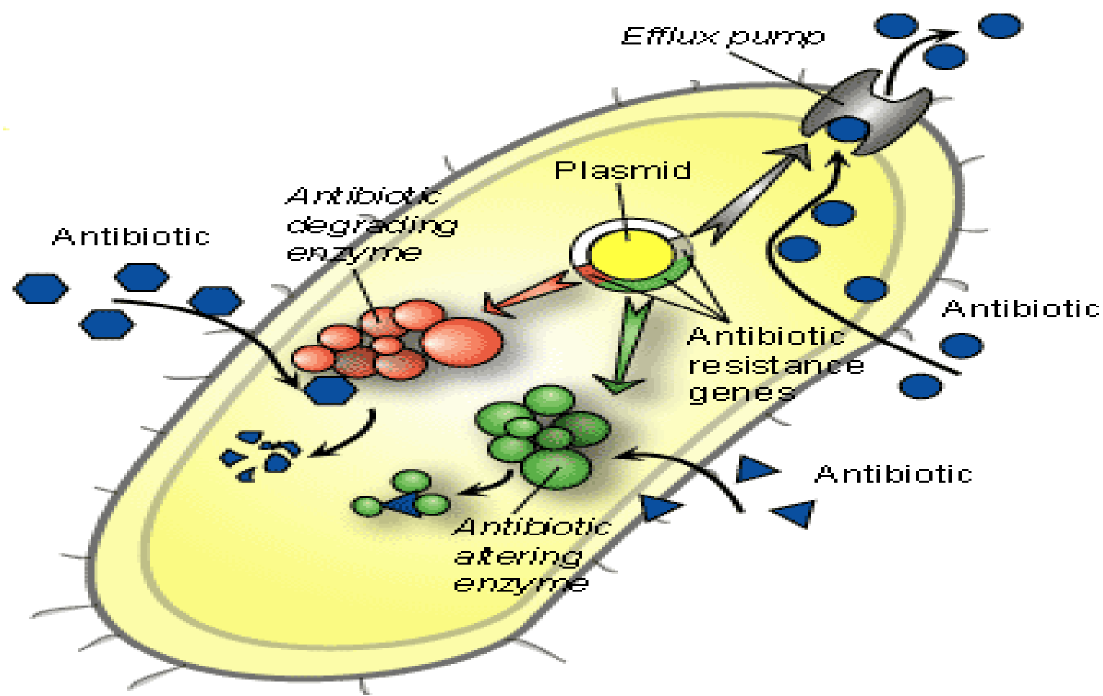

Figure 1.2: Illustration of bacterial mechanisms of antibiotic resistance in *Staphylococcus aureus*, *Escheriachia coli*, *Pseudomonas aeroginosa* and *lactobacillus latis* (Yim, 2007)

1.9 Reduced permeability

Gram-negative bacteria have a unique outer membrane outside the peptidoglycan that acts as an intrinsic permeability barrier against external influences, protecting them against host factors such as lysozyme. The porins in the outer membrane may also prevent or reduce the penetration of antibiotics (Pitout *et al*., 1997). The porin proteins form non-specific trans-membrane diffusion channels that allow exchange of nutrients and other substances such as antibiotics between the extra-cellular environment and the periplasmic space (Hancock, 1987; Hernandez-Alles *et al*., 1999). B-Lactam antibiotics have to penetrate porin channels in the outer membrane and transverse the periplasmic space in order to bind to PBPs (Hindler and Howard, 1994; Pitout *et al*., 1997). Changes in outer membrane permeability may consequently decrease binding to PBPs located on

the inner membrane as a result of the limited influx of antibiotics (Dever and Dermody, 1991). Reduced permeability in Gram-negative bacteria is mainly mediated by the loss or modification of the outer membrane proteins of 35-50 kD (Moosdeen, 1997), although the number and size of porins vary among different Gram-negative organisms (Prober, 1998). Two outer membrane proteins (Omps) of mutant *E. coli* K-12 have been identified and characterized as OmpF and OmpC (Reguera *et al*., 1991). Porin deficient mutants are known as Omp R mutants, and there is a possibility that decreased porin content could be caused by some R plasmids (Nikaido, 1989). In *K. pneumoniae*, resistance to cephamycins, such as cefoxitin, can be attributed to a loss of one or both of the two major outer membrane porins OmpK35 or OmpK36 produced by these organisms. It is also known that most ESBL producing *K. pneumoniae* strains lack OmpK35, which may result in the selection of additional mechanisms of resistance, including the loss of OmpK36 or efflux

1.10 Efflux

Many different bacteria are able to pump out antibiotics. In "pumping" out the antibiotic from the cell, the organism can prevent it from reaching the concentration necessary for effective action. Gram-negative efflux pumps were first identified and characterized in strains of *Escherichia coli* and *P. aeruginosa*. Many similar efflux pumps have since been discovered in nearly all clinically relevant gram-negative bacteria. This can cause resistance not only to the prescribed antibiotic but also to other antibiotics, even different classes of antibiotics (Doménech-Sanchez *et al*., 2003).

Physiologically these pumps appear to be part of the natural defense mechanisms of bacteria against toxic compounds that exist in the environment (Nikaido, 1989). The sequence of proven and putative multi-drug efflux pumps from complete genome of *E. coli* has been recently analysed (Doménech-Sanchez *et al*., 2003).

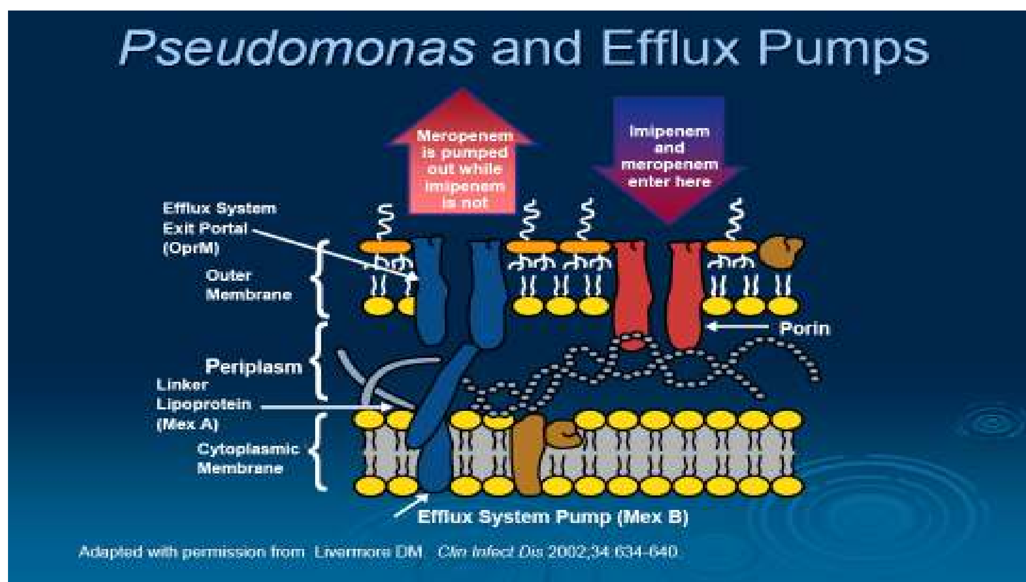

Figure 1.3: Illustration of mechanisms of antibiotic resistance.

1.11 The extended–spectrum ß-Lactamases and ß-Lactam antibiotics in bacteria

Beta-lactam antibiotics form a large group of different compounds containing a β-lactam nucleus. In 1929, the discovery of the antibiotic penicillin and its subsequent development into a stable form radically changed the treatment of disease. Today, penicillin and the other β-Lactam antibiotics are still widely used. The β-Lactam antibiotics include the penicillins, cephalosporins, clavams, carbapenems, and monocyclic β-Lactam (monobactams). All of the members in this family possess the highly reactive β-Lactam ring. These antibiotics interfere with the transpeptidation and transglycosylation reactions of cell wall synthesis; that is, β-Lactam prevents the attachment of new peptidoglycan to the pre-existing cell wall in actively growing cells (Denome and Sylvia, 1999). Although the mechanism of this reaction is not completely understood, it seems that the β-Lactam may act as an analogue to the peptidoglycan substrate. This is because the nitrogen-carboxyl (N-CO) bond of the ring is similar in structure and position to the peptide D-alanyl-D-alanine bond that is cleaved in the transpeptidation reaction, Figure 1.10 (Rogers *et al.*, 1980). There are slight differences between the two molecules in conformation, such as differences in bond angles and

lengths. The β-Lactam may act either as a transition-state analogue or as an allosteric inhibitor (Rogers *et al*., 1980).

Different groups within the family are distinguished by the structure of the ring and the side chain attached to the β-lactam nucleus. Penicillins differ from cephalosporins in that penicillins contain a 5- membered thiazolidine ring complex and cephalosporins contain a 6-membered dihydrothiazine β-Lactam ring complex (Dever and Dermody, 1991) Since the discovery of β-lactam antibiotics in 1928 by Alexander Fleming (Rolinson, 1998), developments have led to the synthesis of semi-synthetic compounds that can be divided into bicyclic penicillins, cephalosporins, monocyclic monobactams, and β-lactamase inhibitor combinations (Hamilton- Miller, 1999) . The inhibition of the PBP/transpeptidase enzymes is due to penicilloylation of the enzyme, which is irreversible, covalent binding of the β-Lactam by the protein. This results in the inability of the cell to synthesize more cross-linked peptidoglycan and lysis of the cell (Rogers *et al*., 1980). This irreversible binding explains why the lethal concentrations of β-Lactam that target these PBPs are low. The inhibition of the carboxypeptidases and endopeptidases is due to reversible binding, and higher concentrations of the antibiotics specific to these PBPs are needed to lyse the cells. Although the majority of the β-Lactam antibiotics are more effective against Gram-positive cells, there are β-Lactams that are effective against both Gram-positive and Gram-negative cells. The targets of the β-Lactams are determined by the structures that are attached to the β-Lactam ring.

The penicillins contain a five-membered thiazolidine ring fused to the β-Lactam ring (Demain *et al*., 1999) and an R-group (Figure 2.7), and they are synthesized from L-alpha-aminoadipic acid, L-cysteine, and L-valine). The R-group determines the type of penicillin and affects the activity of the molecule. In the fermentation method used to produce penicillins, the liquid that the Penicillium mold is bathed in determines the R-group of the antibiotic produced. The most common mass produced penicillins are penicillin G (benzylpenicillin) and penicillin V (phenoxymethylpenicillin) (Demain *et al*., 1999) (figure 2.8). Hydrophobic penicillins are most effective against Gram-positive

bacteria while hydrophilic penicillins (such as penicillin N, Figure 2.9) are broad-spectrum antibiotics (Demain *et al.*, 1999).

The cephalosporins, like the hydrophilic penicillins, are broad-spectrum antibiotics. Unlike penicillin, the cephalosporins do not have a five-membered thiazolidine ring; instead, they have a six-membered dihydrothiazine ring (Figure 2.4) (Demain *et al.*, 1999). An added advantage of the cephalosporins is that they are relatively stable in the presence of β-lactamases. An example of a potent, naturally occurring cephalosporin is cefoxitin (Figure 2.10) (Rogers *et al.*, 1980). Clavams contain the β-Lactam ring, but are not very effective antibiotics. Like the penicillins, they contain a five-membered ring fused to the β-lactam. Clavams, however, replace the sulfur atom of penicillin's thiazolidine ring with an oxygen atom (making the ring an oxazolidine ring). Even though they are not good antibiotics, clavams are extremely useful because they are excellent β-lactamase inhibitors. The clavam clavulanic acid (Figure 2.6) is usually combined with other, more effective β-lactams to make them more effective (Demain *et al.*, 1999).

The most powerful and least toxic natural antibiotic that has been developed is thienamycin (Figure 2.5), a carbapenem B-lactam. The ring fused to the β-lactam in the carbapenems does not contain a double bond. The carbapenems are resistant to B-lactamases, and they are effective against both Gram-positive and Gram-negative bacteria (Demain *et al.*, 1999).

There are also β-lactam antibiotics that do not have a bicyclic ring structure. There are two categories of monocyclic β-lactams, the nocardicins and the monobactams. Both types are more effective against gram-negative than gram-positive bacteria, and both are resistant to β-lactamases (Demain *et al.*, 1999). The nocardicins have not been developed into commercial products, nocardicin A. They are synthesized from L-methionine, L-serine, and L-tyrosine (Demain *et al.*, 1999). The monobactams have a relatively simple structure, and are marketed commercially (Demain *et al.*, 1999). β-

lactam antibiotics are commonly prescribed and have a wide spectrum clinical use because of low toxicity and strong bactericidal activity (Gruneberg, 1994; Petrosino *et al.*, 1998). They can be used in higher dosages for treatment of more severe infections (Sanders and Sanders, 1992). The primary targets for β-lactam antibiotics are the penicillin-binding proteins (PBPs), consisting of transpeptidases and carboxypeptidases responsible for creating cross-linkages between peptide chains. Following penetration of the bacterial cell surface, β-lactam antibiotics attach to the PBPs, to form a β-lactam-PBP complex. Catalytic activity is lost and cell wall synthesis and division interrupted (Richmond, 1981; Essack, Alexander and Pillay, 1994; Prober, 1998; Wright, 1999). The anti-bacterial effect of all β-lactam antibiotics depends on the capacity of the antibiotic to diffuse through the cell membrane of the bacterial cell, the affinity of the antibiotic for its target proteins (the PBPs anchored in the cytoplasmic membrane of the bacterium) and the stability of the antibiotic against bacterial degradation complex system (Dever and Dermody, 1991; Pitout *et al.*, 1997). β-Lactam antibiotics inhibit the cross linking (final stage) of peptidoglycan or murein synthesis of actively dividing bacterial cells (Gould and Mackenzie, 1997). The inhibition of cell wall synthesis by β-lactam antibiotics does not alter the activity of these enzymes; therefore, bacterial autolysis can result from the effects of osmotic pressure on the cell wall damaged by murein hydrolases (Wright, 1999).

Introduced in the 1960s cephalosporins are widely used routinely in many preoperative procedures, because of their broad-spectrum effectiveness and low toxicity (SAML supplement). Cephalosporins are classified according to the route of administration (Bannister *et al.*, 2000). Currently there are four generations of agents, each succeeding a generation possessing a greater spectrum of activity. The antibacterial activity of cephalosporins depends on their ability to penetrate the bacterial cell wall, resist inactivation by β-lactamases and bind to and inactivate PBPs. Resistance can, however, develop at each of these steps (Prober, 1998).

Expanded-spectrum cephalosporins were specifically designed and introduced into clinical practice in the early 1980s, being resistant to hydrolysis by the older broad-spectrum β-lactamases commonly encountered at that time.

The most important and widespread mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria is due to enzyme mediated antibiotic degradation (Neuwirth, 2001). Three classes of enzymes that can hydrolyse β-lactam antibiotics are (1), β-lactamases (2) acylases, and (3) esterases (Dever and Dermody, 1991). The enzyme hydrolyses the β-lactam antibiotic to acidic derivatives without antibacterial properties (Ho *et al*., 1998). β- Lactamase production is mediated by genes carried on a plasmid or on the chromosome and more than one type may be produced by same species at the same or different times (Hindler, Howard and Keiser, 1994). Chromosomal β-lactamases can be constantly produced (constitutive β-lactamases of *E. coli*, *Shigella* species, *Proteus mirabilis*) or only in the presence of β-lactam antibiotics (inducible β-lactamases of *Pseudomonas aeruginosa*, *Enterobacter* species, *Citrobacter* species, *Serratia* species, *Morganella* species and *Providencia rettgeri*). Cefoxitin, imipemen and first generation cephalosporins are potent inducers of chromosomal β-lactamases (Gorbach *et al*., 1997; Prober, 1998). The expression of chromosomally mediated β-lactamases is usually not constitutive, but can be induced or derepressed by exposure to β-lactam antibiotics (Dever and Dermody, 1991).

1.11.1 Types of ESBLs in bacteria

Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid. Typically, they derive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases. This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. An increasing number of ESBLs not of TEM or SHV lineage have recently been described. The name "TEM" is a contraction of Temoniera, the name of the patient from whom resistant bacteria were isolated, whereas SHV is a contraction of sulfhydryl variable; a description of the biochemical properties of this β-lactamase (Heritage *et al.*, 1999). The presence of ESBLs carries tremendous clinical significance. The ESBLs are frequently plasmid encoded. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (for example, aminoglycosides). Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited.

Carbapenems are the antibiotics of choice for serious infections due to ESBL-producing organisms, yet carbapenem-resistant isolates have recently been reported. ESBL-producing organisms may appear susceptible to some extended-spectrum cephalosporins. However, treatment with such antibiotics has been associated with high failure rates. There is substantial debate as to the optimal method to prevent this occurrence. It has been proposed that cephalosporin breakpoints for the *Enterobacteriaceae* should be altered so that the need for ESBL detection would be obviated. At present, however, organizations such as the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) provide guidelines for the detection of ESBLs in *klebsiellae Pneumonia* and *Escherichia coli* (Baker, 1999). In common to all ESBL detection methods is the general principle that the activity of extended-spectrum cephalosporins against ESBL-producing organisms will be enhanced by the presence of clavulanic acid. ESBLs represent an

impressive example of the ability of Gram-negative bacteria to develop new antibiotic resistance mechanisms in the face of the introduction of new antimicrobial agents.

The introduction of the third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against β-lactamase-mediated bacterial resistance to antibiotics. These cephalosporins had been developed in response to the increased prevalence of β-lactamases· Not only were the third-generation cephalosporins effective against most β-lactamase-producing organisms but they had the major advantage of lessened nephrotoxic effects compared to aminoglycosides and polymyxins (Heritage *et al*., 1999). The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983 (Knothe *et al*., 1983). The gene encoding the β-lactamase showed a mutation of a single nucelotide compared to the gene encoding SHV-1. Other β-lactamases were soon discovered which were closely related to TEM-1 and TEM-2, but which had the ability to confer resistance to the extended-spectrum cephalosporins (Brown *et al*., 2000; Sirot *et al*., 1987). Hence these new β-lactamases were coined extended-spectrum β-lactamases (ESBLs). In the first substantial review of ESBLs in 1989, it was noted by Philippon, Labia, and Jacoby that the ESBLs represented the first example in which β-lactamase-mediated resistance to β-lactam antibiotics resulted from fundamental changes in the substrate spectra of the enzymes (Philippon *et al*., 1989)

In 2001, the ESBLs were reviewed (Bradford, 2001). The total number of ESBLs now characterized exceeds 200 Research articles on ESBLs have now originated from more than 30 different countries, reflecting the truly worldwide distribution of ESBL-producing organisms.

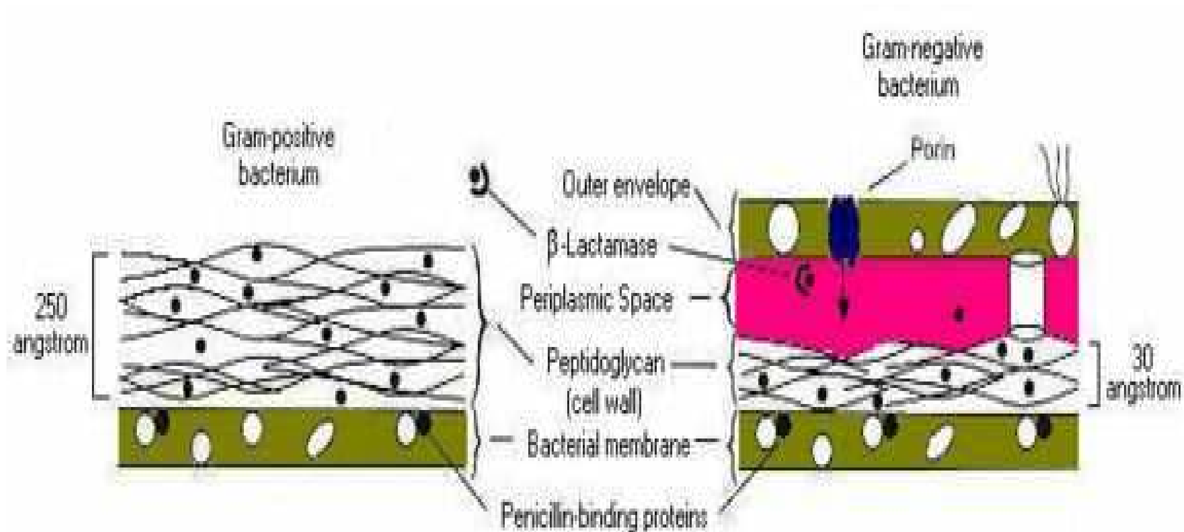

Figure 1.4: Illustration different aspects of beta-lactamases in Gram-positive and Gram-negative cell wall structures

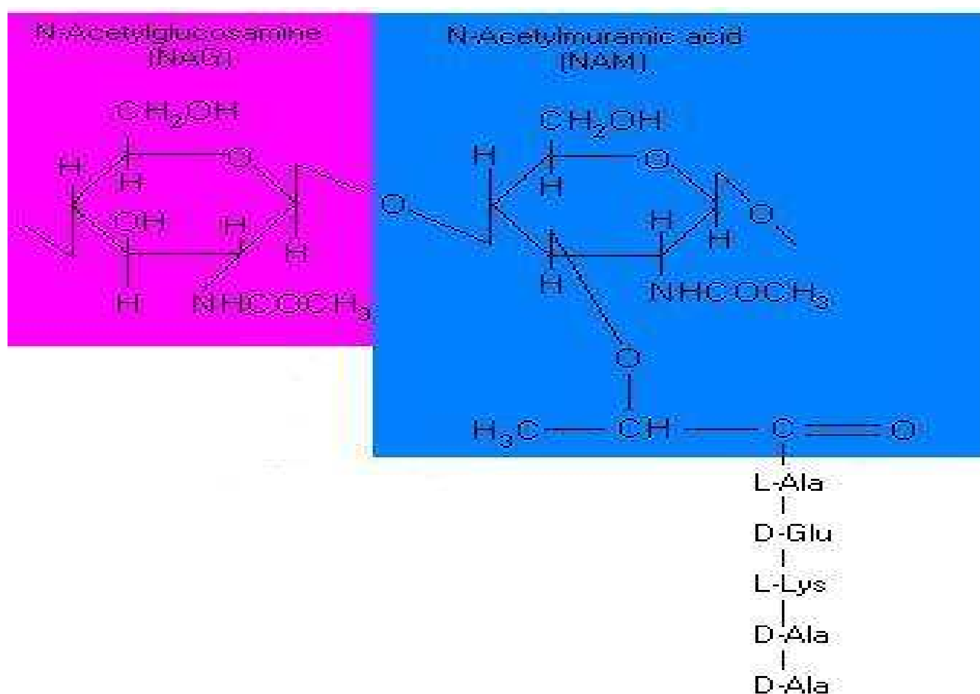

Figure 1.5: Illustration the Structure of Peptidoglycan in beta-lactam antibiotic

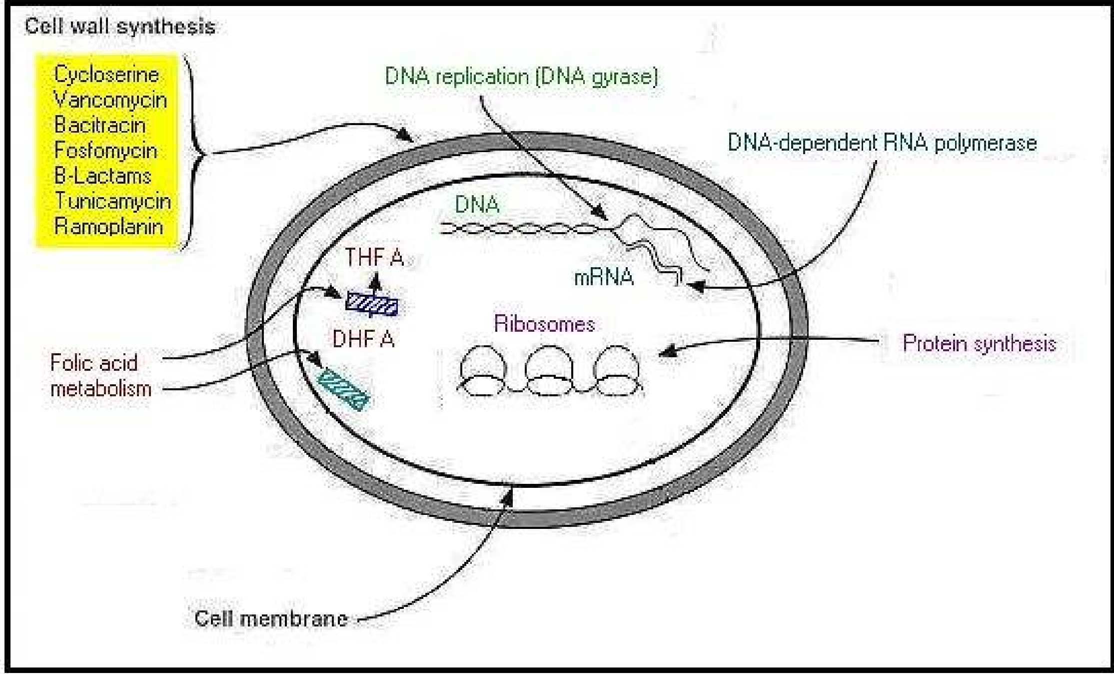

Figure 1.6: Illustration of Antibiotic Targets Sites

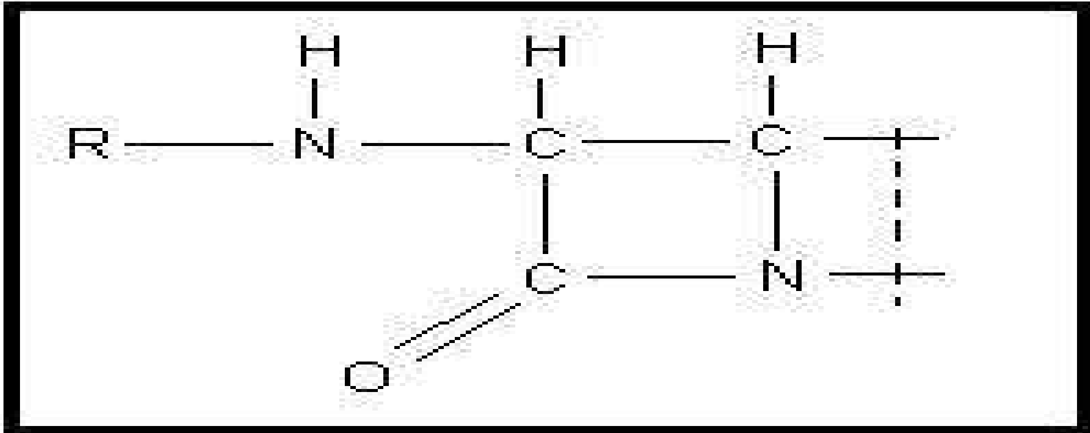

Figure 1.7: The β-Lactam Ring

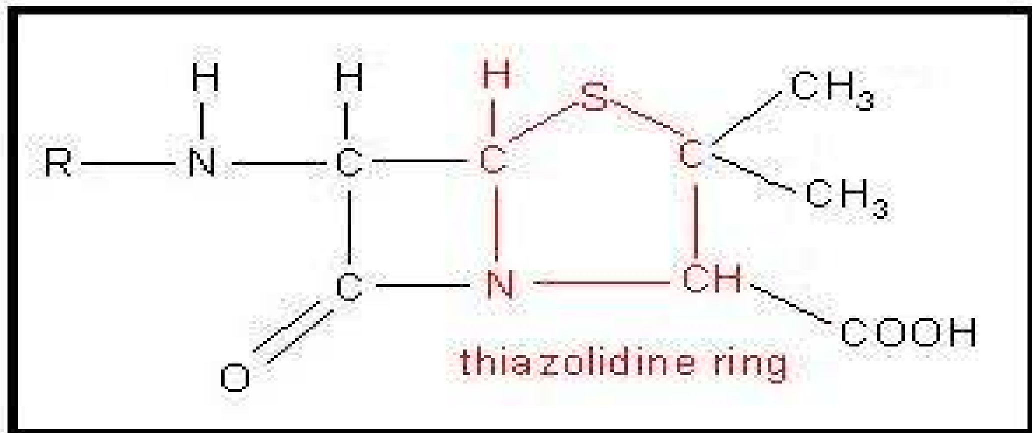

Figure 1.8: The Structure of Penicillin

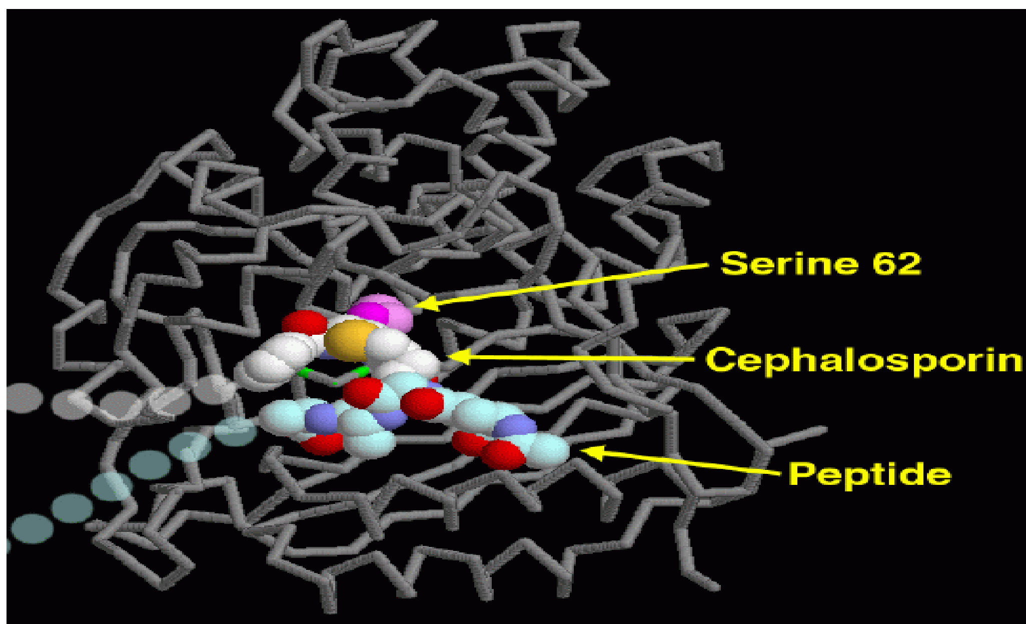

Figure 1.9: Structure of Penicillin-binding Proteins by Kelley *at al*., 1998

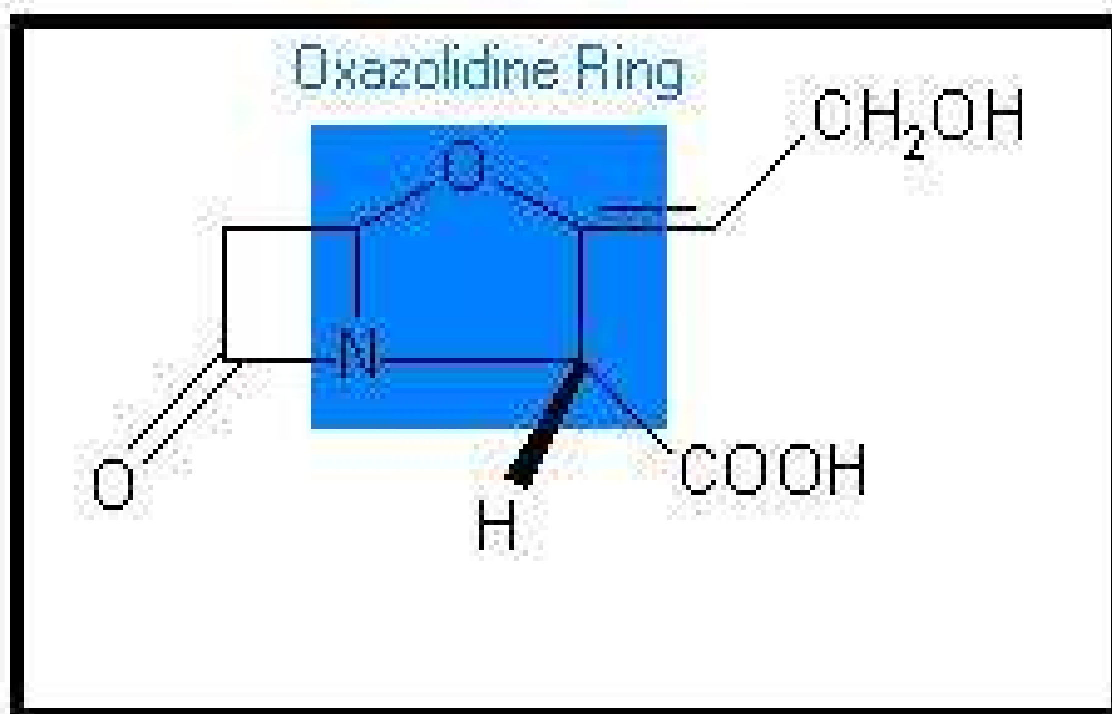

Figure 1.10: The Structure of Clavulanic Acid

1.12 Role of plasmids in resistance

Acquired resistance may occur as a result of spontaneous chromosomal mutations or by acquisition of extra-chromosomal elements (called plasmids) through conjugation (Pitout *et al.*, 2004; Abdel-Rahman and Kearns, 1998). Plasmid-mediated resistance to β-lactam antibiotics is of major concern in hospitals. Studies show that for both nosocomial and community acquired infections, the mortality and morbidity in prolonged hospitalisation, are twice as high in patients infected with antibiotic-resistant strains than with susceptible strains (Pitout *et al.*, 1997; Silva *et al.*, 1999; Lautenbach *et al.*, 2001). Resistance consequently requires the use of more toxic or expensive antibiotics (Hindler, Howard and Keiser, 1994). Plasmids are self-replicating circular DNA, smaller and separate from the bacterial genome, that can be transferred into another bacterial strain or species (Baker, 1999). They encode multiple resistance

phenotypes and carry genetic information that may provide selective advantage to the bacteria (Mims *et al*., 1993). Bacterial plasmids that encode proteins responsible for antibiotic resistance are referred to as resistance (R) factors (Dever and Dermody, 1991; Hindler *et al*., 1994). Plasmid-mediated resistance can be passed to distantly relate bacterial species by conjugation, and the expression of these enzymes is usually constitutive. Bacteria can possess plasmids that can code for more than one β-lactamase in addition to their expression of chromosomal enzyme. Due to carriage of plasmids and promiscuous exchange of such material between bacteria, these resistance genes have spread widely and are also subject to mutation (Lee, Yuen and Kumana, 2001). Plasmid mediated β-lactamases were first recognized in Gram negative bacteria in the early 1960s, shortly after the introduction of ampicillin (Livermore, 1993). Transposons are genetic elements capable of transfer among a wide-variety of plasmids and of jumping between plasmids and bacterial chromosomes (Heritage *et al*., 1999). Three of the TEM-like β-lactamases are encoded by transposons. TEM-1 is determined by Tn3 and TEM-2 by Tn1, while SHV-1 is encoded by a transposon unrelated to Tn1 (Medeiros, 1984). The occurrence of *bla*TEM genes on mobile genetic elements undermines attempts to classify these elements by genetic location as transposons may jump between plasmids and the bacterial chromosome (Heritage *et al*., 1999). Transposons that encode ESBL activity have also been described (Heritage *et a*l., 1999).

1.13 β-Lactames Inhibitor Combinations

In an effort to overcome the hydrolytic action of β-lactamases, different therapeutic approaches such as the use of (a) β-lactamase stable β-lactam antibiotics, (b) metal ion chelators such as EDTA, (c) amino acid modifiers such as boronic acid, and (d) active-site-directed irreversible inhibitors such as clavulanic acid, sulbactam and tazobactam were attempted (Bush and Sykes, 1986; Sougakoff and Jarlier, 2000). It was found that the combination of a β-lactamase inhibitor such as clavulanic acid with a β-lactam antibiotic offered not only stability against inactivating β-lactamases, but also expanded the spectrum of activity of the primary antibiotic (Chaibi *et al*., 1999). Clavulanic acid is produced by a strain of *Streptomyces clavuligerous* and is a potent inhibitor of plasmid mediated β-lactamases produced by both Gram-positive and Gram-negative bacteria. It is effective against broad spectrum β- lactamases but not enzymes that are primarily cephalosporinases (Bryan and Godfrey, 1991; Livermore and Williams, 1996; Gorbach *et al*., 1997). Clavulanic acid is structurally similar to penicillins and cephalosporins in that it contains a β- lactam ring and is able to fit into the catalytic centre of the β-lactamase enzyme (Rolinson, 1991), although the β-lactam ring is fused to an oxazolidine ring . Clavulanic acid can penetrate the bacterial cell the same way as a β-lactam antibiotic and bind to the catalytic site of intra- and extra-cellular β- lactamases, including those that are plasmid encoded (Richmond-Sykes classes III and V) and the chromosomally encoded β-lactamases (Richmond-Sykes classes II and IV) of Gram-negative and Gram-positive bacteria (Todd and Benfield , 1990; Dever and Dermody, 1991). This binding is a complex physiochemical process. The end result of this binding is the prevention of inactivation of the accompanying β-lactam antibiotic (Lee *et al*., 2001).

The discovery of clavulanic acid and its introduction into clinical practice led to the discovery of other compounds that could function as β-lactamase inhibitors (Chaibi *et al*., 1999). These include derivatives of penicillanic acid, β-lactam sulfones such as sulbactam and tazobactam (Lee *et al*., 2001). Enzyme inhibitors may function via a number of mechanisms, including competitive (reversible), non-competitive and

terminal (suicide) inhibition (Abdul-Rahman and Kearns, 1998). The overall antibacterial spectrum of these combinations depends on microbiological factors such as (i) the effectiveness of the enzyme inactivation, (ii) the amount of β-lactamase produced (iii) the intrinsic properties of the β-lactam in the combination, (v) the permeability and intrinsic susceptibility of bacteria to the inhibitor, (v) the physiochemical conditions such as pH, and (vi) the characteristics of the inhibitor (Sanders *et al*., 1988; Livermore, 1993; Ho *et al*., 1998). Although many investigators feel that the combination of amoxicillin and clavulanic acid should be tested at a fixed concentration of clavulanic acid (Thompson, Miles and Amyes, 1995), it has not yet been specifically decided whether a β-lactamase inhibitor should be used in a fixed concentration or titrated in a fixed ratio with the antibiotic (Greenwood, 1996). For effective inhibition β- lactamase inhibitors must penetrate to the same extent as the β-lactam antibiotic and be present for a long enough period. The ratio of β-lactam to inhibitor normally ranges from 1:1 to 30:1 in terms of weight per dose (Lee *et al*., 2001).

1.14 Inhibitor Resistant β-Lactamases

Until 1989 all plasmid-mediated β-lactamases identified were susceptible to clavulanic acid and could theoretically be controlled by the use of β-lactam inhibitor therapy (Essack, Alexander and Pillay, 1994). However, from the mid- 1980s to early 1990s, inhibitor resistant isolates of members of the family *Enterobacteriaceae* were noted (Thompson *et al*., 1990; Thompson and Sanders, 1992; Zhou *et al*., 1994; French, Shannon and Simmons, 1996). Failure to effectively inhibit these β-lactamases may be attributed to:

(1) Type of β- lactamase involved (Thompson *et al*., 1990)

(2) Modification of the kinetic properties of the TEM β-lactamase due to amino acid substitutions (Bouthors, Jarlier and Sougakoff, 1998; Therrien and Levesque, 2000)

(3) Decreased permeability to β-lactams

(4) Level at which a β-lactamase is produced (Brun *et al.*, 1994)

(5) Decreased uptake of the antibiotic due to modification of the outer membrane protein of Gram-negative organisms (Espinasse *et al.*, 1997; Simpson and Durodie, 1998)

(6) Production of β-lactamase not readily inhibited by suicide inhibitors like most chromosomal class C inducible β- lactamases (Tenover *et al.*, 1999)

(7) Production of OXA-type enzymes and/or hyperproduction of cephalosporinases that is less sensitive than TEM to inhibition by clavulanic acid (Stapleton *et al.*, 1995)

(8) A combination of overproduction and decreased uptake (Stapleton *et al.*, 1995; Espinasse *et al.*, 1997).

1.15 Characterization of Extended Spectrum β-Lactamases (ESBLs) types implicated in *Aeromonas* species

Beta-lactamases are most commonly classified according to two general schemes: the Ambler molecular classification scheme and the Bush-Jacoby-Medieros functional classification system (Ambler *et al.*, 1991; Bush *et al.*, 1995; Rasmussen *et al.*, 1997). The Ambler scheme divides β-lactamases into four major classes (A to D). The basis of this classification scheme rests upon protein homology (amino acid similarity), and not phenotypic characteristics. In the Ambler classification scheme, β-lactamases of classes A, C, and D are serine β-lactamases. In contrast, the class B enzymes are metallo- β - lactamases. The Bush-Jacoby-Medeiros classification scheme groups β-lactamases according to functional similarities (substrate and inhibitor profile). There are four main groups and multiple subgroups in this system. A commonly used working definition is that the ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid. In this exposition the term ESBL will be taken to mean those β-

lactamases of Bush-Jacoby-Medeiros group 2be and those of group 2d which share most of the fundamental properties of group 2be enzymes (Bush *et al.*, 1995). Two inducible β-lactamases A1 and A2 have been described in *Aeromonas* species. A1 is an Ambler class C, Bush group 1 inducible cephalosporinase and A2 (CphA) is an Ambler class B, Bush group 3 zinc-metallo carbapenemase that hydrolyses imipenem and meropenem. A1 is readily identified by the adjacent disc testing used to detect an inducible cephalosporinase and the enzyme is usually found in *A. hydrophila* and *A. caviae*. These species are considered resistant to cephalosporins (except cefpirome and cefepime) and cephamycins (cefoxitin and cefotetan). Aztreonam, cefpirome and cefepime therefore can be tested. *A. sobria* usually lacks the inducible cephalosporinase A1 as is indicated by the absence of a flattened edge of the inhibitory zone around a cefotaxime 5μg disc. This species can be tested against cephalosporins, cephamycins and aztreonam. By contrast, the expression of the A2 carbapenemase may be heterogeneous and resistance to imipenem and meropenem may not be detectable by any conventional method including determination of the MIC. As a result, false susceptibility to carbapenems might be reported. *Aeromona*s species should be reported as resistant to carbapenems with the exception of *A. caviae* since strains of this species do not possess a carbapenemase (Bush *et al*., 1995).

1.15.1 SHV-Type ESBLs (class A) in bacteria

The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (Jacoby *et al.*, 2003). This designation was made because it was thought that the inhibition of SHV activity by p-chloromercuribenzoate was substrate-related, and was variable according to the substrate used for the assay (Sykes and Bush, 1982). Reviews focusing on the SHV-type β-lactamases summarizing kinetic properties of β-lactamases of this family have been published (Tzouvelekis and Bonomo, 1999; Heritage *et al*., 1999).

In 1983, a *Klebsiella ozaenae* isolate from Germany was discovered which possessed a β-lactamase which efficiently hydrolyzed cefotaxime, and to a lesser extent ceftazidime (Knothe *et al*., 1983). Sequencing showed that the β-lactamase differed from SHV-1, by replacement of glycine by serine at the 238 position. This mutation alone accounts for the extended-spectrum properties of this β-lactamase, designated SHV-2. Within 15 years of the discovery of this enzyme, organisms harboring SHV-2 were found in every inhabited continent (Paterson *et al*., 2003), implying that selection pressure from third-generation cephalosporins in the first decade of their use was responsible. SHV-type ESBLs have been detected in a wide range of *Enterobacteriaceae* (Bradford, 2001; Weldhagen *et al*., 2003; Huang *et al*., 2004; Poirel *et al*., 2004)

1.15.2 TEM-Type ESBLs (class A) in bacteria

The TEM-type ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was first reported in 1965 from an *Escherichia coli* isolate from a patient in Athens, Greece, named Temoneira (hence the designation TEM) (Danel *et al*., 1998). TEM-1 is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid. TEM-2 has the same hydrolytic profile as TEM-1, but differs from TEM-1 by having a more active native promoter and by a difference in isoelectric point (5.6 compared to 5.4). TEM-13 also has a similar hydrolytic profile to TEM-1 and TEM-2 (Jacoby and Medeiros, 1991).

TEM-1, TEM-2, and TEM-13 are not ESBLs. However, in 1987 *Klebsiella pneumoniae* isolates detected in France as early as 1984 were found to harbor a novel plasmid-mediated β-lactamase coined CTX-1 (Brun-Buisson *et al*., 1987; Sirot *et al*., 1987). The enzyme was originally named CTX-1 because of its enhanced activity against cefotaxime. The enzyme, now termed TEM-3, differed from TEM-2 by two amino acid substitutions (Sougakoff *et al*., 1988).

In retrospect, TEM-3 may not have been the first TEM-type ESBL. *Klebsiella oxytoca*, harboring a plasmid carrying a gene encoding ceftazidime resistance, was first isolated in Liverpool, England, in 1982 (Jacoby, 1997). The responsible β-lactamase was what is now called TEM-12. Interestingly, the strain came from a neonatal unit which had been stricken by an outbreak of *Klebsiella oxytoca* producing TEM-1. Ceftazidime was used to treat infected patients, but subsequent isolates of *Klebsiella oxytoca* from the same unit harbored the TEM-type ESBL (Jacoby, 1997). This is a good example of the emergence of ESBLs as a response to the selective pressure induced by extended-spectrum cephalosporins.

Well over 100 TEM-type β-lactamases have been described, of which the majority are ESBLs. Their isoelectric points range from 5.2 to 6.5. A number of TEM derivatives have been found which have reduced affinity for ß-lactamase inhibitors. These enzymes have been reviewed elsewhere (Chaibi *et al*., 1999). Complex mutants of TEM (CMT-1 to -4) (Fiett *et al*., 2000; Neuwirth *et al*.,2001; Poirel *et al*.,2004; Sirot *et al*.,1997) has been discovered. A unique TEM-derived enzyme, TEM-AQ, has been found in Italy (Perilli *et al*., 1997). TEM 12 and TEM 24 has been descrided resently from *Aeromomas.* This enzyme has an amino acid deletion not seen in other TEM enzymes plus several amino acid substitutions.

1.15.3 Cefotaximase (CTX-M)-Type ESBLs (class A) and Toho β-Lactamases in bacteria

The CTX-M enzymes have been previously reviewed in detail (Bonnet, 2004). The name CTX reflects the potent hydrolytic activity of these β-lactamases against cefotaxime. Organisms producing CTX-M-type β-lactamases typically have cefotaxime MICs in the resistant range (>64 µg/ml), while ceftazidime MICs are usually in the apparently susceptible range (2 to 8 µg/ml). However, some CTX-M-type ESBLs may actually hydrolyze ceftazidime and confer resistance to this cephalosporin (MICs as high as 256 µg/ml) (Baraniak *et al*., 2002; Poirel *et al*., 2002; Sturenburg *et al*., 2004). Aztreonam MICs

are variable. CTX-M-type β-lactamases hydrolyze cefepime with high efficiency (Tzouvelekis *et al.*, 2000), and cefepime MICs are higher than observed in bacteria producing other ESBL types (Yu *et al.*, 2002). Tazobactam exhibits an almost 10-fold greater inhibitory activity than clavulanic acid against CTX-M-type β-lactamases (Bush *et al*, 1993). It should be noted that the same organism may harbor both CTX-M-type and SHV-type ESBLs or CTX-M-type ESBLs and AmpC-type β-lactamases, which may alter the antibiotic resistance phenotype (Yan *et al.*, 2000).

Toho-1 and Toho-2 are β-lactamases related structurally to CTX-M-type β-lactamases. (Toho refers to the Toho University School of Medicine Omori Hospital in Tokyo, where a child was hospitalized who was infected with Toho-1 β-lactamase-producing *Escherichia coli*) Like most CTX-M-type β-lactamases, the hydrolytic activity of the Toho-1 and Toho-2 enzymes is more potent against cefotaxime than ceftazidime (Labia, 1999; Ma *et al.*, 1998).

It appears that the CTX-M-type β-lactamases are closely related to β-lactamases of *Kluyvera* spp. (Decousser *et al.*, 2001; Di Conza *et al.*, 2002; Humeniuk *et al.*, 2002; Oliver *et al.*, 2001). For example, a chromosomally encoded β-lactamase gene of *Kluyvera georgiana* encoded an extended-spectrum β-lactamase, KLUG-1, which shares 99% amino acid identity with CTX-M-8 (Decousser *et al.*, 2001). CTX-M-type β-lactamases have 40% or less identity with TEM and SHV-type ESBLs.

The number of CTX-M-type ESBLs is rapidly expanding. They have now been detected in every populated continent (Alobwede *et al.*,2003; Baraniak *et al.*,2002; Bou *et al.*,2002; Brenwald *et al.*,2003; Cao *et al.*,2002; Chanawong *et al.*,2002; Coque *et al.*,2002; Dutour *et al.*,2002; Gierczynski *et al.*,2003; Kariuki *et al.*,2001; Ma *et al.*,2002; Moland *et al.*,2003; Pai *et al.*,2001; Paterson *et al.*,2003; Petroni *et al.*,2002; Radice *et al.*,2003; Saladin *et al.*,2002; Wang *et al.*,2003; Yamasaki *et al.*,2003; Yu *et al.*,2002). For some years, CTX-M ESBLs were predominantly found in three geographic areas: South America, the Far East, and Eastern Europe. In Western Europe and North America, CTX-M-type β-lactamases have

previously appeared to be infrequent (De Champs *et al*., 2000). However, in recent years, a number of authors have reported the advent of CTX-M-type ESBLs in these regions (Alobwede *et al*., 2003; Bonnet, 2004; Moland *et al*., 2003, Mushtaq *et al*., 2003). Given the widespread findings of CTX-M-type ESBLs in China and India, it could be speculated that CTX-M-type ESBLs are now actually the most frequent ESBL type worldwide.

The relationship between antibiotic consumption and occurrence of CTX-M-type β-lactamases has not been studied, although the prevalence of the enzymes in agents of community-acquired diarrhea raises speculation that oxyimino cephalosporins available outside the hospital (such as ceftriaxone) may be important. Interestingly, identical β-lactamases have been discovered in widely separated parts of the world (for example, CTX-M-3 has been discovered in Poland and Taiwan), suggesting independent evolution of these enzymes (Gniadkowski *et al*., 1998). Clonal spread of CTX-M-type β-lactamase producing bacteria has been well-documented.

1.15.4 Oxacillinase (OXA)-Type ESBLs (class D) in bacteria

The OXA-type β-lactamases are so named because of their oxacillin-hydrolyzing abilities. These β-lactamases (group 2d) are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50% that for benzylpenicillin (Bush *et al*., 1995). They predominantly occur in *Pseudomonas aeruginosa* (Weldhagen *et al*., 2003) but have been detected in many other gram-negative bacteria. Most OXA-type β-lactamases do not hydrolyze the extended-spectrum cephalosporins to a significant degree and are not regarded as ESBLs. However, OXA-10 hydrolyzes (weakly) cefotaxime, ceftriaxone, and aztreonam, giving most organisms reduced susceptibility to these antibiotics. Other OXA ESBLs include: OXA-11, -14, -16, -17, -19, -15, -18, -28, -31, -32, -35, and-45 (Toleman *et al*., 2003). These confer frank resistance to cefotaxime and sometimes ceftazidime and aztreonam (Danel *et al*., 1995; 1997; 1998). The simultaneous production of a carbapenem-hydrolyzing metalloenzyme and an aztreonam hydrolyzing OXA enzyme can readily lead to resistance to all β-lactam antibiotics (Toleman *et al.,* 2003).

In France, a novel derivative of OXA-10 (numbered OXA-28) was found in a *Pseudomonas aeruginosa* isolate (Poirel *et al*., 2001). A novel ESBL (OXA-18) and an extended-spectrum derivative of the narrow-spectrum OXA-13 β-lactamase (numbered OXA-19) have also been discovered in France in *Pseudomonas aeruginosa* isolates (Philippon *et al*., 1989). The evolution of ESBL OXA-type β-lactamases from parent enzymes with narrower spectra has many parallels with the evolution of SHV- and TEM-type ESBLs. OXA 24 was isolated in several species of *Aeromonas.*

1.15.5 Novel ESBL group in bacteria

A variety of other β-lactamases which are plasmid-mediated or integron-associated class A enzymes have been reported (Bonnet *et al*.,2000; Giakkoupi *et al*.,2000; Matsumoto and Inoue,1999; Mavroidi *et al*.,2001; Poirel *et al*.,2000; Poirel *et al*.,1999; Poirel *et al*.,2001; Silva *et al*.,2000). They are remarkable for their geographic diversity. Novel chromosomally encoded ESBLs have also been described (Bellais *et al*., 2001).

VEB-1 has greatest homology with PER-1 and PER-2 (38%) Poirel *et al*., 1999. It confers high-level resistance to ceftazidime, cefotaxime, and aztreonam, which is reversed by clavulanic acid. The gene encoding VEB-1 was found to be plasmid mediated; such plasmids also confer resistance to non- β-lactam antibiotics. The patient from whom the β-lactamase was originally described was a Vietnamese infant hospitalized in France (Poirel *et al*., 1999). An identical β-lactamase has also been found in *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Pseudomonas aeruginosa* and *Aeromonas jandaei* isolates in Thailand (Girlich *et al*., 2001; Karadenizli *et al*., 2001; Naas *et al*., 1999; Poirel *et al*., 2001). Other VEB enzymes have also been detected in Kuwait and China (Naas *et al*., 1999; Poirel *et al*., 2001).

Guiana extended spectrum (GES) (Poirel *et al*.,2000; Poirel *et al* 2001; Vourli *et al*.,2004 ; Wachino *et al*.,2004; Weldhagen, 2004; Weldhagen and Prinsloo, 2004), BES (Bonnet *et al*.,2000, TLA (Alcantar-Curiel *et al*.,2004), SFO (Matsumoto and Inoue, 1999), and IBC

(Giakkoupi *et al.*,2000; Kartali *et al.*,2002; Lebessi *et al.*,2003; Mavroidi *et al.*,2001; Vourli *et al.*,2004; Vourli *et al.*,2003) are other examples of non-TEM, non-SHV ESBLs and have been found in a wide range of geographic locations.

1.16 Clinical microbiological techniques for ESBL detection

Routine antimicrobial susceptibility testing of significant isolates are performed to make reasonable predictions of the treatment outcome and to facilitate selection of an appropriate antibiotic (Greenwood, 1996; Olsson-Liljequist and Forsgren, 1997; Jorgensen and Ferraro, 1998; Walker and Thornsberry, 1998; Gould, 2000). Factors that should be considered in susceptibility testing include (1) predictability of susceptibility to drug(s) of choice, (2) body sites from where the organism was isolated, (3) quantity of organisms present (quantitative cultures), (4) presence of other organisms and quantitation of each, and (5) presence of any unique host factors (Hindler *et al*., 1994).

1.17 Phenotypic based techniques for ESBL detection

Cephalosporin/clavulanate combination disks: The CLSI advocates use of cefotaxime (30 µg) or ceftazidime disks (30 µg) with or without clavulanate (10 µg) for phenotypic confirmation of the presence of ESBLs (National Committee for Clinical Laboratory Standards, 2005). Disks for use in phenotypic confirmatory tests are available from several suppliers (Becton Dickinson, Oxoid, and MAST). Prior to the combination disks becoming available, it was recommended that clavulanic acid solution be applied to the cephalosporin disks within one hour before they are applied to the agar plates. The CLSI recommends that the disk tests be performed with confluent growth on Mueller-Hinton agar. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk is taken to be phenotypic confirmation of ESBL production (National Committee for Clinical Laboratory Standards, 2005).

Broth microdilution: Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25 to 128 µg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4 µg/ml), cefotaxime (0.25 to 64 µg/ml), and cefotaxime plus clavulanic acid (0.25/4 to 64/4 µg/ml). Broth microdilution is performed using standard methods. Phenotypic confirmation is considered as a ≥3-twofold-serial-dilution decrease in MIC of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone.

Hadziyannis *et al.*, (2000) evaluated a set of 12 isolates with well-characterized β-lactamases using this method. Five isolates with known ESBLs and five without ESBLs were correctly identified. There was one false-positive, a *Klebsiella pneumoniae* isolate lacking ESBLs but hyperproducing SHV-1 with a ceftazidime MIC of 4 µg/ml reducing to ≤0.025 µg/ml with ceftazidime/clavulanate. One *Klebsiella oxytoca* isolate which hyperproduced the chromosomal K1 β-lactamase and was resistant to aztreonam was negative for ESBL production.

Steward and colleagues (2001), in their analysis of 139 *Klebsiella pneumoniae* isolates with ceftazidime or cefotaxime MICs of ≥2 µg/ml, found that the definition for a positive phenotypic confirmatory test using broth microdilution was met in 114 (82%) of isolates. Of the 114 isolates, 108 (95%) met the criteria for a positive phenotypic confirmatory test with both ceftazidime and cefotaxime and 6 (5%) with ceftazidime only. For five of the 139 isolates, the results were indeterminate because no ratio of ceftazidime to ceftazidime/clavulanic acid MICs was calculable. Such a situation arises when the ceftazidime MIC was above the highest dilution tested and the ceftazidime plus clavulanic acid MICs were 64 µg/ml or greater. Testing cefepime and cefepime plus clavulanic acid or utilizing CLSI ESBL disk diffusion confirmatory tests, may sometimes determine whether a clavulanic acid effect truly occurs in such isolates (Steward and colleagues, 2001).

Steward and colleagues have devised a useful algorithm for ESBL testing using phenotypic methods (Steward and colleagues, 2001). They suggest using cefoxitin susceptibility in isolates with positive screening tests but negative confirmatory tests as a means of deducing the mechanism of resistance. Cefoxitin resistant isolates may produce AmpC-type enzymes or possess porin changes, although it must be recognized that these can coexist with ESBL production.

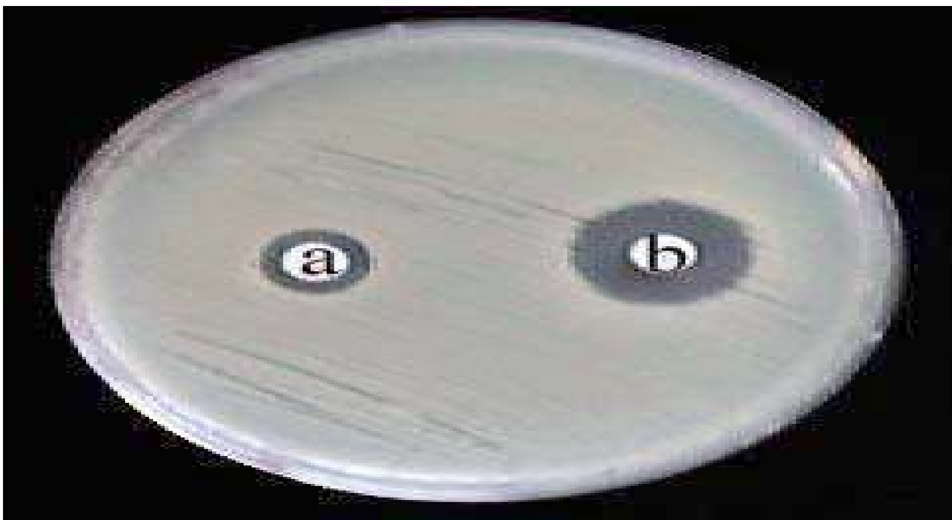

Figure 1.11: NCCLS-style disc diffusion confirmatory test for ESBL production using cefpodoxime. The strain tested on this plate is the ESBL-positive control strain *Klebsiella pneumoniae* ATCC 700603. A ≥5mm increase in the zone of inhibition for the cefpodoxime/clavulanic acid-containing disc versus the zone for the disc containing cefpodoxime alone is considered confirmation of ESBL production. Abbreviations: a, cefpodoxime (10µg); b, cefpodoxime/clavulanic acid (10µg/1µg) (Vercauteen *et al.*, 1997)

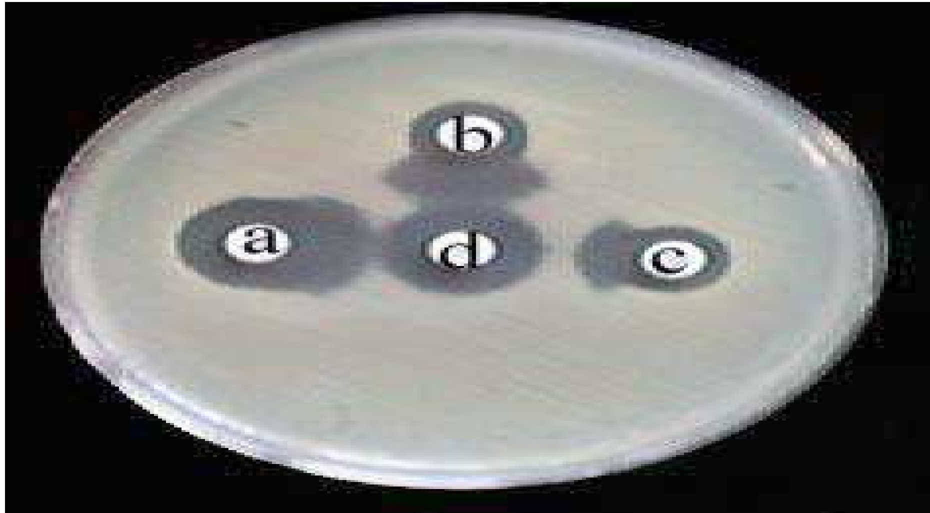

Figure 1.12: Double-disc diffusion test for the detection of ESBL production in *Klebsiella pneumoniae* ATCC 700603 (ESBL-positive control strain). Enhancement of the zone of inhibition around one or more of the β-lactam-containing discs towards the clavulanic acid-containing disc is indicative of ESBL production. Precise placement of discs is important (a distance of 15mm between the discs is recommended) and interpretation is subjective. Abbreviations: a, ceftriaxone (30µg); b, aztreonam (30µg); c, cefpodoxime (10µg); d = amoxycillin/clavulanic acid (20µg/10µg) (Vercauteen *et al.*, 1997)

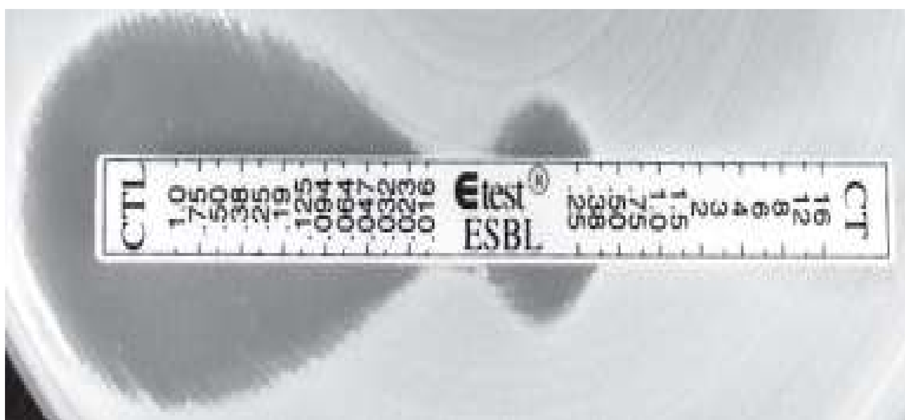

Figure 1.13: Clear cut **ESBL** positive: MIC ratio of ceftazidime/ceftazidime + clavulanate ≥32/0.064≥512**(Vercauteen *et al.*, 1997**)

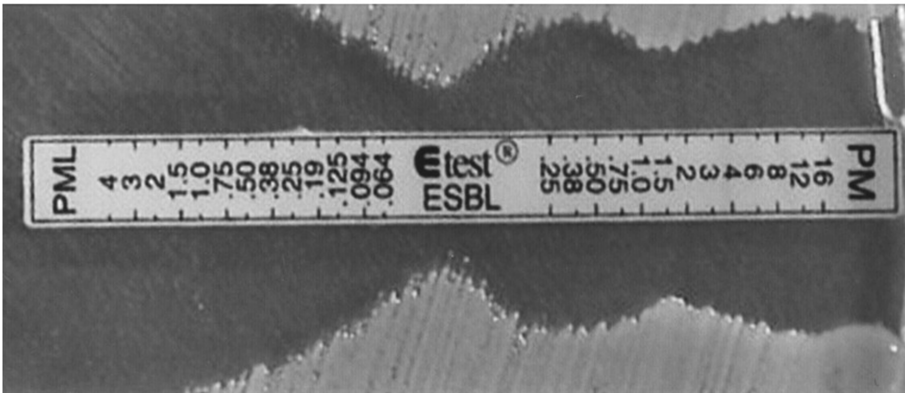

Figure 1.14: A rounded 'phantom' inhibition zone below cefepime end indicative of **ESBL (Vercauteen *et al.*, 1997)**

Figure 1.15: When MIC values were above the test device range, interpretation was 'non-determinable (Vercauteen *et al.*, 1997)

Figure 1.16: Deformation of the cefepime inhibition ellipse indicative of ESBL: **Abrivation: TZ, ceftazidime; TZL, ceftazidime plus clavulanate; PM, cefepime; PML, cefepime plus clavulanate; CT, cefotaxime; CTL, cefotaxime plus clavulanate (Vercauteen *et al.*, 1997**).

1.18 Molecular techniques for ESBL detection

The clinical microbiology techniques mentioned above can only presumptively identify the presence of an ESBL (Bradford, 2001). The identification of specifiec ESBLs in clinical isolates is more complicated (Bradford, 2001). Initial isoelectric point determination was sufficient to identify the ESBL present (Bradford, 2001). However, with various ESBLs present today, many with the same isoelectric point, this method is no longer useful (Bradford, 2001). Currently, various molecular techniques are available to identify specific ESBLs (Bradford, 2001).

1.19 The polymerase chain reaction for ESBL detection

Advances made in polymerase chain reaction (PCR) technology and other molecular based DNA signal and target amplification techniques in the last 20 years , have resulted in these techniques becoming key procedures in molecular diagnostics (Elnifro a *et al*.,2000). These techniques are conceptually simple, sensitive and specific and can be automated (Elnifro *et al*., 2000). Polymerase chain reaction–based techniques are now

common in research laboratories and their use in the diagnostic laboratotory is increasing (Elnifro *et al*., 2000).

The PCR reaction involves two oligonucleotide primers, which flank the target DNA sequence that is to be amplified (Sambrook and Russell, 2001). After the target DNA has been denatured the primers hybridise to opposite strands of the DNA in such an orientation that systhesis by the polymerase enzymes proceeds through the region between the two primers (Sambrook and Russell, 2001). Extension creates two double-stranded target regions, each of which can again be denatured, ready for the second cycle of hybridisation and extension (Sambrook and Russell, 2001). The third cycle generates two double–stranded molecules that comprise precisely the target region (Sambrook and Russell, 2001). Repeated cycles of denatuartion, hybridisation and extension create a rapid exponential accumulation of the specific target fragment of DNA (Sambrook and Russell, 2001). Conventioanl PCR uses end point detection of amplification products ,which relies upon gel electrophoresis of the specific nucleic acids in the presence of ethidium bromide (DNA intercalating dye) followed by visual or densitometric analysis of the resulting bands after irradiation with ultraviolet light (Mackay, 2004).

The use of PCR in the diagnostic laboratory is limited by cost and sometimes the availability of adequate test sample volume if the sample is to be tested for a range of different pathogens (Elnifro *et al*., 2000). A variant of PCR, multiples PCR has been descrided to overcome these shortcomings and to increase the diagnostic capacity of PCR (Elnifro *et al*., 2000). Multiplex PCR is perfomed when more than one target sequence is amplified during the same PCR reaction by icluding more than one pair of primers in the reaction (Elnifro *et al*., 2000). Multiplex PCR has the potential of considerable savings of time and has been applied in many areas of nucleic acid diagnostics, including gene deletion anaysis, mutation and polymorphism analysis, quantitative analysis and RNA detection (Elnifro *et al*., 2000).

1.19.1 Application of molecular methods for detection of ESBL in Gram-negative bacteria

The phenotypic methods may be limited by poor sensitivity, slow-griwth or poorly viable organisms, narrow detection windows, complex interpretation, immunosuppression, antimicrobial therapy, high levels of background and non-specific cross-reactions (Mackay, 2004). Molecular methods based on the genotypic characteristics of microorganisms may overcome some of these limitations.

The increasing number of additional subtypes within each ESBL-family has placed strict limitations on molecular techniques with regard to their ability to cover the whole range of variants within each family (Sundsfjord *et al.*, 2004). Nucleic acid sequencing techniques could probably make this approach more readily available and cost–effective for ESBL-typing (Sundsfjord *et al.*, 2004). Apart from conventional sequencing, pyrosequencing has been used for the identification of GES type ESBLs (Poirel *et al.*, 2006). Pyrosequencing was described as a reliable technique that allows fast identification of short DNA sequences (Poirel *et al.*, 2006).

Table 1.1: Summary of important molecular techniques that can be used for the detection of ESBLs in Gram-negative bacteria (Bradford, 2001)

Test	Advantages	Disadvantages
DNA probes	Specific for a gene family e.g TEM or SHV	Labour intensive. Cannot distinguish between the enzyme variants
PCR	Easy to perform. Specific for a gene family TEM or SHV	Cannot distinguish between the enzyme variants. Quality of template is important to avoid false negative results
Oligotyping	Detects specific TEM variants	Labour intensive .Require specific oligonucleotide probes. Cannot detect new variants.
PCR-Restriction fragment length polymorphism(RFLP)	Easy to perform .Able to detect specific nucleotide changes	to be able to detect nucleotide change ,the change in sequence must result in altered restriction
PCR-Single–strand conformational polymorphism (SSCP)	Able to distinguish between a number of SHV variants	Require special electrophoresis
Ligase chain reaction (LCR)	Able to distinguish between a number of SHV variants	Require a large number of oligonucleotide
Nucleotide sequencing	The Gold standard .Can detect all variants	Labour intensive and expensive. Technically challenging. Manual methods can be difficult to interpret

1.20 Genomic methods for *Aeromonas hydrophila*

Genomic fingerprinting methods are considered the most accurate methods for the typing of microorganisms for epidemiological purposes (Speijier *et al.,* 1999).Typing is an important epidemiological tool utilized for recognizing outbreaks of infection, detection , recognizing particularly virulent strains and monitoring vaccination programmes (Olive and Bean ,1999).

Genomic fingerprinting methods include pulsed-filed gel electrophoresis (PFGE), ribotying (RFLP analysis of Rrna genes for differentiating between species and strains) and PCR-based fingerprinting methods (Speijier *et al*., 1999). Due to the availability of various typing methods there is no general agreement on the optimal typing strategy to be used for a given pathogen (Renders *et al*., 1996). Phenotypic methods such as antibiogram patterns and pyocin typing have been used (Menon *et al.,* 2003). These phenotypic methods however, are time consuming and inconsistent (Menon *et al*., 2003).

The methods used currently are mostly PCR-based and include random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis and amplified fragment length polymorphism (AFLP) analysis (Olive and Bean, 1999).

Table 1.2: Various techniuqe, principle, adventages and disadvantages as outlined by (Holt and Cote, 1998).

Technique and principal	Advantages	Disadvantages
RAPD: Incorporates a single arbitrary designed oligonucleotide primer in an amplification reaction to generate DNA fragment polymorphisms (Annealing site variation)	Least hands-on time. Suitable for high throughput situations. High resolving power when using polyacrylamide-urea gels. Detects differences along whole genome making it useful over long periods. Reproducible, simple and robust	Discriminatory power less than AFLP and Macrorestriction analysis. Moderateintralaboratory reproducibility. Standardization is difficult
RFLP: Use polymorphism seen in restriction enzyme recognition sites, within a particular genetic locus of interest, to form gel bands that differ in size between unlike strains	Simple and reproducible	Moderate discrimination
Macrorestriction analysis: Isolates are digested with SpeI in agarose blocks and the resulting fragments separated by PFGE (Restriction site variation)	Highly reproducible. High discriminatory power. Considered reference method for majority of nosocomial pathogens	Time consuming. Use agarose gels resulting in lower resolving power. Difficult
AFLP:Based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion	High resolving power when using polyacrylamide-urea gels. Highly discriminatory and reproducible	Require purified DNA. Expensive setup and running cost

1.21. Medicinal plants as an alternative means of treatment

Traditional medicine refers to the knowledge, skills, and practices based on the theories, beliefs, and experiences that are indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2000). The WHO reports that traditional medicine is the primary health care system for 80% of the population in developing countries. In Latin America, the WHO Regional Office for the Americas reported that 71% of the populations in Chile and 40% of the population in Colombia had used traditional medicine (WHO, 1999). The WHO indicated that in many Asian countries traditional medicine was widely used, even though Western medicines were often readily available, and in Japan, 60-70% of allopathic doctors reportedly prescribed traditional medicines for their patients (WHO, 1999). It was also reported that more than 3.5 billion people relied on plants for the treatment of both human and livestock diseases (FAO, 1997). The majority of the developing countries depend on traditional medicinal plants for their healthcare (Balick and Cox, 1996). This global utilization of medicinal plants has considerably increased in the last two decades (Dawit, 2001). Maintaining health through traditional medicine in general and utilization of medicinal plants in particular is almost as old as the history of humankind. In developing countries dependence on traditional medicinal plants may be due to inaccessibility of modern medical system, economic and cultural factors (Abbiw, 1996). According to Konno (2004), easy accessibility, efficacy on treatment and affordable cost in getting health services were cited as the main reasons for the preference of traditional medicine over modern medication. Medicinal plants are an integral part of South African culture.

In South Africa, 21st century drug therapy is used alongside traditional African medicines to heal the sick. While plants have been used in traditional medicine to treat various ailments, scientific analyses of the supposed benefits of many plants are still inadequate especially in southern Africa. *Artemisia afra* Jacq. Ex Willd, commonly

known as 'Umhlonyane' in Xhosa, is a shrubby perennial herb with leafy and hairy stem. The plant is widely used for various medicinal purposes by different communities in different geographical areas. *A. afra* has been traditionally used for respiratory disorders such as coughs, colds, whooping cough, bronchitis and asthma. It is also used in treating fevers, mumps swelling, pneumonia, pimples and skin rashes (Van Wyk and Gericke, 2000). *Carpobrotus edulis* L., also referred to as sour fig or Hottentot's fig, is widely utilized as a traditional remedy for a wide range of bacterial and fungal infections (Smith *et al*., 1998) including the treatment of eczema, burns, wounds, TB, vaginal thrush, toothache and earache (Van Wyk *et al*., 1997).

1.22 The objectives of the study:

1- To characterize *Aeromonas* isolates from water sources and stool samples of HIV positive and negative individuals in the Limpopo Province of South Africa.

2- To determine the prevalence of extended spectrum beta-lactamases (ESBLs) and the genes encoding antibiotic resistance in *Aeromonas hydrophila*.

3- To determine genetic relatedness between isolates from water and stool samples of HIV positive and HIV negative individuals by phylogenetic analysis.

4- To determine the antimicrobial activities of selected medicinal plants against *Aeromonas hydrophila.*

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Chapter two

Antimicrobial susceptibility testing of *Aeromonas hydrophila* isolated from Limpopo Province, South Africa using VITEK 2 system, MicroScan WalkAway, disk diffusion and E-test method

2.1 Abstract

A total of 300 isolates of *Aeromonas hydrophila* isolated from water and stool samples were tested using the Vitek 2 system, disk diffusion, MicroScan Walkaway and E-test for antimicrobial susceptibility testing. For the total of 32 antimicrobial tested, the MICs agreement was 99.7% for isolates from all sources. The aim of this study was to determine antimicrobial susceptibility testing of *Aeromonas hydrophila* isolated from Limpopo Province, South Africa by different methodology. Almost 100% of isolates were resistant to ampicillin using both two methods with the MIC ranging from 1 µg/ml to 64 µg/ml. Overall; the agreement of the (antimicrobial susceptibility testing) AST results among all four methods for the drugs tested was 100% Aminoglycosides, 100% Carbapenems, 100% Monobactams, 93% Cephalosporins and 89.4% Beta-lactam/ Beta-lactam inhibitors. Overall agreement between the disk diffusion, MicroScan Walkaway and Vitek methods was 98%, respectively. This study indicated that AST methods correlated with one another when testing *Aeromonas hydrophila* isolates, with a few exceptions. In general, discrepancies among the methods were due to isolates being interpreted as intermediately susceptible or due to an increased number of resistances detected with disk diffusion and a lower number with Vitek and MicroScan Walkaway.

2.2 Introduction

Aeromonas species are microbial etiological agents of diarrhoea particularly in developing countries, where diarrhoeal diseases constitute a very important cause of morbidity and mortality among children and young adults (WHO, 2002). It has been reported that more than 800 millions cases of diarrhoea occur annually in developing countries particularly in rural areas; accounting for about 4.5 million deaths (Oyofo *et al.,* 2002). Children below the age of five especially those in areas devoid of access to potable water supply and sanitation, immunocompromised patients and elderly people are extremely prone to the devastating effects of diarrhoea which might be transmitted by contaminated food and water (Obi *et al.,* 2003). Classical microbial agents of diarrhoea include viruses namely rotaviruses, norwalk viruses, adenoviruses, calici like viruses; parasites such as *Giardia lamblia*, *Cryptosporidium parvum*, *Entamoeba histolytica* and bacteria such as *Escherichia coli*, S*almonella*, *Shigella*, V*ibrio cholerae*, *Aeromonas, klebsiella* and *Campylobacter* species (Obi *et al.,* 2003, Samie *et al.*,2007). Although viruses, particularly rotaviruses, are frequently incriminated in childhood diarrhoea, bacteria and parasitic agents such as *Campylobacter* and *E. histolytica*, constitute major causes of diarrhea in developing countries (Samie *et al.,* 2006). However, incriminating evidence suggest that some emerging agents of diarrhea, such as *Aeromonas* species accounts for a substantial degree of morbidity and mortality in different age groups. Thus, diarrhoeal agent of concern in this study is *Aeromonas hydrophila*

Aeromonas species are important opportunistic pathogens in HIV/AIDS disease and may cause a septicaemic illness in the absence of enteric disease (Manfredi *et al.*, 2002). *Aeromonas* species have emerged as significant causes of gastroenteritis and when clinical laboratories include screenings for *Aeromonas* in routine enteric culture procedures, the percentage-recovery for this organism often exceeds that of *Salmonella* and *Shigella* combined (Wasf *et al.,* 2000). The isolation rate of *Aeromonas* in many developing countries may range from 5% to 28% in clinical isolates (Oberhelman and Taylor, 2000). In food samples particularly poultry , *Aeromonas* has been isolated in

rates as high as 82% in broilers in Senegal (Cardinale *et al.,* 2003) and in 77% of chicken samples in Kenya (Osano and Arimi, 1999). In the Venda region of South Africa, *Aeromonas species* were isolated from clinical and environmental samples (Obi *et al*., 2007).

However studies on *Aeromonas* species have received little attention in South Africa. The management of diarrhoea may depend on the use of antibiotics for bacterial agents such as *Aeromonas* species. Macrolides, cephalosporins and fluoroquinolones are commonly used drugs in the treatment of severe *Aeromonas* infections. However, resistance to these antimicrobial agents have been described worldwide (Engberg *et al*., 2001; Cardinale *et al*., 2003; Upcraft and Upcraft, 2001) and has increased tremendously. Resistance to another macrolide, azithromycin was found in 7% to 15% of *Aeromonas* isolates in 1994 and 1995 in Thailand (Hoge *et al.,* 1998). Cardinale *et al.,* 2003 reported an increase in resistance to fluoroquinolone in Senegal. Multidrug resistance has also been described for *Aeromonas* due to over expression of the EHPgp 1 and 5 genes as well as the production of superoxide dismutase (Higgins 1993). The increasing resistance of microorganisms to antimicrobial agents has necessitated the search for novel and more effective antimicrobial compounds (Obi *et al*., 2003). However, the aim of this study was to determine antimicrobial susceptibility testing of *Aeromonas hydrophila* isolated from Limpopo Province, South Africa by the VITEK 2 system and E-test methods

2.3 Materials and Methods

A total of 1,369 samples (660 stool samples and 709 water samples) were collected during 2005 and 2006 in Limpopo Province and were screened for the presence of *Aeromonas* species. Both stool and water specimens were collected from patients as well as their household drinking in locations of Bela-bela (Waterburg district), Madombidzha (Vhembe district) and Mankweng (Capricorn district). Stool specimens with and without diarrhea were cultured on blood agar (Oxoid Ltd, Basingstoke, UK) and MacConkey agar (Difco/BD Diagnostics Systems, Sparks, MI, USA) and water

samples were plated on Cysteine Lactose Electrolytes Deficient (CLED) agar and MacConkey agar (Difco/BD Diagnostics Systems, Sparks, MI, USA). A total of 300 isolates were used in this study of which 150 were isolated from stool samples and 150 isolated from water samples respectively.

Isolated strains were stored in tubes containing 1.5 ml Brain Heart Infusion broth with 10% v/v glycerol at -70°C until further analysis. The isolates were identified using biochemical tests and confirmed using the API 20E and API 20 NE identification systems (bioMerieux, Marcy-l'Etoile, France). The isolates were further identified by the VITEK 2 system.

2.3.1 Antibiotic susceptibility testing

Microdilution and disk diffusion methods were performed as described by the National Committee for Clinical Laboratory Standards. Susceptibility of *Aeromonas hydrophila* to antimicrobial agents was examined by an agar diffusion method using paper disks containing the following antibiotic concentrations: amikacin (30µg), ampicillin (10µg), gentamicin (10µg), cefalotin (30µg), cefotaxime (30µg), cefoxitin (30µg), ceftazidime (30µg) , piperacillin/tazobactam (100/10µg), amoxicilin/clavulanic acid (20/10µg), ofloxacin (5µg), imipenem (10µg), cefuroxime (30µg) , cefepime (30µg), meropeenem (10µg), cefpdoxime (10µg), trimethoprim/sulfathoxazole (1.25/23.75µg), nitrofurantoin (300µg), norfloxacin (10µg), ofloxacin (5µg), piperacillin (100µg), tobramycin (10µg), colistin (10µg), aztreonam (30µg), cefpirome (30µg), isepamicin (30µg), netilmicin (30µg), pefloxacin (30µg) , ticarcillin (75µg), ticarcillin/clavulanic acid (75/10µg), cefaclor (30µg) , nalidixic acid (30µg) and ertapenem (10µg). These antimicrobial agents were selected on the basis of antimicrobial agents which could be measured by the VITEK 2 system card according to NCCLS guideline M7-A5 (NCCLS, 2000).

2.3.2 VITEK 2 system susceptibility tests

Antimicrobial susceptibilities of the test organisms were determined using the VITEK 2 system (software version 1.02) (bioMérieux) according to the manufacturer's recommendations. The test organisms from colonies grown on 5% horse blood agar after 18 h incubation were suspended in sterile physiological saline to 0.5 McFarland standards. Approximately 2 ml of this suspension was automatically loaded using sterile multichannel pipette into the VITEK 2 ID GNB (Identification-Gram-negative bacilli) and AST (antimicrobial susceptibility testing)-GN04 cards (for Gram-negative bacilli).

2.3.3 MicroScan WalkAway susceptibility tests

MicroScan (Dade Behring, Inc., W. Sacramento, Calif.) susceptibility tests were performed according to the manufacturers' directions. The identity of the bacteria was determined using the MicroScan WalkAway−96 system with conventional Gram−negative breakpoint panels (NBPC 11). Briefly, bacterial suspension was prepared by inoculating 3 ml sterile water with colony isolates and adjusting the suspension to a 0.5 McFarland Standard. The prepared plates were then incubated at 37° C for 24 hours, and zones of inhibition were calculated by measuring the diameter (mm) of the inhibited growth zone.

2.3.4 E-Test susceptibility tests

E-test was performed according to the manufacturer’s instruction. Briefly, an overnight culture of the bacteria diluted to a 0.5 McFarland turbidity standard was used to inoculate Mueller-Hinton agar plate (Oxoid, Basingstoke, UK). After drying, the E-test strips were applied on the plates and incubated overnight at 37 °C. The MICs on both ends were read on the intersection of the inhibition ellipse and the E test-strip edge. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as

positive controls. These tests were performed according to NCCLS M7-A5 guidelines (NCCLS, 2003) and M100-S10 guidelines (NCCLS, 2003), respectively. The MICs were interpreted using the recommended NCCLS thresholds.

2.4 Data analysis

All data analysis was performed by using the SAS System for Windows, release 6.12 (SAS Institute, Cary, N.C.). The resistance breakpoints used in this study was those according to National Committee for Clinical Laboratory Standards. These breakpoints were used to calculate very major, major, and minor errors between the E-Test, MicroScan, and Vitek results. Very major errors occurred with organisms for which MICs indicated resistance by Vitek and susceptibility by the Microscan and E-Test method. Major errors occurred with organisms for which MICs indicated susceptibility by Microscan and resistance by the E-Test and Vitek method. Minor errors occurred with organisms for which MICs indicated intermediate resistance by one or two methods and susceptibility or resistance by the other method. Denominators for calculating error rates, were as follows: the number of resistant isolates (very major error rate), the number of susceptible isolates (major error rate), and the total number of isolates tested (minor error rate).

2.5 Statistical analysis

Simple linear regression analysis was applied to define linear functions correlating the zone of inhibition (mm) with MICs obtained by E-test (mg/l). The E-test and agar dilution variables were linearized by logarithmic conversions.

The E-test results were also compared to the zones of inhibition using the method of least squares as applied to computers. The strength of the linear association between pairs of variables was determined by coefficients of determination (*R*-square): *R*-square ≥50%, strong correlation; *R*-square ≥25–<50%, moderate correlation; and *R*-square < 25%, weak correlation.

The validation of these linear models was carried out by *F*-test. All *P* values reported were two-tailed and values lower than 0.05 were considered significant. The data were analyzed with the Minitab statistical package.

2.6 Results

Three hundred isolates of *Aeromonas hydrophila* of which 150 were isolated from stool samples and the other 150 isolated from water samples were tested against various types of antimicrobials using different methodologies such as E-Test, disk diffusion, MicroScan conventional panels, and Vitek cards (Table 2.1-2.6).

In a comparison of the overall error rates among the different methods and antimicrobial agents for isolates from stool samples, there were a limited number of very major errors for most of the agents tested (Table 2.1-2.2). The exceptions were with the Vitek, in which three (2%) very major errors for Cefuroxime and thirteen (8.7%) for Piperacillin/tazobactam were detected. Only one (n=2; 1.3%) major error was detected for Imipenem in the Vitek; however, there were major errors in the results obtained by MicroScan Walkaway and E-Test. The major error rate for MicroScan Walkaway was highest for Piperacillin/tazobactam (n=5; 3.3%) and Norfloxacin (n=1; 90.7%), and in the E-Test, the major error rate was highest for Tobramycin (n=3; 2%). The highest minor error rate was detected in the Vitek with Aztreonam (n=38; 25.3%), in MicroScan Walkaway with Norfloxacin (n=12; 8%) and in E-Test with Aztreonam (n=43; 28.7%). Overall, there was more than 98.0% agreement with E-Test, MicroScan Walkaway and the Vitek methods, respectively (Table 2.1-2.2).

In a comparison of the overall error rates among the different methods and antimicrobial agents for isolates from water samples, there were no very major and major errors for all the agents tested. The highest minor error rate was detected in the Vitek with Trimethoprim/sulfathoxazole (n=3; 2%), in MicroScan Walkaway with Norfloxacin (n=3; 2%) and in E-Test with Cefepime (n=2; 1.3%). Overall, there was more

than 99.7% agreement with E-Test, MicroScan Walkaway and the Vitek methods, respectively.

In comparison of the overall antimicrobial and interpretation among the different methods and antimicrobial agents for isolates from stool samples, there was resistance to most of the agents tested. The exceptions were with the Disk diffusion, in which most Quinolones were resistant which ranged from 4% to 15%. Only two of five test aminoglycosides showed some resistance (amikacin 7% and Gentamicin 5 %.) Only ampicillin 62% showed resistance amongst all Beta-lactam penicillins tested. There was no resistance was detected on Beta-lactam/ Beta-lactam inhibitors, Carbapenems, Monobactams, Folate antagonists and other such as colistin. However, Cephalosporins showed some resisitance which ranges from 1% to 18%. The Vitek, MicroScan Walkaway and E-Test also show some resistance Quinolones with the E-Test showing less resistance. The aminoglycosides also showed some resistacne for both three methods used ranging from 1% to 23% respectively. (Amongst all tested Beta-lactam penicillins by Vitek, MicroScan Walkaway and E-Test ampicillin showed to be resistance 97% to 100% respectively.). There were no resistances detected on Beta-lactam/ Beta-lactam inhibitors, Carbapenems, Monobactams, Folate antagonists and other antimicrobials such as colistin and Nitrofurantoin tested. However, Cephalosporins showed some resisitance which ranges from 1% to 16%. Overall, there was more than 98.0% agreement with E-Test, MicroScan Walkaway and the Vitek methods with only about 2.0% disagreement with disk diffusion. In a comparison of the overall percentage resistance among the different methods and antimicrobial agents for isolates from water samples, there was some resistance to antimicrobial agents tested, with the exception of ampicilin which showed resistance of 94% to 100%. Overall, there was more than 99.9% agreement with Disk diffusion, E-Test, MicroScan Walkaway and the Vitek methods, respectively.

The MICs for Quinolones range between 1 μg/ml to 64 μg/ml with the exception of Nalidixic Acid which ranged from 1 μg/ml to 128 μg/ml. The MICs of all tested

Aminoglycosides was ≤1 μg/ml to 64 μg/ml with the exception of Tobramycin which ranged from1 μg/ml to 64 μg/ml (Table 2.3-2.6). The MICs of ampicilin was the hights which range from 128 μg/ml to ≥512 μg/ml. The MICS of Beta-lactam/ Beta-lactam inhibitors ≤1 μg/ml to 64 μg/ml, Carbapenems's MICs ranges from 1 μg/ml to 64 μg/ml**.** Cephalosporins MICS ranged from 1 μg/ml to 64 μg/ml with the exception of Ceftazidime which ranged from 1 μg/ml to 128 μg/ml. The MICs ranges of water isolates were 98 to 100% similar to stool isolates.

2.7 Discussion

In this study, the interpretation from four different antimicrobial susceptibility tests (AST) against three hundred isolates of *Aeromonas hydrophila* we compared. In general, the results indicated that the AST methods correlated with one another, with a few exceptions. The most significant discrepancies among the methods generally fell into two categories; the first was the detection of an errors. Overall, the number of very major were with the Vitek, in which three (2%) very major errors for Cefuroxime and thirteen (8.7%) for Piperacillin/tazobactam were detected. Only one (n=2; 1.3%) major errors detected for Imipenem in the Vitek; however, there were major errors in the results obtained by MicroScan Walkaway and E-Test. The major error rate for MicroScan Walkaway was highest for Piperacillin/tazobactam (n=5; 3.3%) and Norfloxacin (n=1; 90.7%), and in the E-Test, the major error rate was for Tobramycin (n=3; 2%). There were also a number of minor errors detected in the study that were more widely distributed among the various typing methods. Of the minor errors, the highest minor error rate was detected in the Vitek with Aztreonam (n=38; 25.3 %). These discrepancies, in part, may be due to the interpretation of the results, because in a number of cases the resistance detected was just over the MIC resistance breakpoint, and the susceptible isolates were detected just below the intermediate-susceptible range with other methods. While there were some discrepancies, overall there was a greater than 98% agreement between each testing method.

When the results of this study were compared to other AST comparison studies, the results were relatively similar. The error rates reported by Guthrie *et al*, 1999 and Rajesh Nayak *et al*., 2007 had a similar pattern to those the present study. Our results were also similar with the results by Guthrie *et al*. (1999) on for trimethoprim/sulfamethoxazole. The pattern of these findings was similar to what was seen for Cefuroxime and Piperacillin/tazobactam in the present study, which had higher error rates compared to the rest of the drugs tested. Study that examined susceptibility testing in Gram negative organisms also had similar results, with overall categorical error rates of around 2% for Vitek and broth microdilution testing, which was similar to the 2.1% to 3.3% range in our study (Karlowsky *et al*., 2003).

The results of this study also demonstrated that the MICs of the quinolones, aminoglycosides and cephalosporins ranged between 1µg/ml to 64µg/ml. Overall, the study confirmed that the interpreted results of the methods were similar for susceptibility testing of *A. hydrophila* isolated from water and stool samples.

Table 2.1: Comparison of three different susceptibility methods on isolates of *Aeromonas hydrophila* from stool samples

Antimicrobial agent	VITEK 2 system			MicroScan WalkAway			E Test		
	Error type			Error type			Error type		
	Very major	Major	Minor	Very major	Major	Minor	Very major	Major	Minor
Quinolones									
Ciprofloxacin	0	0	2	0	0	0	0	0	0
Norfloxacin	0	0	0	0	1	12	0	0	23
Ofloxacin	0	0	0	0	0	0	0	0	0
Pefloxacin	0	0	0	n/a	n/a	n/a	0	0	14
Nalidixic Acid	0	0	0	n/a	n/a	n/a	0	0	0
Aminoglycosides									
Amikacin	0	0	3	0	0	0	0	0	1
Gentamicin	0	0	0	0	0	0	0	0	0
Netilmicin	0	0	0	n/a	n/a	n/a	0	0	0
Isepamicin	0	0	0	n/a	n/a	n/a	0	0	0
Tobramycin	0	0	0	0	0	0	0	3	0
Beta-lactam penicillins									
Ampicillin	0	0	21	0	0	7	0	0	0
Piperacillin	0	0	0	n/a	n/a	n/a	0	0	0
Ticarcillin	0	0	0	n/a	n/a	n/a	0	0	3

Beta-lactam/ Beta-lactam inhibitors									
Piperacillin/tazobactam	13	0	3	0	5	0	0	0	0
Amoxicillin/clavulanic acid	0	0	0	0	0	0	0	0	17
Ticarcillin/calvunic acid	0	0	0	n/a	n/a	n/a	0	0	0
Carbapenems									
Imipenem	0	2	0	0	0	0	0	0	15
Meropenem	0	0	26	n/a	n/a	n/a	0	0	31
Ertapenem	0	0	0	0	0	0	0	0	0
Cephalosporins									
Cefalotin	0	0	10	0	0	1	0	0	0
Cefotaxime	0	0	0	0	0	0	0	0	23
Cefoxitin	0	0	0	0	0	0	0	0	12
Ceftazidime	0	0	13	0	0	0	0	0	0
Cefuroxime	3	0	0	0	0	0	0	0	0
Cefepime	0	0	5	0	0	0	0	0	0
Cefpirome	0	0	0	n/a	n/a	n/a	0	0	0
Cefaclor	0	0	0	n/a	n/a	n/a	0	0	0
Cefpodoxime	0	0	0	n/a	n/a	n/a	0	0	0
Monobactams									
Aztreonam	0	0	38	0	0	0	0	0	43

Folate antagonists									
Trimethoprim/sulfathoxazole	0	0	0	0	0	0	0	0	0
Others									
Colistin	0	0	0	n/a	n/a	n/a	0	0	12
Nitrofurantoin	0	0	0	0	0	0	0	0	0

Table 2.2: Percentage susceptibility of isolates of *Aeromonas hydrophila* from stool samples

Antimicrobial	Disk diffusion			VITEK 2 system			MicroScan WalkAway			E Test		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Quinolones												
Ciprofloxacin	73	17	10	88	2	10	78	12	10	100	0	0
Norfloxacin	67	23	10	93	1	6	77	16	7	100	0	0
Ofloxacin	80	5	15	84	1	15	89	2	9	98	1	1
Pefloxacin	96	0	4	100	0	0	n/a	n/a	n/a	100	0	0
Nalidixic Acid	87	3	10	87	5	8	n/a	n/a	n/a	97	2	1
Aminoglycosides												
Amikacin	67	26	7	71	23	6	84	2	14	76	1	23
Gentamicin	78	17	5	83	14	3	76	3	20	77	4	19
Netilmicin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Isepamicin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Tobramycin	89	3	0	92	5	3	88	3	9	91	3	6
Beta-lactam penicillins												
Ampicillin	25	13	62	0	3	97	0	0	100	0	0	100
Piperacillin	98	2	0	93	2	5	n/a	n/a	n/a	100	0	0
Ticarcillin	99	1	0	100	0	0	n/a	n/a	n/a	100	0	0
Beta-lactam/ Beta-lactam inhibitors												

Piperacillin/tazobactam	100	0	0	100	0	0	100	0	0	100	0	0
Amoxicillin/clavulanic acid	100	0	0	100	0	0	100	0	0	100	0	0
Ticarcillin/calvunic acid	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Carbapenems												
Imipenem	100	0	0	98	2	0	100	0	0	100	0	0
Meropenem	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Ertapenem	100	0	0	100	0	0	99	1	0	100	0	0
Cephalosporins												
Cefalotin	78	12	10	88	9	3	82	5	13	86	2	12
Cefotaxime	71	10	19	86	6	8	77	8	15	81	7	12
Cefoxitin	90	6	4	100	0	0	n/a	n/a	n/a	98	1	1
Ceftazidime	98	1	1	100	0	0	100	0	0	100	0	0
Cefuroxime	79	13	8	92	3	5	88	9	2	73	11	16
Cefepime	100	0	0	100	0	0	100	0	0	97	2	1
Cefpirome	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Cefaclor	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Cefpodoxime	73	9	18	70	16	14	n/a	n/a	n/a	89	5	6
Monobactams												
Aztreonam	100	0	0	98	2	0	100	0	0	100	0	0
Folate antagonists												

Trimethoprim/sulfathoxazole	100	0	0	100	0	0	100	0	0	100	0	0
Others												
Colistin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Nitrofurantoin	88	12	0	94	6	0	93	4	3	98	0	2

Table 2.3: MICs of isolates of Aeromonas *hydrophila* from stool samples

Antimicrobial agent	No. of isolates per MIC(µg/ml)									
	≤1	2	4	8	16	32	64	128	256	≥512
Quinolones										
Ciprofloxacin	ND	112	31	4	1	1	1	0	0	0
Norfloxacin	ND	98	42	6	3	1	0	0	0	0
Ofloxacin	ND	108	33	3	6	0	0	0	0	0
Nalidixic Acid	ND	89	7	4	10	13	5	22	0	0
Aminoglycosides										
Amikacin	75	46	8	22	15	14	9	0	0	0
Gentamicin	16	31	52	14	9	11	17	0	0	0
Tobramycin	ND	96	17	4	12	21	0	0	0	0
Beta-lactam penicillins										
Ampicillin	0	0	0	0	0	0	0	48	13	89
Beta-lactam/ Beta-lactam inhibitors										
Piperacillin/tazobactam	ND	49	54	3	19	25	0	0	0	ND
Amoxicillin/clavulanic acid	ND	56	51	9	22	7	5	0	0	ND

Carbapenems										
Imipenem	56	38	19	17	7	9	4	0	0	0
Ertapenem	43	49	21	4	2	9	14	8	0	0
Cephalosporins										
Cefalotin	ND	31	100	8	5	4	2	0	0	0
Cefotaxime	ND	101	40	6	5	1	0	0	0	0
Cefoxitin	ND	93	38	9	10	0	0	0	0	0
Ceftazidime	ND	89	7	4	10	13	5	22	0	0
Cefuroxime	17	11	49	17	9	31	16	0	0	0
Cefepime	ND	89	23	21	11	5	0	0	0	0
Monobactams										
Aztreonam	ND	78	26	2	19	8	17	0	0	ND
Folate antagonists										
Trimethoprim/sulfathoxazole	ND	66	74	3	3	1	0	0	0	ND
Others										
Nitrofurantoin	44	24	40	7	11	22	0	0	0	0

ND =Not Done

Table 2.4: Comparison of three different methods for the characteraztion of *Aeromonas hydrophila* from water samples

	Very major	Major	Minor	Very major	Major	Minor	Very major	Major	Minor
Quinolones									
Ciprofloxacin	0	0	0	0	0	0	0	0	0
Norfloxacin	0	0	0	0	0	0	0	0	0
Ofloxacin	0	0	0	0	0	0	0	0	0
Pefloxacin	0	0	0	n/a	n/a	n/a	0	0	0
Nalidixic Acid	0	0	0	n/a	n/a	n/a	0	0	0
Aminoglycosides									
Amikacin	0	0	0	0	0	0	0	0	0
Gentamicin	0	0	0	0	0	0	0	0	0
Netilmicin	0	0	0	n/a	n/a	n/a	0	0	0
Isepamicin	0	0	0	n/a	n/a	n/a	0	0	0
Tobramycin	0	0	0	0	0	0	0	0	0
Beta-lactam penicillins									
Ampicillin	0	0	0	0	0	0	0	0	0
Piperacillin	0	0	0	n/a	n/a	n/a	0	0	0
Ticarcillin	0	0	0	n/a	n/a	n/a	0	0	0

Beta-lactam/ Beta-lactam inhibitors									
Piperacillin/tazobactam	0	0	0	0	0	0	0	0	0
Amoxicillin/clavulanic acid	0	0	0	0	0	0	0	0	0
Ticarcillin/calvunic acid	0	0	0	n/a	n/a	n/a	0	0	0
Carbapenems									
Imipenem	0	0	0	0	0	0	0	0	0
Meropenem	0	0	0	n/a	n/a	n/a	0	0	0
Ertapenem	0	0	0	0	0	0	0	0	0
Cephalosporins									
Cefalotin	0	0	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0	0	0
Cefoxitin	0	0	0	0	0	0	0	0	1
Ceftazidime	0	0	0	0	0	0	0	0	0
Cefuroxime	0	0	0	0	0	0	0	0	0
Cefepime	0	0	0	0	0	0	0	0	2
Cefpirome	0	0	0	n/a	n/a	n/a	0	0	0
Cefaclor	0	0	0	n/a	n/a	n/a	0	0	0
Cefpodoxime	0	0	0	n/a	n/a	n/a	0	0	0
Monobactams									
Aztreonam	0	0	0	0	0	3	0	0	0

Folate antagonists									
Trimethoprim/sulfathoxazole	0	0	3	0	0	0	0	0	0
Others									
Colistin	0	0	0	n/a	n/a	n/a	0	0	0
Nitrofurantoin	0	0	0	0	0	2	0	0	0

Table 2.5: Percentages of antibiotic susceptibility patterns of *Aeromonas hydrophila* isolated from water samples

Antimicrobial	Disk diffusion			VITEK 2 system			MicroScan WalkAway			E Test		
	S	I	R	S	I	R	S	I	R	S	I	R
Quinolones												
Ciprofloxacin	25	75	0	97	3	0	100	0	0	100	0	0
Norfloxacin	98	2	0	93	7	0	n/a	n/a	n/a	100	0	0
Ofloxacin	99	1	0	100	0	0	n/a	n/a	n/a	100	0	0
Pefloxacin	87	13	0	87	12	0	n/a	n/a	n/a	97	3	0
Nalidixic Acid	81	20	5	83	17	0	76	23	0	77	23	0
Aminoglycosides												
Amikacin	100	0	0	100	0	0	100	0	0	100	0	0
Gentamicin	100	0	0	100	0	0	100	0	0	100	0	0
Netilmicin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Isepamicin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Tobramycin	89	3	0	92	8	0	88	12	0	91	9	0
Beta-lactam penicillins												
Ampicillin	0	3	97	0	0	100	0	0	100	0	0	100
Piperacillin	100	0	0	100	0	0	100	0	0	100	0	0
Ticarcillin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Beta-lactam/ Beta-lactam inhibitors												

Piperacillin/tazobactam	100	0	0	100	0	**0**	100	0	0	100	0	0
Amoxicillin/clavulanic acid	100	0	0	100	0	0	98	2	0	100	0	0
Ticarcillin/calvunic acid	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Carbapenems												
Imipenem	100	0	0	100	0	0	100	0	0	97	3	0
Meropenem	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Ertapenem	100	0	0	100	0	0	100	0	n/a	100	0	0
Cephalosporins												
Cefalotin	100	0	0	100	0	0	100	0	0	100	0	0
Cefotaxime	100	0	0	100	0	0	100	0	0	100	0	0
Cefoxitin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Ceftazidime	100	0	0	100	0	0	100	0	0	100	0	0
Cefuroxime	100	0	0	100	0	0	99	0	1	100	0	0
Cefepime	100	0	0	100	0	0	100	0	0	100	0	0
Cefpirome	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Cefaclor	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Cefpodoxime	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Monobactams												
Aztreonam	100	0	0	100	0	0	100	0	0	100	0	0
Folate antagonists												

Trimethoprim/sulfathoxazole	100	0	0	100	0	0	100	0	0	100	0	0
Others												
Colistin	100	0	0	100	0	0	n/a	n/a	n/a	100	0	0
Nitrofurantoin	94	6	0	100	0	0	96	4	0	100	0	0

Table 2.6: MIC (µg/ml) of *Aeromonas hydrophila* isolated from water samples

Antimicrobial agent	No. of isolates per MIC(µg/ml)									
	≤1	2	4	8	16	32	64	128	256	≥512
Quinolones										
Ciprofloxacin	ND	126	6	3	5	7	4	0	0	0
Norfloxacin	ND	106	23	6	4	8	3	0	0	0
Ofloxacin	ND	108	33	3	6	0	0	0	0	0
Nalidixic Acid	ND	49	72	5	23	1	0	0	0	0
Aminoglycosides										
Amikacin	34	66	18	30	0	0	0	0	0	0
Gentamicin	66	45	4	7	9	11	8	0	0	0
Tobramycin	ND	18	95	7	11	19	0	0	0	0
Beta-lactam penicillins										
Ampicillin	0	0	0	0	0	0	0	0	37	113
Beta-lactam/ Beta-lactam inhibitors										
Piperacillin/tazobactam	ND	100	33	17	0	0	0	0	0	ND
Amoxicillin/clavulanic acid	ND	112	14	8	16	0	0	0	0	ND
Carbapenems										

Imipenem	132	15	3	0	0	0	0	0	0	0
Ertapenem	143	2	3	1	0	0	0	0	0	0
Cephalosporins										
Cefalotin	ND	101	29	6	10	4	0	0	0	0
Cefotaxime	ND	83	44	18	5	0	0	0	0	0
Cefoxitin	ND	100	33	4	13	0	0	0	0	0
Ceftazidime	ND	111	8	2	15	10	4	0	0	0
Cefuroxime	88	6	47	9	0	0	0	0	0	0
Cefepime	ND	134	16	0	0	0	0	0	0	0
Monobactams										
Aztreonam	ND	133	12	2	3	0	0	0	0	ND
Folate antagonists										
Trimethoprim/sulfathoxazole	ND	99	8	41	2	0	0	0	0	ND
Others										
Nitrofurantoin	90	23	20	10	6	0	0	0	0	0

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Chapter three

Extended-Spectrum β-Lactamases in *Aeromonas hydrophila* isolated from stool samples in the Limpopo Province, South Africa

3.1 Abstract

Aeromonas species producing extended-spectrum beta-lactamases (ESBLs) have been reported in many countries, but information on their prevalence in Africa are scanty. A total of 230 isolates of *Aeromonas hydrophila* were isolated from 660 stool samples and 709 water samples collected in different municipalities in Limpopo Province, South Africa over a period of 18-months. Isolates were screened for production of ESBLs by the double disk diffusion test and for AmpC production by assessing resistance to cefoxitin. *Bla*SHV, *bla*TEM, *bla*CTX-M, and *bla*CMY-2 were sought from all ESBL-positive and cefoxitin-resistant isolates. Only 21 isolates were found to be ESBL producers. All 21 isolates were screened for production of SHV, TEM and CMY-2 ESBL and, they were all (100%) found to be producers of TEM 63 and CMY-2. In conclusion, the results indicated that *Aeromonas hydrophila* produce extended-spectrum beta-lactamases which is an essential enzyme coding for antibiotic resistance.

3.2 Introduction

Resistance to the extended-spectrum cephalosporins among members of the family Enterobacteriaceae has become a growing problem worldwide due to the occurrence of extended-spectrum beta-lactamases (ESBLs) and AmpC-type beta-lactamases (Massinda *et al*., 1991). Two most common inducible β-lactamases A1 and A2 have been found in *Aeromonas* species (Bush *et al*., 1995). A1 is an Ambler class C, Bush group 1 inducible cephalosporinase and A2 (CphA) is an Ambler class B, Bush group 3 zinc-metallo carbapenemase that hydrolyses imipenem and meropenem (Ambler *et al*.,1980 ;Bush *et al*.,1995) . A1 has been found in *A. hydrophila* and *A. caviae* and was identified by the adjacent disc testing which is used to detect an inducible cephalosporinase and the enzyme (Bush *et al*., 1995). These organisms are usually considered resistant to cephalosporins except cefpirome and cefepime and cephamycins such as cefoxitin and cefotetan. An antibiotic which lacks the inducible cephalosporinase such as aztreonam, cefpirome and cefepime may be used for *A. sobria* and this may be indicated by the absence of a flattened edge of the inhibitory zone around a cefotaxime disc (Bush *et al*., 1995).

Although reports of ESBLs associated with *A. hydrophila* are relatively rare but, the number of reported cases is steadily increasing *Aeromonas* spp have been found to express a wide variety of ESBL types, including TEM and SHV (Fosse *et al*., 2004, Marchandin *et al*., 2003). The prevalence of ESBLs and AmpC beta-lactamases in *A. hydrophila* species is of paramount importance in developing countries where infections with these organisms are common (Massinda *et al*., 1991).

Resistance profiles to ampicillin, aztreonam, cefixime, cefepime, cefoxitin, cefotaxime, cefpodoxime, ceftazidime, ceftriaxone, amoxicillin-clavulanic acid and chloramphenicol are rapidly increasing, necessitating the use of fluoroquinolones or extended-spectrum cephalosporins for the treatment of extraintestinal infections (Crump *et al*., 2003).Widespread fluoroquinolone use in children has been discouraged because of the potential adverse effects on cartilage development. Therefore, extended-spectrum cephalosporins such as cefotaxime or ceftriaxone are

becoming the option of treatment of enteric bacterial infections in children. ESBLs production has considerable implications for medical health practitioners in this era of HIV/AIDS as Aeromonas species are gradually becoming ESBLs producers. ESBLs have been found in many enterobacterciae including enteric bacteria such as *Salmonella* in South Africa (Belle *et al*., 2002; Hansen *et al*., 2001).

The study determine the prevalence of ESBLs in 21 *Aeromonas hydrophila* isolates from stool samples of HIV/AIDS individuals in Limpopo Province of South Africa by the use of a competitive PNA-based multiple PCR. Deoxyribonucleic acid sequencing was used to confirm the identity of blaTEM 63 and blaCMY2 genes identified during the study.

3.3 Materials and Methods

Detection of isolates for the production of ß-lactamases was perfomed as follows:

(1) The iodometric tube method, using Penicilin G as the substrate (Livermore & Williams, 1996) was employed. Crude extracts of the ß-lactamases were obtained by sonication and centrifugation of overnight cultures of the test isolates. Twenty µl of starch indicator (containing 1% soluble starch), 20 µl iodine reagent (containing 2% iodine in 53% potassium iodide) and 100µl benzyl penicillin were pipetted into a glass test tube. Crude enzyme (100µl) was added and the mixture vigorously shaken at room temperature. When β-lactamase was present, the blue-black colour of the mixture disappeared and the solution became milky white within 5 min.

(2) The Nitrocefin method where cells were mixed directly onto moist nitrocefin on filter paper and left to stand for one (1) hour for any colour reaction to occur was also used. A change from yellow to red indicated the production of ß lactamase

3.3.1 Detection of ESBL production

Production of ESBLs was studied by the double disc synergy and inhibition potentiated disc diffusion tests as described by Jarlier *et al.*, (1988). Isolates were inoculated on Muellar –Hinton (MH) -agar plates. Discs containing respectively ceftazidime (30µg), cefazolin (30µg), cefoxitin (30µg) and aztreonam were placed 25 mm (centre to centre of the discs), from a disc containing ampicillin/ clavulanic acid (20µg). After overnight incubation at 37°C, the diameters of inhibition zones around the antibiotic discs were measured using a Venier Caliper. A clear extension of the edges of the inhibition zone of any of the antibiotics towards the disc containing clavulanic acid was regarded as a phenotypic confirmation of the presence of ESBL (Jarlier et al., 1988; Acar & Goldstein, 1996). Isolates positive for ESBL production were subjected to polymerase chain reaction (PCR) amplification using primers designed for the detection of *bla*TEM, *bla*CMY2, *blaCTX*-M and *bla*SHV genes.

3.3.2 Crude enzyme extraction

Overnight cultures in brain heart infusion broth were centrifuged at 15 000g for 30 min at 4°C. The pellet was washed with 0,1M phosphate buffer (pH7) and resuspended in the same buffer. Cells were disrupted by French Pressure Cell Press (American Instrument company, Maryland, USA) and the cell debris removed by centrifugation (40 000g, 30 min, 4°C) [Beckman, model L3-50 ultracentrifuge, USA]. The supernatant was used as the crude extract containing the β- lactamase

3.3.3 Whole-cell DNA extraction

Extraction of whole-cell DNA was perfomed by a precipitation –based method, as descrided previously (Philippon and Arlet, 2006). Briefly; one single colony-forming unit (CFU) of *Aeromonas hydrophila* was resuspended in 5 ml brain heart infusion broth (Oxoid Ltd., Hampshire, UK) and incubated overnight at 37°C (WBM SPL 25 Labcon shaking water bath , Laboratory Marketing Services , Marisburg) while shaking at 50 revolutions per minute (RPM) . One ml of the overnight culture was pelleted in a centrifuge at 3 000x g (Laboguge 400r, Heraeus Instruments, Germany)

for 10min at 4°C. The pellet was resuspended in 1 ml sterile distilled water and re-pelleted as descrided above. The resulting pellet was resuspended in 500µl STE buffer containing 75 mM NACL (Promega, Madison, WI), 20 mM Tris (Sigma Chemical Co. St.Louis, MO) and 25 mM EDTA (Promega, Madison, WI) at pH 7.5.

Cells were lysed by adding 25µl of a 20% SDS (Promega, Madison, WI) solution (pH 12.45) and 1 µl of a 50mg/ml stock solution lysozyme (Sigma Chemical Co. St.Louis, MO) with incubation at 37°C (QBT2 heating block , Grant Instruments Ltd ., Cambridge , United Kingdom) for one hour. On completion of cell lysis, 220ul of 5 M NACL (Promega, Madison, WI) was added followed by the addition of 700 µl chloroform/isoamyalcohol (24:1) (Sigma Chemical Co. St.Louis, MO) to separate DNA and protein phases . Following centrifugation at 5 000 x g (Z233 m-2 , Hermle Labortechnik , Wehingen , Germany) for 10min at room temperature (25°C), the upper phase containing whole-cell DNA was removed and transferred to a new tube.

The DNA was precipitated with 700 µl isopropanol (Merck, Darmstadt, Germany) at -20°C for 1 hour or overnight. The precipitated DNA was pelleted by centrifugation at 5 000 x g (Z233 m-2, Hermle Labortechnik, Wehingen, Germany) for 10min at room temperature (25°C) followed by two washing steps with 800 µl of a 70% ethanol solution (Merck, Darmstadt, Germany). After air-drying, the DNA pellet was resuspended in 1 ml TE buffer (Promega, Madison, WI), containing 10 mM Tris/HCL and 1 mM EDTA at pH 7.4. 500 µl of the extracted DNA was stored at 4°C and -20°C until further analysis.

3.3.4 PCR amplification of *bla*TEM 63, *bla*SHV and *bla*CMY2 genes

Polymerase chain reaction analysis was perfomed with the *bla*TEM 63, *bla*SHV and *bla*CMY2 genes specific primer set using whole-cell DNA extracted from 21 ESBLs producing *A.hydrophila* isolates as template. Primer synthesis and purification was conducted by Inqaba Biotechnology Industries Pty.Ltd., South Africa. The reaction consisted of 12.5 µl GoTag Green Master Mix (Promega, Madison, WI), 0.8 µl of each primer (20 mM), 4 µl whole–cell DNA as template and molecular garde water (Promega, Madison, WI) prepared to a final reaction volume of 25 µl. The amplicons obtained were analysed by gel electrophoresis.

3.3.5 Sequence –specific competitive PNA-based muliplex PCR

Two sets of primer pairs were used in a multiplex assay together with a *bla*TEM 63, *bla*SHV and *bla*CMY2 genes specific PNA-probe (Applied Biosystems, Rotkreuts, Switzerland). The selected primer pairs were specific for the detection of *bla*TEM 63, *bla*SHV and *bla*CMY2 genes and amplified 306-and 505 bp products respectively. The multiplex PCR mix (reaction volume 50 µl) consisted of 10 x Mg-free PCR buffer, 1.5 mM MgCl2, a 200µM concentration of each deoxynucleoside triphosphate ,1.25 U of Taq DNA polymerase(Promega, Madison, WI) , a 0.32 mM concentration of each primer ,0.32 µM PNA –probe and 2µl DNA template.

Multiplex PCR amplification was performed on a GeneAmp 9600 thermocycler (Perkin Elmer Cetus, Emeyville, CA). The PCR cycle programme consisted of an initial denaturation step at 95°C for 2 min, followed by 35 amplification cycles each at comprising a danaturation step at 95°C for 30s ,followed by an annealing step at 50°C for 1 min and an extension step at 72°C for 1 min. After the completion of 35 amplification cycles, a final extension step was performed at 72°C for 5 min.

3.3.6 Verification of DNA extractions and PCR products

All DNA extractions were verified by gel electrophoresis at 2 V/cm (Eilte -300 Power supply Wealtec Corp., Kennesaw ,GA) for 1 h in a 1% agarose gel (Pronadisa , Madrid , Spain) containing ethidium bromide (Promega, Madison, WI) (0.5µl/ml) at 4 V/cm (Eilte -300 Power supply Wealtec Corp., Kennesaw ,GA) for 45 min in 1 xTBE running buffer (pH8.3) ,with a 100bp DNA ladder (Promega, Madison, WI) as molecular size marker. All agarose gels were visualized under UV illumination (TFM -26 Ultarviolet Transilluminator, UVP, Upland, CA) and the images captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland, CA).

3.3.7 DNA sequencing of *bla*TEM 63, *bla*SHV and *bla*CMY2 genes

The *bla*TEM 63, *bla*SHV and *bla*CMY2 specific PCR amplicons were sequenced with the forward primer using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix , State College , PA), incorporating the ABI Bih Dye Terminator Cycle Sequencing kit version 3.1 (Aplied Biosystems ,Foster City ,CA) to determine the *bla*TEM 63, *bla*SHV and *bl*aCMY2 identity . Sequencing was performed by Inqaba Biotechnical Industries Pty.Ltd., South Africa. Electropherograms of the sequences generated were inspected using Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia). PCR products obtained with the *blaTEM 63, blaSHV* and *blaCMY2* specific PCR from *enterobacteriaceae* (Hanson *et al*., 2001) and non-typhodial salmonella (Kruger *et al*., 2004) were used as sequencing controls.

Table 3.1. Primers used in the study

Primers	Sequence (5′-3′)	Nucleotide positions	Expected size of amplification product
SHV (F)	**GGGTTATTCTTATTTGTCGC**	1-20	846
SHV(R)	**TTAGCGTTGCCAGTGCTC**	865-846	
CTX-M(F)	**CGCTTTGCGATGTGCAG**	264-280	550
CTX-M(R)	**ACCGCGATATCGTTGGT**	814-798	
CMY(F)	**ATGATGAAAAAATCGTTATGCTGC**	2132-2149	1.145
CMY(R)	**TTATTGCAGCTTTTCAAGAATGCGCCA**	2762-2745	
TEM(F)	**ATAAAATTCTTGAAGACGAAA**	1-22	840
TEM(R)	**GACAGTTACCAATTAATCA**	861-840	

3.4 Results and Discussion

The sequence-selective competitive PNA-based multiplex PCR assay was used to determine the prevalence of *bla*TEM, *bla*SHV, *blaCTX*-M and *bla*CMY2 type genes in clinical isolates of *A.hydrophila* as indicated in figure 3.1-3.4. The amplification products generated during the PNA-based multiplex PCR were subjected to gel electrophresis after which two distinct patterns were detected. The results indicated that one (4.76%) each of the 21 *A.hydrophila* isolates tested positive for a possible *bla*CMY and *bla* TEM respectively. when analysed with competitive PNA-based multiplex PCR.

Sequencing of the amplicons obtained from the possible *bla*TEM and *bla*CMY positive isolates revealed these genes to be *bla*TEM63 and *bla*CMY2 with a 100% identify to the *bla*TEM63 gene (GenBank accession number AF 332513) reported by Katherene *et al* .,2006 and *bla* CMY2 gene (GenBank accession number AY 581207) as also reported (Svetlana egorova *et al* .,2007) .

Attempts to amplify the *bla*TEM and *bla*CMY genes as outlined in tables 3.2 to 3.5 Premega Taq in Buffer A (Promega, Madison, WI) produced little product. Sufficient amplification was achieved using GoTaq Green Master Mix (Promega, Madison, WI). GoTaq Green Master Mix (Promega, Madison, WI) gave better results compared to Premega Taq in Buffer A. Optimised master mixes were thus a better choice of PCR reagents to be used in screening projects, resulting in greater sensitivity. The PNA-based multiplex PCR technique has been reported to be sensitive to template quality which might have resulted in the false-negative results.

The advent of resistance to expanded –spectrum cephalosporins antibiotics in *Aeromonas* species and in other natural AMPC beta-lactamase producing members of the family Enterobacteriaceae is due mainly by a constitutive over expression of chromosomal enzymes (Ehrhart *et al*.,1993). A lot of ESBLs including TEM 24 have also been described in *Aeromonas* species and have been responsible for several diarrheal diseases (Mohammad and Hassan, 2004, Alberto *et al*., 1996, Wang *et al*, 2002, Marchandin *et al*., 2003). Extended-spectrum beta-lactamases among *Aeromonas*

species are of great concern since they are causative agents of diarrhea and other extra-intestinal infections.

It should be noted that the ESBLs, CMY 2 as well as TEM 63 endowed isolates observed in this study are in harmony with published reports on nontyphoidal Salmonella and other organisms in South Africa and elswhere (Kruger *et al*., 2004 , Canton *et al*., 2002), suggesting that *Aeromonas* might have acquired the enzymes from other species of the family *Enterobacteriaceae* . The absence of the CTM and SHV enzymes noted in this study may be related to the mode of transfer of these enzymes from the environment to the patients.

The occurrence of ESBLs in *A.hydrophila* emphasizes the importance of constant surveillance of clinical isolates to determine the prevalence of antibiotic resistance genes (figures 3.1-3.4). The dissemination of beta-lactamases may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of diarrheal infections due to ESBL-producing *A.hydrophila*. Continuous monitoring and evaluation of emerging antibiotic resistance genes in bacteria such as *A.hydrophila* is of great importance in enforcing adequate control measures including policy imperatives on antibiotic usage. Guidelines should also be developed and employed to regulate the use of antibiotics in order to balance the need to provide adequate initial antimicrobial coverage to patients who are at higher risk, taking into account unnecessary utilization of antibiotics. The most important and widespread mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria is due to enzyme mediated antibiotic degradation. β- Lactamase production is mediated by genes carried on a plasmid or on the chromosome. The genes encoding β-lactamases can reside on bacterial chromosomal DNA or on plasmids. The TEM-type and SHV-type are widespread enzymes that attack the narrow-spectrum cephalosporins and most of the penicillins.

Resistance to expanded-spectrum cephalosporins have appeared in clinically significant Gram-negative bacteria, also as a result of the production of β-lactamases.

Among the first of the extended spectrum β-lactamases to cause significant clinical problems, were mutants derived from narrow-spectrum SHV-1 or TEM-1 β-lactamases (Canton *et al*., 2002). Low level TEM-1 causes resistance to ampicillin, amoxicillin and ticarcillin, while higher levels can result in resistance to piperacillin, mezlocillin, cephalothin, cefamandole and cefoperazone. SHV-1 is a narrow spectrum β-lactamase with activity against penicillins. There is a considerable geographical spread of these β-lactamases and they have become a global problem in treating infections.

In this study, antimicrobial susceptibility profiles revealed high level resistance to β-lactam agents such as amoxicillin, cefotaxime, cefuroxime, piperacillin and cefoxitin. ESBL production was indicated in 17% of the isolates by the double-disc diffusion method, the method routinely used in susceptibility testing in most diagnostic laboratories. 19% of the selected isolates produced enzymes expressing TEM-related β-lactamases and 5 % of the isolates produced CMY-2.

All strains had MICs for amoxicillin ranging from 32->128 µg/ml, a value two-fold higher than the susceptibility breakpoint (≤8µg/ml) and for piperacillin ranging from 16->128 µg/ml, also indicating resistance transfer. A plasmid of 9-40 kb that was detected may be responsible for the production of ESBLs in these strains. The *bla*TEM gene was detected in 19% of the strains. The *bla*SHV gene was not detected in all 21/21 of the strains. β-lactam resistance could be attributed to the presence and action of β-lactamases such as the TEM and CMY-2 type enzymes and this resistance can be transmitted between bacteria, causing problems specifically in the health environment with ripple effects on health care delivery (Canton *et al*., 2002).

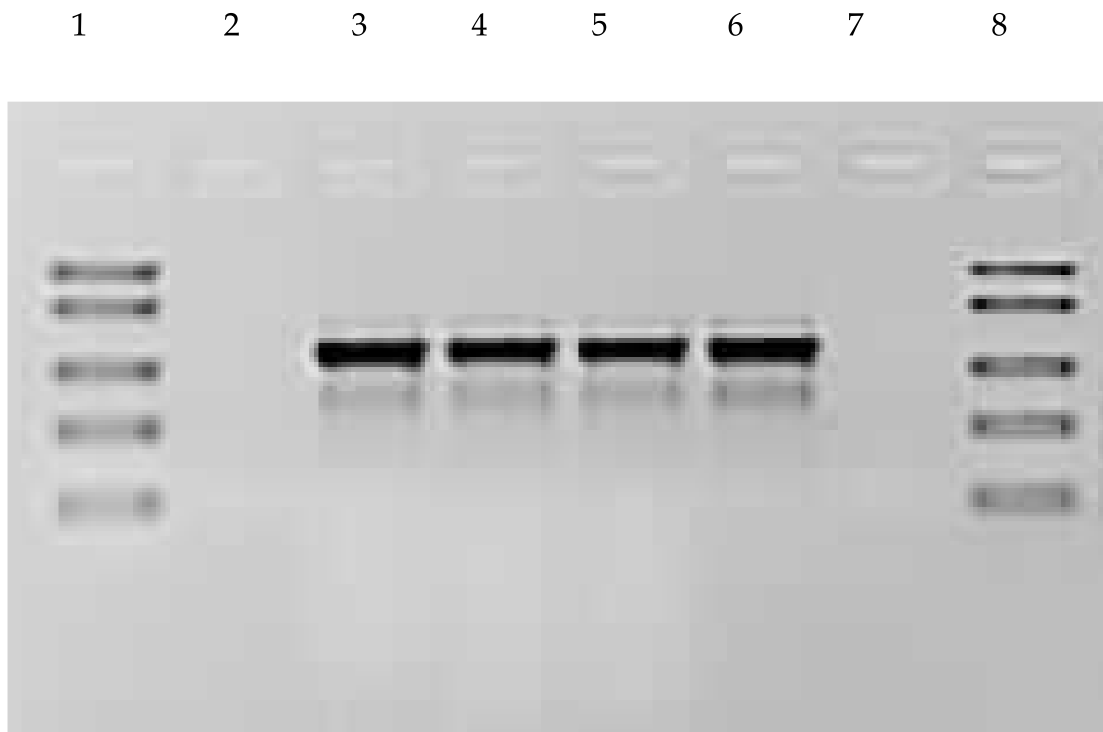

Figure 3.1. Detection and identification of *A. hydrophila* Tem 63 genes by amplification of fragments in the PCR. Lanes 1 and 8,100-5000 bp ladder (invitrogen); Lanes 2 and 7 is water PCR negative control and Lanes 3 ,4 ,5 and 6 different isolates of *A. hydrophila* showing the TEM 63 genes

Table 3.2 PCR products after amplification with the TEM primers

Isolates	***bla*TEM gene**
Sesbl 0001	**-ve**
Sesbl 0002	**+ve**
Sesbl 0003	**-ve**
Sesbl 0004	**-ve**
Sesbl 0005	**-ve**
Sesbl 0006	**-ve**
Sesbl 0007	**+ve**
Sesbl 0008	**-ve**
Sesbl 0009	**-ve**
Sesbl 0010	**-ve**
Sesbl 0011	**-ve**
Sesbl 0012	**-ve**
Sesbl 0013	**-ve**
Sesbl 0014	**+ve**
Sesbl 0015	**-ve**
Sesbl 0016	**+ve**
Sesbl 0017	**+ve**
Sesbl 0018	**-ve**
Sesbl 0019	**-ve**
Sesbl 0020	**-ve**
Sesbl 0021	**-ve**

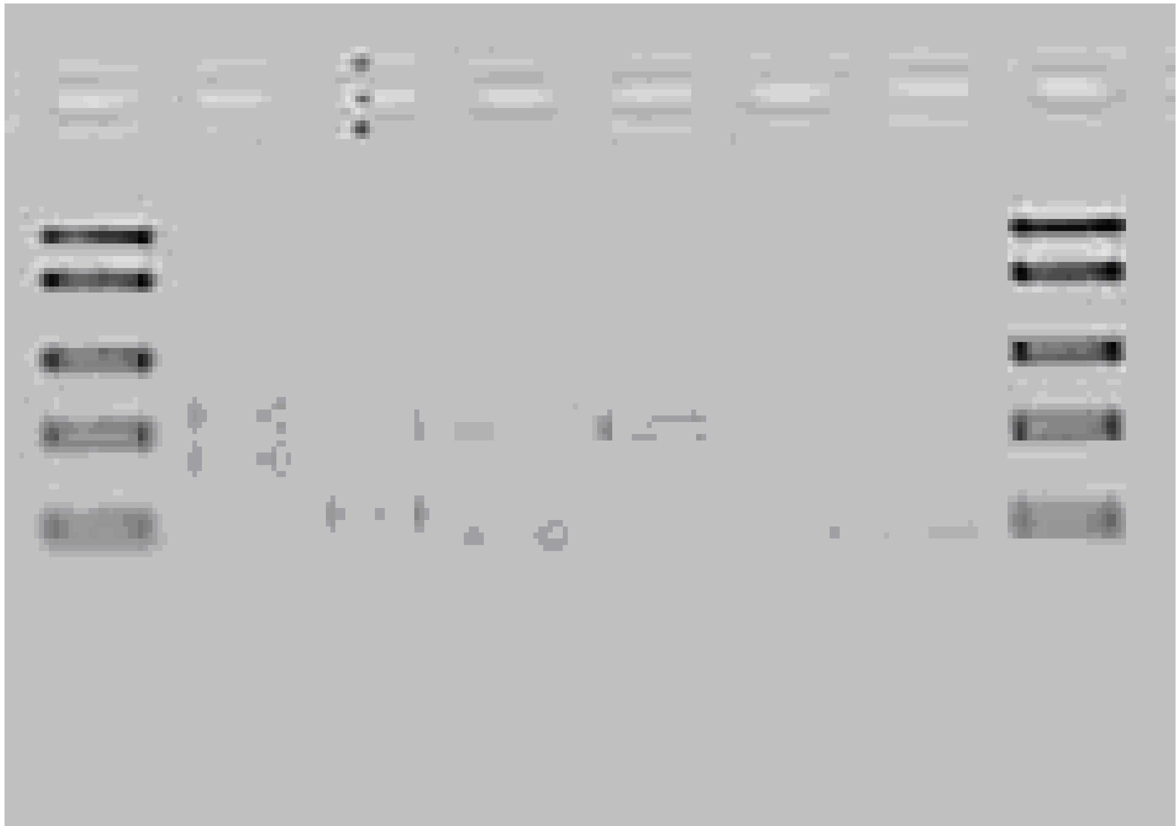

Figure 3.2: Detection and identification of *A. hydrophila* SHV genes by amplification of fragments in the PCR. Lanes 1 and 8,100-5000 bp ladder (invitrogen); Lanes 2 and 7 is water PCR negative control and Lanes 3 ,4 ,5 and 6 different isolates of *A. hydrophila* showing the absence of SHV genes

Table 3.3 PCR products after amplification with the SHV primers

Isolates	***BlaSHV*gene**
Sesbl 0001	**-ve**
Sesbl 0002	**-ve**
Sesbl 0003	**-ve**
Sesbl 0004	**-ve**
Sesbl 0005	**-ve**
Sesbl 0006	**-ve**
Sesbl 0007	**-ve**
Sesbl 0008	**-ve**
Sesbl 0009	**-ve**
Sesbl 0010	**-ve**
Sesbl 0011	**-ve**
Sesbl 0012	**-ve**
Sesbl 0013	**-ve**
Sesbl 0014	**-ve**
Sesbl 0015	**-ve**
Sesbl 0016	**-ve**
Sesbl 0017	**-ve**
Sesbl 0018	**-ve**
Sesbl 0019	**-ve**
Sesbl 0020	**-ve**
Sesbl 0021	**-ve**

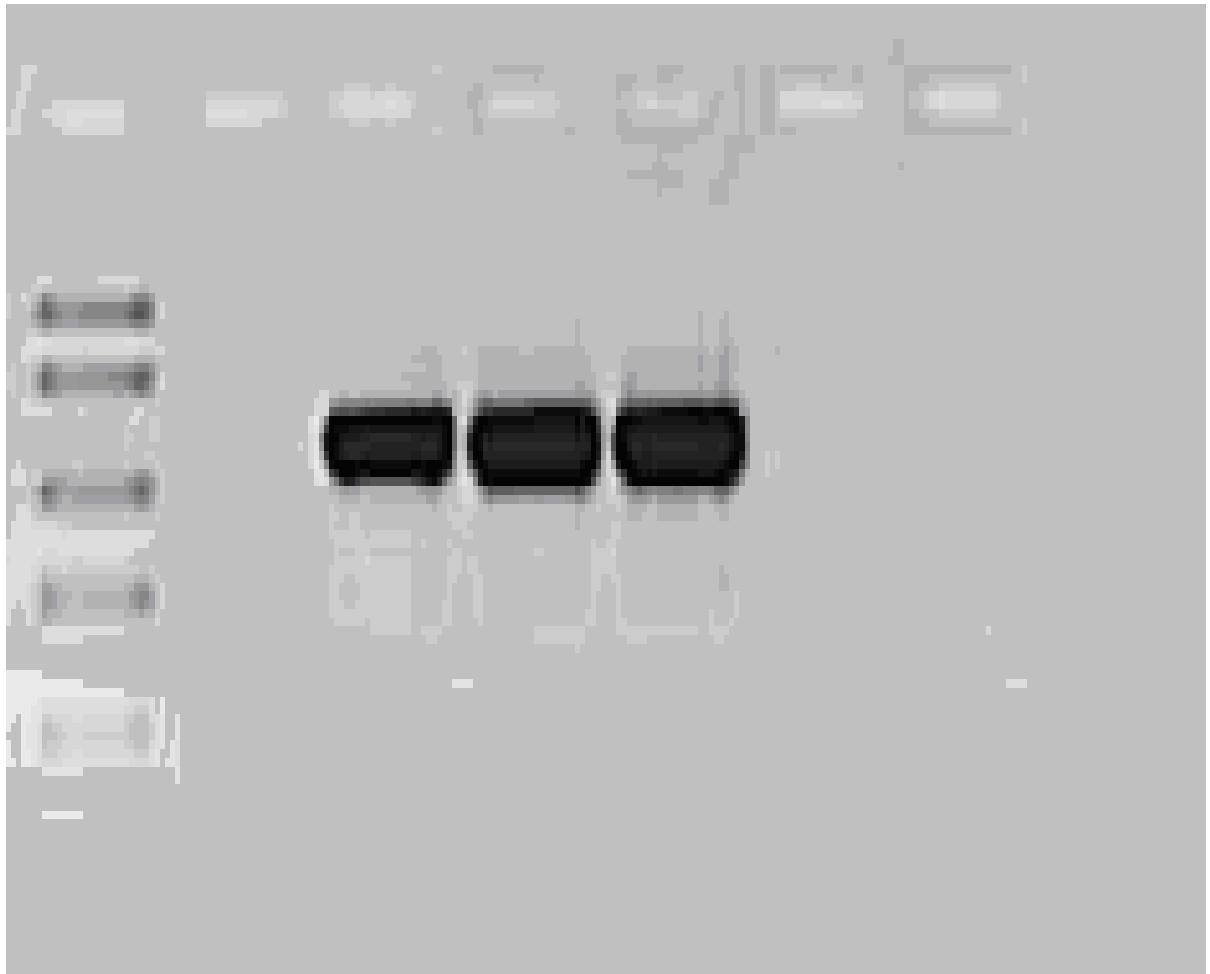

Figure 3.3: Detection and identification of *A. hydrophila* CMY-2 genes by amplification of fragments in the PCR. Lanes 1 ,100-5000 bp ladder (invitrogen); Lanes 2 and 7 is water PCR negative control and Lanes 3 ,4 and 5 different isolates of *A. hydrophila* showing the CMY-2 genes

Table 3.4 PCR products after amplification with the CMY-2 primers

Isolates	***BlaCMY2* gene**
Sesbl 0001	**-ve**
Sesbl 0002	**-ve**
Sesbl 0003	**-ve**
Sesbl 0004	**-ve**
Sesbl 0005	**-ve**
Sesbl 0006	**-ve**
Sesbl 0007	**-ve**
Sesbl 0008	**-ve**
Sesbl 0009	**-ve**
Sesbl 0010	**-ve**
Sesbl 0011	**-ve**
Sesbl 0012	**-ve**
Sesbl 0013	**-ve**
Sesbl 0014	**+ve**
Sesbl 0015	**-ve**
Sesbl 0016	**-ve**
Sesbl 0017	**-ve**
Sesbl 0018	**-ve**
Sesbl 0019	**-ve**
Sesbl 0020	**-ve**
Sesbl 0021	**-ve**

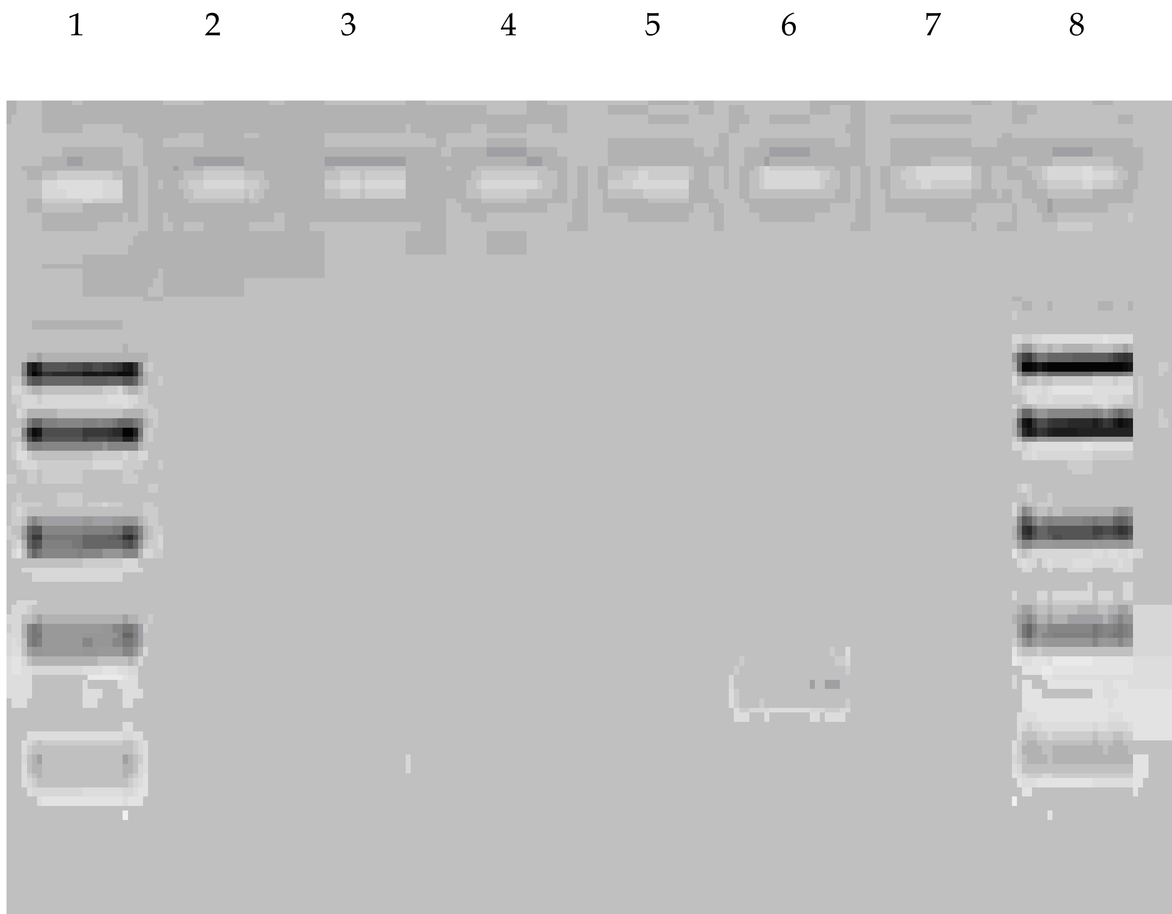

Figure 3.4: Detection and identification of *A. hydrophila* CTX-M genes by amplification of fragments in the PCR. Lanes 1 and 8 ,100-5000 bp ladder (invitrogen); Lanes 2 and 7 is water PCR negative control and Lanes 3 ,4 and 5 different solates of *A. hydrophila* showing the absence CTX-M genes

Table 3.5 PCR products after amplification with the CTX-M primers

Isolates	***BlaCTX-M*gene**
Sesbl 0001	**-ve**
Sesbl 0002	**-ve**
Sesbl 0003	**-ve**
Sesbl 0004	**-ve**
Sesbl 0005	**-ve**
Sesbl 0006	**-ve**
Sesbl 0007	**-ve**
Sesbl 0008	**-ve**
Sesbl 0009	**-ve**
Sesbl 0010	**-ve**
Sesbl 0011	**-ve**
Sesbl 0012	**-ve**
Sesbl 0013	**-ve**
Sesbl 0014	**-ve**
Sesbl 0015	**-ve**
Sesbl 0016	**-ve**
Sesbl 0017	**-ve**
Sesbl 0018	**-ve**
Sesbl 0019	**-ve**
Sesbl 0020	**-ve**
Sesbl 0021	**-ve**

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Appendices

Primer used for detection reaction for forward and reverse reaction

CMY Forward Primer 1A

gmmmcrckgggyaygamtcayaaaacssccrgggtaktrgwwwmaswmctcccargatssccaaaraatga
aaatgaaaskkkgggawkrwccccsgsssrwgascwwccaacrggktttttsccagggkkmsggaccsrwggg
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Cmy forward Primer 1B

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at

Cmy forward Primer 1C

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CMY Reverse Primer 1A

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CMY Reverse Primer 1B

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acasaca

CMY Reverse Primer 1C

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CMY Reverse Primer 1D

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SHV forward primer 1A

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SHV forward primer 1B

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SHV Reverse primer 1A

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SHV Reverse primer 1B

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TEM forward primer 1A

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TEM Forward 1B

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TEM Forward 1C

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TEM Forward 1D

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TEM Forward 1E

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TEM Forward 1F

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TEM Forward 1G

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TEM Reverse 1A

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TEM Reverse 1B

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TEM Reverse 1C

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TEM Reverse 1D

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TEM Reverse 1E

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TEM Reverse 1F

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g

TEM Reverse 1G

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Chapter four

Isolation and characterization of class 1 integron genes in clinical isolates of *Aeromonas hydrophila* from Limpopo Province, South Africa

4.1 Abstract

Extended–spectrum beta-lactamases are considered to be some of the most common and important resistance mechanisms against beta-lactam, penicillin and cephalosporins antibiotics today. The genes encoding for these enzymes are usually carried by multidrug-resistance plasmids and are capable of horizontal transfers among different bacterial species. CMY and TEM type ESBL enzymes have been found mainly in members of enterobactericae around the world and in South Africa the enymes were isolated from *Klebsiella pneumonia* and non-typhoidal salmonellae. Studies on the presence of integron genes among isolates of Aeromonas hydrophila in South Africa have received a cursory attention.

This study investigated the occurrence of integrons in local isolates of *Aeromonas hydrophila*. Results indicated that 5% of ESBL producing isolates harboured integron class 1 enzyme, located on the plasmids.

This study has therefore revealed the presence of integron genes in clinical isolates of *Aeromonas hydrophila* and this is of great epidemiological importance.

4.2 Introduction

Bacteria are able to acquire antibiotic resistance genes to provide protection against most antibiotics. The dissemination of antibiotic resistance genes by horizontal gene transfer has led to the rapid emergence of antibiotic resistance among bacteria (Ploy *et al.,* 2000). Extensive studies have shown that mobile genetic elements such as plasmids and transposons are able to facilitate the spread of genetic material between species or genera of bacteria. In the 1980s, genetic elements termed integrons were identified on these mobile elements **(**Hall and Stokes, 1993).

Integrons are gene-capture and expression systems characterized by the presence of an *intI* gene encoding an integrase, a recombination site (*attI*), and a promoter (Ploy *et al.*, 2000). Integrons are able to capture gene cassettes from the environment and incorporate them by using site-specific recombination. To date, at least eight classes of integrons have been described (Nield *et al*., 2001). Each class is distinguished by differences in the sequences of the integrase genes. Integron classes 1 and 2 contain antibiotic resistance gene cassettes and have been the focus of numerous and widespread studies (Roe *et al*., 2003; Goldstein *et al*., 2001). Class 4 integrons have been termed superintegrons and are found on the small chromosome of *Vibrio cholerae*. Superintegrons harbor hundreds of gene cassettes which encode adaptations that extend beyond antibiotic resistance and pathogenicity (Rowe-Magnus *et al*., 2003). The remaining classes of integrons may also contain antibiotic resistance gene cassettes, but their worldwide prevalence remains low (Nield *et al*., 2001 and Poole *et al*., 2006). Integrons play a major role in the dissemination of antibiotic resistance genes in gram-negative bacteria and are commonly associated with members of the family *Enterobacteriaceae* (Fluit and Schmitz**,** 1999).

Antibiotic uses in clinical and nonclinical settings are tangential to the development of antibiotic-resistant bacteria worldwide. The use of antibiotics as prophylactics and growth promoters in food-producing animals and its effect on the development of antibiotic-resistant bacteria is one area that has become topical in recent times. Of particular concern is the potential for antibiotic-resistant bacteria or the antibiotic

resistance genes carried by these strains to enter the food chain and, consequently, to be passed on to humans (Fluit and Schmitz, 1999). Evaluation of integrons containing antibiotic resistance genes affords the opportunity to provide a means to rapidly assess the potential reservoir of one form of antibiotic resistance that may be present in bacterial populations associated with meat production animals. Methods capable of detecting, isolating, and characterizing integron-containing antibiotic-resistant bacteria may allow the assessment of this form of antibiotic resistance gene transfer between bacterial hosts.

However, different bacterial genera and species exhibit various levels of intrinsic antibiotic resistance, conferred by an active antibiotic resistance mechanism, while others will show inherent resistance due to factors such as decreased uptake of particular antibiotics (Fluit and Schmitz, 1999). In such cases, substantial efforts will be necessary to discriminate between isolates which show inherent resistance and those which have active resistance mechanisms gained through antibiotic resistance genes. The study of integron-borne antibiotic resistance in members of the family *Enterobacteriaceae* has been dominated by clinic-based research and has centered on the phenotypic and genotypic screening of bacteria isolated from symptomatic patients (Schmitz *et al.*, 2001; White *et al.*, 2001). While this approach is useful for monitoring the development of antibiotic resistance within clinically important bacteria, it almost certainly underestimates the variety of integron-associated antibiotic resistance genes that exist and hence the development of antibiotic-resistant bacteria.

The class 1 integron may have originated from the Tn*402* transposon with conservation of the 5_ conserved segment (5_-CS), where the integrase gene and the *attI* site are located (Roe *et al.*, 2003). Downstream of the cassettes there is a 3_ conserved segment (3_-CS) encoding a truncated *qacE* gene, a *sul1* gene, and sometimes, one or two open reading frames, orf5 and orf6. However, the 3_-CS may vary considerably between the class 1 integrons. The genes and open reading frames of the 3_-CS are considered to be trapped gene cassettes, while the original segment from Tn*402* with transposition genes is often completely lost.

Class 1 integrons carry integrase gene (intI1), which code for the site-specific recombinase responsible for cassette insertion (Soler Bistue *et al.*,2006), and include the attI1 site where the cassettes are integrated and a promoter, P_c, is responsible for the transcription of the cassette-encoded genes (Partridge *et al.*, 2000 and Yu *et al.*,2004). Gene cassette contains a single antibiotic resistance gene and a 59-base element (or attC site) downstream of the gene, which is responsible for recombination events (Collis *et al.*, 2002).

Inspite of the clinical significance of Aeromonas only a few studies have, to date, addressed the prevalence of class 1 integrons among isolates (Rosser and Young, 1999 and Schmidt *et al.*, 2001). The role of class 1 integrons in conferring antibiotic resistance to clinical isolates is well documented (Leverstein-van Hall *et al.*, 2003, Segal *et al.*, 2003, White and Rawlinson, 2001). Studies of the incidence of class 1 integrons in bacterial pathogens associated with agriculture and fish farming, such as *Escherichia coli* and *Aeromonas salmonicida,* have also shown a link between integrons and antibiotic resistance. Incidence of integrons in environmental bacteria were undertaken by Rosser and Young (1999) who studied the incidence of class 1 integrons in isolates from the Tay estuary, where 3.6% of isolates carried class 1 integrons. Nield *et al.,* 2001 identified three new integron classes in total community DNAs extracted from Australian soils by using primers for a conserved region of the integrase gene and the 59-be site.

In this study we investigated the occurrence, distribution, and cassette content of integrons among all different ESBL producing *Aeromonas hydrophila* clones isolated from patients in Limpopo Province during 2006 and 2008. The evaluation of the contribution of class 1, class 2, and class 3 integrons to the dissemination of the different ESBL types such as TEM, SHV, CTX-M and the antibiotic resistance genes frequently associated with ESBL-producing organisms was undertaken .

4.3 Material and methods

4.3.1 Bacterial strains

Twenty-one clinical isolates of *Aeromonas hydrophila* were used in this study. The isolates were ESBL producing.

4.3.2 Media and antimicrobial susceptibility testing

Aeromonas hydrophila was grown on 5% bovine blood agar plates at 36°C and incubated for 18 hours. Isolated strains were stored in tubes containing 1.5 ml Brain Heart Infusion broth with 10% v/v glycerol at -70°C for further analysis. The isolates were identified using biochemical tests and confirmed using the API 20E and API 20 NE identification systems (bioMerieux, Marcy-l'Etoile, France).

The susceptibility of the *Aeromonas hydrophila* to antimicrobial agents was examined by using agar diffusion method paper disks containing the following antibiotics concentrations: Amikacin (30µg) , Ampicillin (10µg) , Gentamicin (10µg) , Cefalotin (30µg) , Cefotaxime (30µg) , Cefoxitin (30µg) , Ceftazidime (30µg), Piperacillin/tazobactam (100/10µg) , Amoxicilin/clavulanicacid (20/10µg) , Ofloxacin (5µg), Imipenem (10µg), Cefuroxime (30µg) , Cefepime (30µg), Meropenem (10µg) , Cefpdoxime (10µg) , Trimethoprim/sulfathoxazole (1.25/23.75µg) , Nitrofurantoin (300µg) , Norfloxacin (10µg) , Piperacillin (100µg) , Tobramycin (10µg) , Colistin (10µg) , Aztreonam (30µg) , Cefpirome (30µg) , Isepamicin (30µg) , Netilmicin (30µg) , Pefloxacin (30µg) , Ticarcillin (75µg) , Ticarcillin/clavulanic acid (75/10µg) , Cefaclor (30µg) , Nalidixic acid (30µg) and Ertapenem (10µg) .

Disks were purchased from Oxoid and antimicrobial susceptibilities were interpreted according to National Committee for Clinical Laboratory Standards (NCCLS). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains. Etest strips with gradient concentrations of cefotaxime, ceftazidime, and cefepime at one end and cefotaxime, ceftazidime, cefepime with

Clavulanic Acid (CA) at the other end were used in accordance with the guidelines of the manufacturer (catalogue no. 16V03228, 16V03258, and 16V03478; AB BIODISK, Solna, Sweden). The isolates were also tested for the production of ESBL, ESBL production was determined by a ≥3 twofold-concentration decrease in any MIC of cefotaxime, ceftazidime, or cefepime combined with CA versus its MIC when tested alone. Extended–spectrum beta-lactamases production was also identified by the presence of a phantom zone or a deformation of the cefotaxime, ceftazidime, or cefepime inhibition zone independent of the MIC ratios. A result was considered indeterminate when the MICs were outside the range of MICs of the respective E-test ESBL test strip, and a MIC ratio could therefore not be calculated. *E. coli* ATCC 25922 was used as the control organism.

4.3.3 Plasmid DNA extraction

One single colony-forming unit (CFU) of *Aeromonas hydrophila* was suspended in 5 ml brain heart infusion broth (Oxoid Ltd., Hampshire, UK) and incubated overnight at 37°C (WBM SPL 25 Labcon shaking water bath ,Laboratory Marketing Services ,Marisburg) while shaking at 50 revolutions per minute (RPM) . One ml of the overnight culture was pelleted in a centrifuge at 3 000x g (Laboguge 400r, Heraeus Instruments, Germany) for 10min at 4°C. The pellet was resuspended in 1 ml TE buffer (Promega, Madison, WI) containing 10 mM Tris/HCL (Promega, Madison, WI) AND 1 mM EDTA (Promega, Madison, WI) at pH 7.4 and pelleted as above.

Cells were lysed by adding 600µl of a 4% SDS (Promega, Madison, WI) solution (pH 12.45) with incubation at 37°C (QBT2 heating block, Grant Instruments Ltd., Cambridge, United Kingdom) for 30 min and on n completion of cell lysis 30µl of 2 M Tris (Sigma Chemical Co.,St.Louis,MO) at pH 7.0 was added . After neutralization, chromosomal DNA and protein were precipitated by adding 240 µl of 5 M NACL (Promega, Madison, WI) solution and incubated on ice for 4 hours. After incubation the mixture was centrifuged (Z233 M-2 Hermle LABORTECHNIK, Wehingen, Germany) at 5 000 x g for 10min at room temperature (25°C). DNA was precipitated with 700 µl isopropanol (Merck, Darmstadt, Germany) at -20°C for 1

hour or overnight. The precipitated DNA was pelletd by centrifugation (Z233 M-2 Hermle LABORTECHNIK, Wehingen, Germany) at 5 000 x g for 5 min at room temperature (25°C) with 800 µl of 70% ethanol solution (Merck, Darmstadt, Germany). After air-drying, the DNA pellet was resuspended in 1 ml TE buffer (Promega, Madison, WI), containing 10 mM Tris/HCL and 1 mM EDTA at pH 7.4. 500 µl of the extracted DNA was stored at 4°C and -20°C until further analysis.

4.3.4 Whole-cell DNA extraction

Extraction of whole-cell DNA was performed by a precipitation-based method in combination with a repeated final washing step with 70% ethanol to improve template quality by desalting. Extracted DNA was resuspended in TE buffer (pH 7.4) (Promega, Madison, WI) and stored in 500 µl aliquoats at -20°C and -70°C until further analysis.

4.3.5 Chromosomal DNA extraction

Extraction of chromosomal DNA from *A. hydrophila* was accomplished by spooling the chromosomal DNA onto a class rod from extracted whole-cell DNA. Spooled chromosomal DNA was resuspended in 200 µl TE buffer (pH 7.4) (Promega, Madison, WI) and stored in 100 µl aliquot at -20°C and -70°C until further analysis.

4.3.6 Competitive PNA-based multiplex PCR

Peptide nucleic acid-based multiplex PCR amplification and detection of amplicons was peformed as described in the previous chapters. The PNA-based multiplex amplification was conducted on a Gene Amp PCR thermocycler system 9600 (Perkin Elmer Cetus, Emeryville, CA). The primers used for the amplification of these genes and the predicted sizes of the amplification products were: PrimersINT-E(F),(AGGATGCGAACCACTTCATC) and INT-E(R) CGGTGCCTGAGTCAATTCTT plasmids from successful clones were used to determine the sequence of the integron by the dideoxynucleotide-chain termination method ,with an automatic DNA sequencer (ABI 3130 XL).

4.3.7 Class 1 integron specific PCR

The primers INT-E(F) and INT-E(R) were synthesized and purified by Iqaba Biotechnolgies Industries Pty Ltd.,South Africa. The class 1 integron specific PCR reaction mix consisted of 12.5 µl GoTaq Green Master Mix (Promega, Madison, WI) ,0.1µM of each primer (20 µM), 2 µl plasmid DNA as template and water to a final volume of 25 µl .

4.3.8 Verification of DNA extractions and PCR products

DNA extractions and class 1 integron specific PCR products were verified by gel electrophoresis at 2 V/cm (Eilte -300 Power supply Wealtec Corp., Kennesaw ,GA) for 1 h in a 1% agarose gel (Pronadisa ,Madrid ,Spain) containing ethidium bromide (Promega, Madison, WI) (0.5µl/ml) at 4 V/cm(Eilte -300 Power supply Wealtec Corp., Kennesaw ,GA) for 45 min in 1 xTBE running buffer (pH8.3) ,with a 100bp – 10 000 bp DNA ladder (Promega, Madison, WI) as molecular size marker. All agarose gels were visualized under UV illumination (TFM -26 Ultarviolet Transilluminator, UVP, Upland, CA) and the images captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland, CA).

4.3.9 Conditions for amplification using the IntI1 and IntI2

Conditions for amplification using the IntI1 and IntI2 primers were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s; 62 °C for 30 s; and 72 °C for 60 s. Conditions for amplification using the Cal1 primers were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 120 s. If no amplification of the targeted gene was observed, then amplification of a 356 bp fragment of 16S rRNA sequence was used as a positive control and distilled water (without DNA template) as a negative control. The sequence of the primers and the PCR operating conditions for the 16S rDNA are as described by Wang *et al*. (2003).

4.4 Results and Discussion

Sequence–selective, competitive PNA-based multiplex PCR was performed on both plasmids and DNA obtained from twenty-one ESBL positive isolates. Of all the positive isolates, only one (4.76%) carried the integron class 1 gene. A consensus sequence results obtained for class 1 intergron-specific PCR is given in Figure 4.1. The Class 1 integron sequence data maps are presented in Figure 4.2. Sequence analysis of the 50bp cloned PCR amplicon reveaveld the structure of class 1 intergron, such as the 5′-CS element containing an IntI1 intergrase gene with its own promoter region, an atti1 recomination site, and the 3′-CS element containing qacΔ1.

Class 1 integron and gene cassettes have been found in a number of different bacterial genera and appear to be prevalent in nature (Leverstein-van Hall *et al.*, 2003). This result were similar to those reported by Rosser and Young (1999), who demonstrated that 3.6% of Gram-negative bacteria in an estuarine environment contained the class 1 integron in UK. In contrast, to our study clinical isolates from other studies revealed that the prevalence of class 1 integron in other Gram-negative bacteria were 52% in Taiwan (*E. coli)* (Yu *et al*., 2003), 54.6% in Korea (*E. coli*) (Yu *et al*., 2003), 59% in France (*Enterobacteriaceae*) and 9.4% in China (*Shigella* spp.) (Pan *et al*., 2006). This discrepancy may be due to the differences in bacterial genera or sources of isolates

An increasing incidence of multidrug resistance amongst *Aeromonas* spp. isolates, which are emerging, has been observed worldwide. This can be attributed to the horizontal transfer of mobile genetic elements, viz.: plasmids and class 1 integrons. Class 1 integrons are commonly found in antibiotic-resistant clinical isolates of gram-negative bacteria. Each class 1 integron contains up to several gene cassettes encoding drug resistance, and the pool of such cassettes seems to be large. The cassettes are incorporated into the attI site of the integron by the site-specific enzyme integrase, encoded by the intI1 gene, which is identical in all class 1 integrons. The class 1 integron may have originated from the Tn402 transposon with conservation of the 5' conserved segment (5'-CS), where the integrase gene and the attI site are

located (Fluit *et al*., 1999, Kamali-Moghaddam *et al*., 2000, Recchia *et al*., 1997). Downstream of the cassettes there is a 3' conserved segment (3'-CS) encoding a truncated qacE gene, a sul1 gene, and sometimes, one or two open reading frames, orf5 and orf6. However, the 3'-CS may vary considerably between the class 1 integrons. The genes and open reading frames of the 3'-CS are considered to be trapped gene cassettes, while the original segment from Tn402 with transposition genes is often completely lost (Fluit *et al*., 1999, Kamali-Moghaddam *et al*., 2000, Recchia *et al*., 1997). The presence of class 1 integrons reportedly varied from 0 to 90% among species (Kamali-Moghaddam *et al*., 2000).

Integrons and gene cassettes have been identified as primary sources of resistance and are suspected to serve as reservoirs of antimicrobial resistance genes within microbial populations (Bass *et al*., 1999; Ochman *et al*., 2000 and Barlow *et al*., 2004)

Approximately 29% of all the isolates in our study showed a multidrug resistance phenotype. Eighteen percent of the isolates were resistant to cephalothin; however, only 2% were resistant to ceftriaxone, which also belongs to the cephalosporin family. Resistance to ampicillin was prevalent in approximately 11% of the isolates. Resistance to tetracycline (7%), kanamycin (4%), gentamicin (1%), and streptomycin (30%) were also noted rate to the fluoroquinolone, ciprofloxacin was 2%. Three (<1%) of the 21 isolates were resistant to sulfamethoxazole.

4.5 Conclusion

The potential emergence of class integrons in *Aeromonas hydrophilia* may be facilitated by horizontal transfers from related genera of the family enterobacteriaceae and could spell further conundrum in the use of antibiotics for the management of Aeromonas related infections. A more comprehensive study to unravel the extent of integrons associated Aeromonas isolates is therefore warranted.

gcacgatgatcgtgccgtgatcgaaatccagatccttgacccgcagttgcaaaccctcactgatccgcatgcccgttccata

cagaagctgggcgaacaaacgatgctcgccttccagaaaaccgaggatgcgaaccacttcatccggggtcagcaccacc

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cgacttcgctgctgcccaaggttgccgggtgacgcacaccgtggaaacggatgaaggcacgaacccagtggacataag

cctgttcggttggtaagctgtaatgcaagtagcgtatgcgctcacgcaactggtccagaaccttgaccg

← IntI1

aacgcagcggtggtaacggcgcagtggcggttttcatggctt

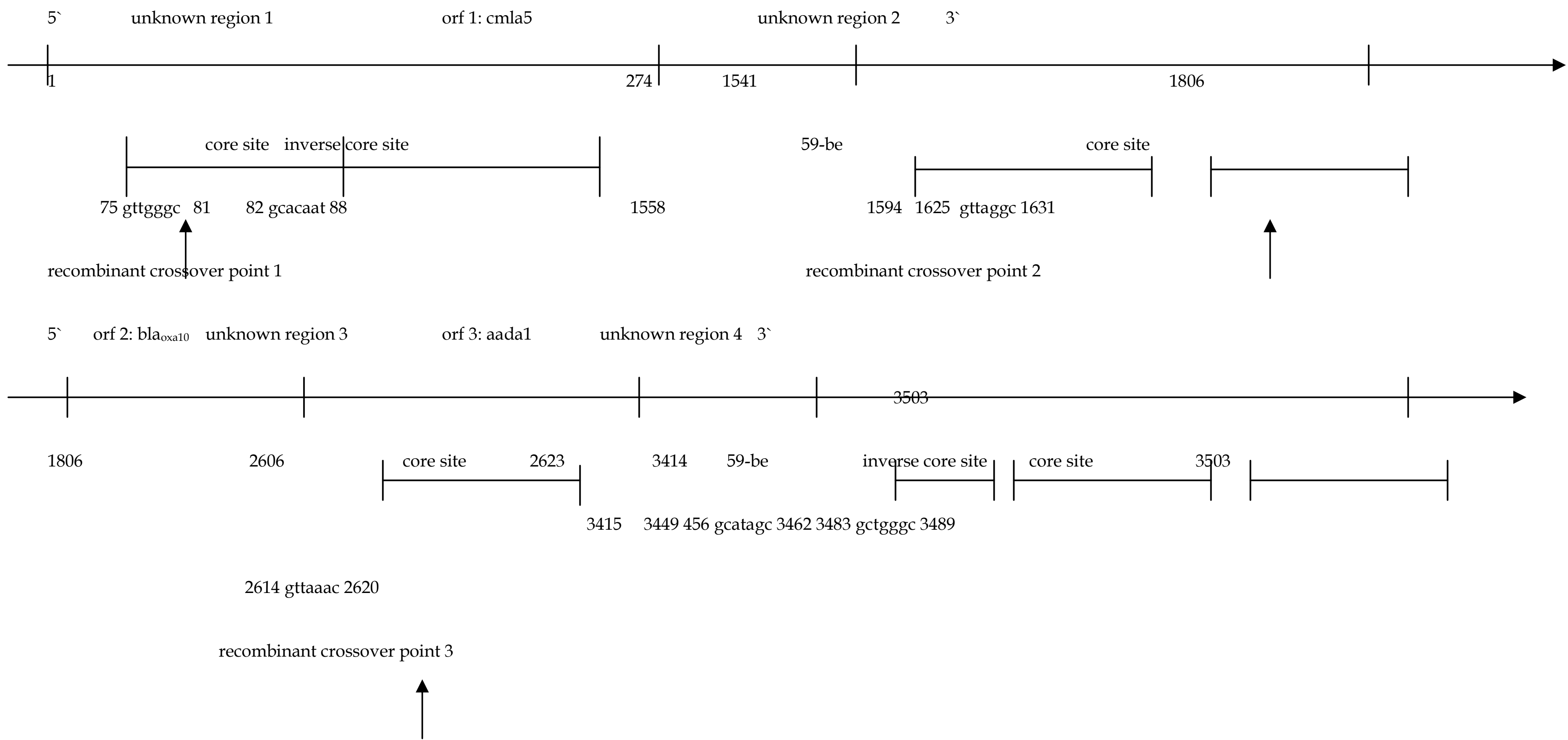
Core site 479-481

gttatgactgtttttttgtacagtctatgcctcgggcatccaagcag

508 - 514

caagcgcgt

Figure 4.1 consensus sequence



closest relatives in genbank, embl, ddbj databases:

1) *aeromonas veronii* – ef034153, 2) *acinetobacter baumannii* – ct025832

Figure 4.2: Class 1 integron sequence data maps

4.5.1 The integron deposited in the genbank under acession number as discribed below , including the origin of the gene

LOCUS EU328347 3503 bp DNA linear BCT 02-JAN-2008
DEFINITION. Aeromonas hydrophila strain 14 class 1 integron, partial sequence.
ACCESSION EU328347
VERSION EU328347.1 GI:163963004
KEYWORDS
SOURCE Aeromonas hydrophila
ORGANISM *Aeromonas hydrophila*
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;aeromonadaceae; Aeromonas.
REFERENCE 1 (bases 1 to 3503)
FEATURES Location/Qualifiers
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/organism="Aeromonas hydrophila"
/mol_type="genomic DNA"
/strain="14"
/db_xref="taxon:644"
/note="PCR_primers=fwd_name: Class 1 integron 5`CS,
rev_name: Class 1 integron 3`CS"
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/note="acquired exogenously for antimicrobial resistance"
/mobile_element="integron: class 1"
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/note="putative core site; recombinant cross-over site"
misc_recomb 82.88
/note="putative inverse core site"
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/gene="cmlA5"
CDS 228.1541
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/protein_id="ABY50545.1"
/db_xref="GI: 163963005"

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GGLAYVVASMGLALTSSAEVFLGLRILQACGASACLVSTFATVRDIYAGREES
NVIYG

ILGSMLAMVPAVGPLLGALVDMWLGWRAIFAFLGLGMIAASAAAWRFWPE
TRVQRVAG

LQWSQLLLPVKCLNFWLYTLCYAAGMGSFFVFFSIAPGLMMGRQGVSQLGFS
LLFATV

AIAMVFTARFMGRVIPKWGSPSVLRMGMGCLIAGAVLLAITEIWASQSVLGFI
APMWL

VGIGVATAVSVSPNGALRGFDHVAGTVTAVYFCLGGVLLGSIGTLIISLLPRNT
AWPV

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misc_recomb 1624..1630
/note="core site; recombinant cross-over point"
gene 1806..2606
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CDS 1806..2606
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EGQLR

ISAVNQVEFLESLYLNKLSASKENQLIVKEALVTEAAPEYLVHSKTGFSGVGTE
SNPG

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gene 2623..3414
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CDS 2623..3414

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VPEQ

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/note="inverse core site"
misc_recomb 3482.3488
/note="putative core site"

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ORIGIN

1 tgacgcgcct gttgcgcatg gcttcgccat tttatgcgca ataggcgcgc caccctgtcg
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2341 cagcatctaa agaaaaccag ctaatagtaa aagaggcttt ggtaacggag gcggcacctg
2401 aatatctagt gcattcaaaa actggttttt ctggtgtggg aactgagtca aatcctggtg
2461 tcgcatggtg ggttgggtgg gttgagaagg agacagaggt ttactttttc gcctttaaca
2521 tggatataga caacgaaagt aagttgccgc taagaaaatc cattcccacc aaaatcatgg
2581 aaagtgaggg catcattggt ggctaaaaca aagttaaaca tcatgaggga agtggtgatc
2641 gccgaagtat cgactcaact atcagaggta gttggcgtca tcgagcgcca tctcgaaccg
2701 acgttgctgg ccgtacattt gtacggctcc gcagtggatg gcggcctgaa gccacacagt
2761 gatattgatt tgctggttac ggtgaccgta aggcttgatg aaacaacgcg gcgagctttg
2821 atcaacgacc ttttggaaac ttcggcttcc cctggagaga gcgagattct ccgcgctgta
2881 gaagtcacca ttgttgtgca cgacgacatc attccgtggc gttatccagc taagcgcgaa
2941 ctgcaatttg gagaatggca gcgcaatgac attcttgcag gtatcttcga gccagccacg
3001 atcgacattg atctggctat cttgctgaca aaagcaagag aacatagcgt tgccttggta
3061 ggtccagcgg cggaggaact ctttgatccg gttcctgaac aggatctatt tgaggcgcta
3121 aatgaaacct taacgctatg gaactcgccg cccgactggg ctggcgatga gcgaaatgta
3181 gtgcttacgt tgtcccgcat ttggtacagc gcagtaaccg gcaaaatcgc gccgaaggat
3241 gtcgctgccg actgggcaat ggagcgcctg ccggcccagt atcagcccgt catacttgaa
3301 gctagacagg cttatcttgg acaagaagaa gatcgcttgg cctcgcgcgc agatcagttg
3361 gaagaatttg ttcactacgt gaaaggcgag atcaccaagg tagtcggcaa ataatgtcta
3421 actcaagcgt tagatgcact aagcacataa ttgctcacag ccaaactatc aggtcaagtc
3481 tgctaggatc cgaattcgtc gac

Primers used for detecting reaction: forward and reverse reactions

Integron forward primer 1A

tkracatgcctccgcacgatgatcgtgccgtgatcgaaatccagatccttgacccgcagttgcaaaccctcactgatcc
gcatgcccgttccatacagaagctgggcgaacaaacgatgctcgccttccagaaaaccgaggatgcgaaccacttc
atccggggtcagcaccaccggcaagcgccgcgacggccgaggtcttccgatctcctgaagccagggcagatccgt
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ctggtccagaaccttgaccgaacgcagcggtggtaacggcgcagtggcggttttcatggcttgttatgactgttttttt
gtacagtctatgcctcgggcatccaagcagcaagcgcgttacgccgtgggtcgatgtttgatgttatggagcagca
wcrmtgttwckcmrcawaacgatttttcatmaaar

Integron forward primer 1B

Tgrcatgacatccgcacgatgatcgtgccgtgatcgaaatccagatccttgacccgcagttgcaaaccctcactgatc
cgcatgcccgttccatacagaagctgggcgaacaaacgatgctcgccttccagaaaaccgaggatgcgaaccactt
catccggggtcagcaccaccggcaagcgccgcgacggccgaggtcttccgatctcctgaagccagggcagatccg
tgcacagcaccttgccgtagaagaacagcaaggccgccaatgcctgacgatgcgtggagaccgaaaccttgcgctc
gttcgccagccaggacagaaatgcctcgacttcgctgctgcccaaggttgccgggtgacgcacaccgtggaaacgg
atgaaggcacgaacccagtggacataagcctgttcggttggtaagctgtaatgcaagtagcgtatgcgctcacgcaa
ctggtccagaaccttgaccgaacgcagcggtggtaacggcgcagtggcggttttcatggcttgttatgactgttttttt
gtacagtctatgcctcgggcatccaagcagcaagcgcgttacgccgtgggtcgatgtttgatgttatggagcagca
wcrmtrttwckc

Integron forward primer 1C

tgacgttgcaccgcacgatgatcgtgccgtgatcgaaatccagatccttgacccgcagttgcaaaccctcactgatcc
gcatgcccgttccatacagaagctgggcgaacaaacgatgctcgccttccagaaaaccgaggatgcgaaccacttc
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ctggtccagaaccttgaccgaacgcagcggtggtaacggcgcagtggcggttttcatggcttgttatgactgttttttt
gtacagtctatgcctcgggcatccaagcagcaagcgcgttacgccgtgggtcgatgtttgatgttatggagcaccaa
cg

Integron forward primer 1D

aycgtacccacggcgtacgcgcttgctgcttggatgcccgaggcatagactgtacaaaaaaacagtcataacaagc
catgaaaaccgccactgcgccgttaccaccgctgcgttcggtcaaggttctggaccagttgcgtgagcgcatacgcta
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aggcgagcatcgtttgttcgcccagcttctgtatggaacgggcatgcggatcagtgagggtttgcaactgcgggtca
aggatctggatttcgatcacggcacgatcatcgtgcgggagggcaagggctccaaggatcgggccttgtttacccc
garaa

Integron forward primer 1E

ttwwrrwkmmgkagctgcwggagcatcggacgttgatcatttcgctgttgccgcgcaacacggcttggccggt
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ggttggaagccagcttagaatttcgcagtatcagtgagttctagagtctctatatttaataatgtcagcatctaagaaac
agctatagtaaagaggctttgtacgagcgacctgaawtctakgcatcaactgatttcyggtkgactgatcatctgag
tccatgtgatggtagtcgaaaggaacaagctactgctactgatgcacgagatgtgc

Integron reverse primer 1A

caatatgtgcttaskgykctarygctkgagaagacattatttgccgasyacytwgrwgatctcttttkaacgtctg
macaaattgttcaaastgagctkckcgcgasgscaaamgatcttcttcttgtscaagataaccctgtctagcttcrw
kwttsacgggctgatrctgggccggcaggcgctccattgcccagtcggttcttgctccttcggcgcgattttgccggtt
actgcgctgtaccrmatgckkgcyracrwaagcactasatttcgctcatgsctwgscmrgtcgggcggcgagtk
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kakkkccyccgccgcyggacykrsctgggcaacgctatgttctcttgcttttgttgtcccryattyggaacartgycg
atccyggctggytcgaagatacctgcaagaatgtcrttgggctgccattctccctgtygsmcctskmkcttacctgs
ataackscacskaatgatgyttaycttgracaaaatgaagacttctacrsctcgsgsaatctcgctctctccraatttgtt
cactacktgaaaaaggtcattgatcargkctcgccgcgttgttgtctaactccttacggtcacgcactaccagcatawt
tgmtcactgtgwgstwtcagarrgyyyysktawta

Integron reverse primer 1B

crtsmgmttwyagtkttwctgmcatgykawkagrsttwytkracgwckggkaggwmagtaaaaaaaaa
ggkactgaasyayyckwacawcwgwgstgkccrckamasrwwctgatcttctkgccwkcttaaaaakcct
gtcmwwatcmagtatagtttctagagtcyctatacgkaaktmkmttyttmgcctatwaagaaaacacsctwt
tcytaaaaaatttttggttactgmggtgtacccwrtgyatstaragcwtwmcactacwtttttttctggtgyggk
aactgaggmggyyagttctataycrttaaggtttcggttagcgcctyaaaaasctcckgtttyckatccggawcy
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srwttyytttsggggyagwatyaaktwtmtctywkwgyatga
gkymtrtgtgcttagtgyatcwammgcttgarttagacrttrktkrmsgackamygtgrwgwtcyctmctta
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sgttyysgayyttaaartkcmtccrsmsmwggaackammrwkgccrckmwraawwyycttgcyywy
swmarymakrkarmswgakgrmwkymtwgsgggsywggmymrarkataccwgcawgargga
mgtggygmtsscmtamkymwmawygcarytmkcgcakgkmtggggmrcsmtmcagagtgatgtc
gwascgamswtgmwggscgacttctwswrcgggtacaatctggatgtctscmtgrraasmcraagtkwc
mtwgakgtgstggatmcragctcgccrmggygtgwcatcacrmcttaccgagctttgatcaassaaatywwt
gkaamtgtgtgcttccc

Intergron reverse primer 1C

ctgccgagascsagattctccgcgctgwatgtacrccattgwtgtgcackacgasatsrtkctcgatgackmtmct
acctctgakaactgagwyktrcwkaatgcgakcaccacttccctcatgatgktawcttygagyyagccacgaty
gayrtystcykkytmwygagytgaswgagcaagatatmttagstgcmytgaytgtccwgtstagaccatsty
twwgaysarawgtracwstatctayttswcasctamygaamccttatgsswmwsagacgtcgactcractss
mtgcagawracgaaatgtwkgactgacgtagatcycagtgctacatcgwrwcacagctmatcrcwctgacgt
agttgctgcgaygggcaaktackcagyyagaccgctctcgaactcatcggtaagctgacaagactactgcatcgac
agctgcatgcgctrtacat

Intergron reverse primer 1D

graagtgcgtccgcacgatgatcgtgccgtgatcgaaatccakatccttgacccgcrgttgcmaccctcactgatcc
gcatgcccgttccatacagaagctgggcgaacaarcsatgctcgccttccagaraaccgaggatgcgarccacttcat
ccggggtcagsrccrccggcragcgccgcgrcggccgaggkcttccgatctcctgaagccmgggcagatccgtgc
acagcaccttgccgtakaagaacagcaaggccgccaatgcctgacgatgcgtggagaccgaaaccttgcgctcgtt
cgccagccaggacagaaatgcctcgacttcgctgctgcccaaggttgccgggkgacgcacaccgtgraaacggat
gaaggcacgaacccmgtggacataagcctgttcggttggtaagctgtaatgcaagtagcrtatgcgctcacgcaac
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wraskgtttttttgyacagtctwatgcctcgggcatccaagcagcaagcgcgttacgccgtgggtcgatgtttgatg
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gggtcttaaaatggtaaaagattggattcccatctctcatgataattacaagcaggtgcaaggaccgttctatcatgga
accaaagccaatttggcgattggtgacttgctaaccacagggttcatctctcatttcgaggacggtcgtattcttaagca
catctactttttcakccttgatggagccagcagtttyggggagctgaacttgctatgtcactgtctggcctcgagggtc
kcgkctacatatacatagytgagccaacagggaccgwtcgaagackatccgaatcttacgaaccaamamatttc
ccggtaatccacacagtcctatasaacctgcgaacccttwgaraacttgttggcgwtgtgagactgkgagggggc
atcctgctgatcaataacgcgatgatggattcgtkagatgactaagcgccgtgttamycrtcatgagactagtcattg
catacaatgcatcgaaccggtacgccaagagattccgccgaccckgwttatgcgccc

Intergron reverse primer 1E

grmawtwtgctgcttagtgcatctaacgcttgagttagacattatttgccgactaccttggtgatctcgcctttcacgt
agtgaacaaattcttccaactgatctgcgcgcgaggccaagcgatcttcttcttgtmcaagataagcctgtctagyttc
aagtatgacgggctgatactgggccggsaggcgctccatkgcccagtmggcagcgacatcctksggsrcgattkt
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ggygagttccatagmgttaaggtttcatttakcgcckcarataratsctgttcrggraccggatcaaagarttcctccg
mcgstggacctaccaaggmaacgctatgktctcktgsttttgtcrgcaagatagccagatcartgtysatcgtggct
ggctcgaagatacctgcaagaatgtcatkgcgctgccattctscaaattgcmgtksgcgsttagctggataacgccr
cggaatgatgtcgtcgtgcacaacaatggtgacttctacagcgcggagaatctcgctctctscaggggaagccgaa
gtttccaaaaggtcgtkgatcamagctcgccgcgttgtttcrtcaagccttacggtcaccgtaascagcaaatcartat
cactgtgtggcttcaggccgccatccactgcggagccgkacaaatgtacggccagcaacgkcagytcgagatggc

gctcgatgacgccmactacctctgatagktgagtckatacttcsgmgatcaccacttcyctcatgatgtttaactttgt
tttasycaccrrtgatgccctcwckttsmrtgattytgkggkgatggaktttcktagcgrcracttactttcgttgtcta
tatcyrtgttaacggcgamraagtaarmctmtgyctactttctcaacccamccaatcycaccrtgmkacacagg
atytgactcagytcscmaccakaaaaacwgttktgatgyacwagaatwgtcagggcygccttcgtaacaaagc
gctwttgactrttagctgattgcattraatgctgmagttattcaatatgagacytagaccttcattgatactgcgaagtc
agctgactcgaccgattgtcagtcacatga

Chapter five

Random Amplified Polymorphic DNA Typing of Clinical and Environmental Strains of *Aeromonas hydrophila* from Limpopo Province, South Africa

5.1 ABSTRACT

The aim of the present study was to determine the genetic relatedness of strains isolated from diarrhoeal stool and water specimens collected from water-storage containers from different geographical areas in the Limpopo province. In total, 32 *Aeromonas* strains isolated from stool specimens collected from HIV/AIDS patients suffering from gastroenteritis and their household drinking-water stored in 20-L and 25-L containers were analyzed by random amplified polymorphic DNA PCR (RAPD). The RAPD fingerprints obtained proved reproducible when repeated on three different occasions using whole-cell DNA isolated from the *Aeromonas* strains. In total, 12 unique RAPD fingerprints were found. The results revealed a tendency of the isolates to cluster according to their origin of isolation (best-cut test 0.80 and bootstrap values >50%). However, a certain degree of similarity was also observed between isolates of water sources and clinical sources which indicated genetic relatedness. There were also genetic similarities between the clinical and the environmental strains of *Aeromonas* spp. isolated from different geographical areas. This study has demonstrated the genetic relatedness of *Aeromonas hydrophila* isolates from household drinking-water and clinical sources in South Africa, which may be due to cross-contaminations from water to patients or vice-versa. This observation is of public health significance, particularly in the era of HIV/AIDS. This study points to the importance of monitoring and evaluating infection-control measures for improved hygiene and to prevent cross- contaminations.

5.2 INTRODUCTION

The genus *Aeromonas* comprises several species of oxidase-negative and catalase-positive, glucose fermenting, facultative anaerobic, Gram-negative, rod-shaped, motile and non-motile bacteria (Howard *et al.*, 1996). They are widely distributed in nature, especially in aquatic environments and have been isolated from various raw foods, such as fish and vegetables. These bacteria are widely found in surface water and sewage; they also occur in untreated and treated drinking-water (Maalej *et al.*, 2003). In humans, *Aeromonas* species are responsible for gastroenteritis, chronic diarrhoea, wound infections, respiratory tract infections, peritonitis, urinary tract infections, and septicaemia (Martínez *et al.*, 2007). *Aeromonas* spp. have been implicated as diarrhoea-causing agents in both HIV-positive and HIV-negative patients in South Africa (Obi *et al.*, 2002). However, no study has determined the possible genetic relationship between environmental and clinical strains.

Genomic fingerprinting methods, such as random amplified polymorphic DNA PCR (RAPD) typing, are regarded as accurate methods for the typing of microorganisms for epidemiological purposes (Alavandi *et al.*, 2001) Although *Aeromonas* spp. have been described in South Africa, there is a paucity of studies that have determined the possible source of human infections, including the phylogenetic relatedness between clinical and environmental isolates. In the present study, RAPD was employed to determine the genetic relatedness of strains isolated from diarrhoeal stool and water specimens collected from water-storage containers from different geographical areas in the Limpopo province, South Africa.

5.3 MATERIALS AND METHODS

5.3.1 Study sites and Bacterial isolates

Stool samples and household drinking-water samples were collected from HIV and AIDS patients in different locations in the Limpopo province, including Belabela, Madombidzha, Mankweng (Polokwane), and Musina. *Aeromonas* spp. were isolated from these samples and characterized as previously described (Obi *et al*., 2007). Thirty-two strains of *Aeromonas hydrophila* from these samples were used in the present study for beta-lactamase production, antibiotic susceptibility as previously described (Obi *et al*.,2007), and in genotyping studies. The geographical origins and sources of the strains used in the study are indicated in the table. Ethical clearance was obtained from the Ethics and Research Committee of the University of Venda, Thohoyandou, Limpopo province, South Africa.

5.3.2 Genomic DNA extraction

Extraction of whole-cell DNA was performed by a precipitation-based method as described previously (Balcázar *et al*., 2007). Briefly, the cells were lysed by the addition of SDS (Promega, Madison, WI) and lysozyme (Sigma Chemical Co. St. Louis, MO, USA) with incubation at 37 °C (QBT2 heating block, Grant Instruments Ltd., Cambridge, United Kingdom) for one hour. The DNA was further isolated using chloroform/ isoamyalcohol (24:1) (Sigma Chemical Co., St. Louis, MO, USA) and precipitated with isopropanol (Merck, Darmstadt, Germany) at -20 °C overnight and stored at -20 °C until further analysis.

5.3.3 Confirmation of *Aeromonas hydrophila* identity by 16S rRNA gene-sequencing

All the *Aeromonas* strains used in the present study were identified as previously described and confirmed by PCR analysis and sequencing of the 16S rRNA gene for two isolates. The 16S rRNA gene sequence was PCR-amplified in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler as previously described (Alavandi et al., 2001) All DNA extractions and PCR products were verified by gel electrophoresis for one hour in a 1% agarose gel (Pronadisa, Madrid, Spain) containing ethidium bromide (Promega, Madison, WI, USA) and the images captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland, CA, USA).

5.3.4 Random amplified polymorphic DNA analysis of strains

Random amplified polymorphic DNA analysis was performed on 32 isolates using whole-cell DNA as template to determine the genetic relationships of isolates according to a modified method previously described (Campbel *et al.*,2000; Zemanová *et al.*,2004) using three different primers to obtain a banding pattern representative of the whole genome. All the samples were run at least twice using each primer.

5.3.5 Analysis of RAPD fingerprints

The amplification products were electrophoresed in 1.5% agarose gel in Tris-borate buffer. Computer analyses were carried out using the GelCompar II software (version 3.0; Applied Maths, Kortrijk, Belgium). Similarity between fingerprints was calculated with the Dice coefficient. Cluster analysis was performed using the unweighted pair-group method with average linkages (UPGMA).

5.4 Results

The fingerprints of the isolates generated between 2 and 17 bands ranging from 100 to 3,500 bp. The banding patterns proved reproducible when repeated on two separate occasions. Random amplified polymorphic DNA fingerprints which possessed more than 90% similarity were considered identical and were assigned an RAPD type. On this basis, 12 unique RAPD types were assigned as shown in the table 5.2. Minimum inhibitory concentration ranges of the isolates to Antibiotics and Antibiotic resistance combination as shown in tables 5.1, 5.2 and figure 5.8.

There was a difference between the clinical and the environmental strains. Type 1, for example, was composed of four clinical strains, and type 4 was composed of five clinical strains while type 7 was composed of all four environmental strains. However, there was a mixture of clinical and environmental strains in the same type.

5.5 Discussion

We had previously isolated and characterized *Aeromonas* spp. from stool and water samples from different areas in the Limpopo province (Obi *et al*., 2007). However, previous studies were not able to fully ascribe any genetic relatedness between the environmental and the clinical isolates. In the present study, three primers were used for RAPD fingerprinting of 32 *A. hydrophila* strains from stools and water samples from HIV and AIDS patients from five different localities approximately 400 km apart throughout the Limpopo province situated in the northern part of South Africa.

The banding patterns obtained in the present study were similar to those obtained by other researchers (Alavandi *et al*., 2001). The results indicate a high genetic diversity in the *A. hydrophila* population in South Africa even though

there was a mixture of environmental and clinical strains in some RAPD types probably due to separate introductions of the strains in the localities and poor health practices (Andargie *et al.*,2008; Eshcol *et al.*,2009).

Other studies around the world, using genetic methods, have described high diversity in the *A. hydrophila* population (Korbsrisate *et al.*, 2007; Lee *et al.*, 2008). Previous studies have indicated that homologs of a gene encoding a protein with lipase activity appeared to be widely distributed in *Aeromonas* strains, probably associating with the evolutionary genetic difference between clinical and environmental isolates of *A. hydrophila* (Watanabe *et al.*, 2004). We have previously shown that clinical strains of *A. hydrophila* possessed pathogenic characteristics, such as haemolytic and haemagglutinating activities (Obi *et al.*, 2007). It is, thus, possible that the environmental strains that had similar RAPD profiles as clinical strains might share pathogenic characteristics as well. Studies of *A. hydrophila* in Japan, using randomly-amplified polymorphic DNA-PCR (RAPDPCR), revealed one specific RAPD pattern group (G) that was associated only with strains from environmental sources. The comparison of isolates with pattern group G with a set of isolates derived from human blood showed low induction of cytotoxicity from those with RAPD pattern group G suggesting low virulence of these strains (Lee *et al.*, 2008).

Although antibiotic resistance was common, statistical analysis using the chi-square test indicated that RAPD type 4 was more associated with amikacin resistance ($\chi 2=3.872$; $p=0.049$) and resistance to cefotaxime ($\chi 2=3.872$; $p=0.049$). Of all the strains tested, four were beta-lactamase-positive, of which two were of RAPD type 6. Statistical analysis indicated that RAPD type 6 was more associated with beta-lactamase production ($\chi 2=5.878$, $p=0.015$). Multiple drug resistance (MDR) defined as resistance of a specific strain to more than two antibiotics was common (41%) among the strains tested. Statistical analysis indicated that RAPD type 4 was more associated with MDR ($\chi 2=4.567$, $p=0.033$). In a previous study, there was a correlation between the antibiotic resistance

profile and the RAPD profiles of specific *Aeromonas* isolates (Gunsalam *et al*., 2006).

In the present study, we did not find a clear differentiation between strains from different localities. RAPD type 1 was not found in Musina area but was found in all the other areas, including Belabela, Mankweng, and Madombidzha.

Some RAPD genotypes included only one strain (Figures 5.1 to 5.7). For example, RAPD genotype 2, type 3, and type 9 were all obtained from Musina area and included the environmental and clinical isolates while type 8, 10, and 12 were obtained from Mankweng, Madombidzha, and Belabela respectively. This diversity indicates the need for regular monitoring of *Aeromonas* strains for pathogenic characteristics and for the possibility of epidemic strains that could be circulating in the population. Studies of clinical and environmental ecotypes of *Aeromonas* in Mexico demonstrated the high intraspecific diversity within *A. hydrophila* and revealed a clear differentiation of strains according to their ecological origins (Aguilera-Arreola *et al*., 2005). The same study also confirmed, based on the distribution of virulence-related genes, that *A. hydrophila* is a genetically-heterogeneous species that harbour ecotypes which have different pathogenic potentials to humans and other animals.

The results indicating the genetic similarity between the clinical and environmental isolates of *A. hydrophila* in this study do not accord with those obtained in Switzerland that showed no genetic relationship between clinical and environmental strains (Moyer *et al*., 1992). Studies in France also found that the water sampled in the hospital was not the source of infections for patients at the University Hospital in Marseille (Davin-Regli *et al*., 1998). With the high prevalence of HIV and AIDS in South Africa, patients might become more susceptible to infection by *Aeromonas,* and the possibility of acquiring the organisms from water is high and represents a great threat to human health.

The results of the present study have revealed the possibility of an intricate web of relationship between isolates from stool and water samples in the Limpopo province of South Africa, indicating cross contaminations and points to the need for effective control measures. The study also showed that specific genotypes might be related to antibiotic resistance. Further studies on the cytotoxicity of strains under investigation are warranted and will further clarify the pathogenic characteristics of *A. hydrophila* strains in the region.

Table 5.1: Minimum inhibitory concentration ranges of the isolates to Antibiotics

Antimicrobial agent	Percentage Susceptible and Range of MIC_{90} (µg/ml) of the isolates to antibiotics				Breakpoints
	Water(n=17)	Water(**MIC_{90}**)	Stool (n=15)	stool(**MIC_{90}**)	**Breakpoints**
Amikacin (AMK)	100	≥32	80	≥32	≤8 ≥32
Ampicillin (AMP)	6	8 ,16,32	0	16,32	≤8 ≥32
Aztreonam (AZT)	100	8 ,16,32	100	8 ,16,32	≤8 ≥32
Cefazolin (CF)	76	8 ,16,32	73	8 ,16,32	≤8 ≥32
Cefepime (CFP)	100	8 ,16,32	100	8 ,16,32	≤8 ≥32
Cefotaxime (CFT)	94	8 ,16,32	87	8 ,16,32	≤8 ≥32
Ceftazidime (CTZ)	100	8 ,16,32	100	8 ,16,32	≤8 ≥32
Ceftriaxone (CTX)	53	8 ,16,32	60	8 ,16,32	≤8 ≥32
Cefuroxime (CFM)	100	8 ,16,32	100	8 ,16,32	≤8 ≥32
Cephalothin (CPN)	100	8 ,16,32	100	8 ,16,32	≤8 ≥32
Ciprofloxacin (CIP)	82	8 ,16,32	93	8 ,16,32	≤1 ≥4
Ertapenem (ERT)	100	0.25, 0.5,1	100	0.5,1	≤4 ≥16
Gentamicin (GM)	41	4 , 8,16	67	8,16	≤4 ≥16
Imipenem (IMI)	100		100		≤4 ≥16
Nitrofurantoin (NI)	53	4 ,8 ,16	67	4 ,8 ,16	≤32 ≥128
Meropenem (MEM)	100	0.25,0.5, 1	100	0.5, 1	≤4 ≥16
Piperacillin (PRL)	100	4,8 ,16	100	8 ,16	≤16 ≥128
Pipemidic acid (PIP)	100	16,32,64	100	16,32,64	≤4 ≥16
Tobramycin (TN)	56	4 ,8 ,16	35	4 ,8 ,16	≤4 ≥16

Table 5.2: Random Amplified Polymorphic DNA profiles

Locality	Isolate*	RAPD type	Beta-lactamase production	Antibiotic resistance combination
Belabela	6165S	1	Negative	Ampicillin
Belabela	6168S	1	Negative	Ampicillin
Belabela	6173S	4	Negative	Amikacin, ampicillin, cefotaxime, nitrofurantoin, tobramycin
Belabela	6197S	4	Positive	Amikacin, ampicillin, cefazolin, cefotaxime
Belabela	6237S	5	Positive	Ampicillin, cefotaxime, nitrofurantoin, tobramycin
Belabela	6164E	5	Negative	Ceftriaxone, ciprofloxacin, gentamicin, ampicillin
Belabela	6249E	6	Positive	Amikacin, ampicillin, cefazolin, cefotaxime, nitrofurantoin, tobramycin
Belabela	6241E	7	Negative	Nitrofurantoin, amikacin, ampicillin
Belabela	6238S	12	Negative	Nitrofurantoin
Belabela	6248E	11	Negative	Ampicillin
Madombidhza	6171S	1	Negative	Ampicillin
Madombidhza	6170S	4	Negative	Ampicillin, nitrofurantoin, tobramycin
Madombidhza	6199S	6	Positive	Ampicillin, cefazolin, nitrofurantoin, tobramycin
Madombidhza	6250E	7	Negative	Ampicillin
Madombidhza	6183S	10	Negative	Ampicillin, cefazolin, ampicillin
Madombidhza	6184S	11	Negative	Ampicillin
Mankweng	6242E	8	Negative	Amikacin, ampicillin, cefazolin,
Mankweng	6258E	7	Negative	cefotaxime, nitrofurantoin, tobramycin
Mankweng	6166S	1	Negative	Ampicillin
Mankweng	6189E	6	Negative	Ampicillin
Mankweng	6182S	5	Negative	Tobramycin
Mankweng	6255E	11	Negative	Ampicillin
Mankweng	6172S	11	Negative	Nitrofurantoin, tobramycin
Musina	6257E	2	Negative	Amikacin, ampicillin, cefazolin,
Musina	6175S	4	Negative	cefotaxime, nitrofurantoin, tobramycin
Musina	6174S	3	Negative	Nitrofurantoin
Musina	6239E	5	Negative	Ampicillin, cefazolin
Musina	6192E	5	Negative	Amikacin, ampicillin, cefazolin, cefotaxime, nitrofurantoin, tobramycin
Musina	6190E	6	Negative	Ampicillin
Musina	6240E	7	Negative	Ampicillin
Musina	6247E	9	Negative	Ampicillin
Musina	6198S	4	Negative	Ampicillin

Isolate* E: Enivironmtal samples

S: Stool samples

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

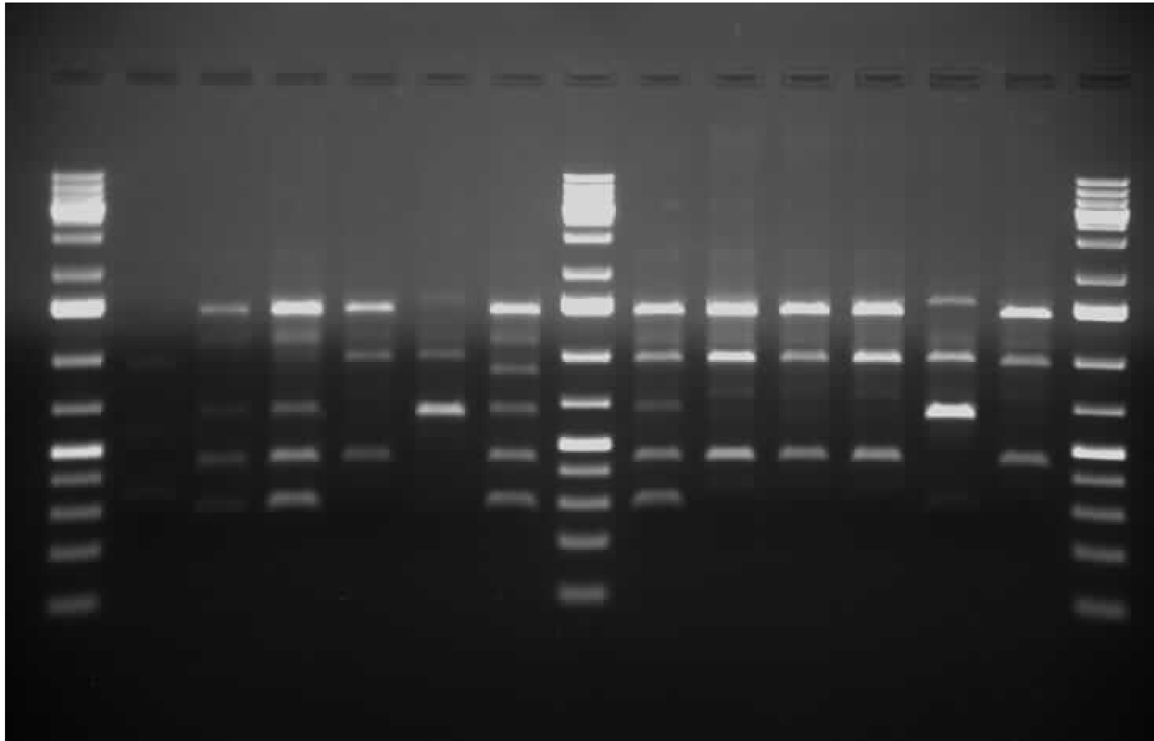
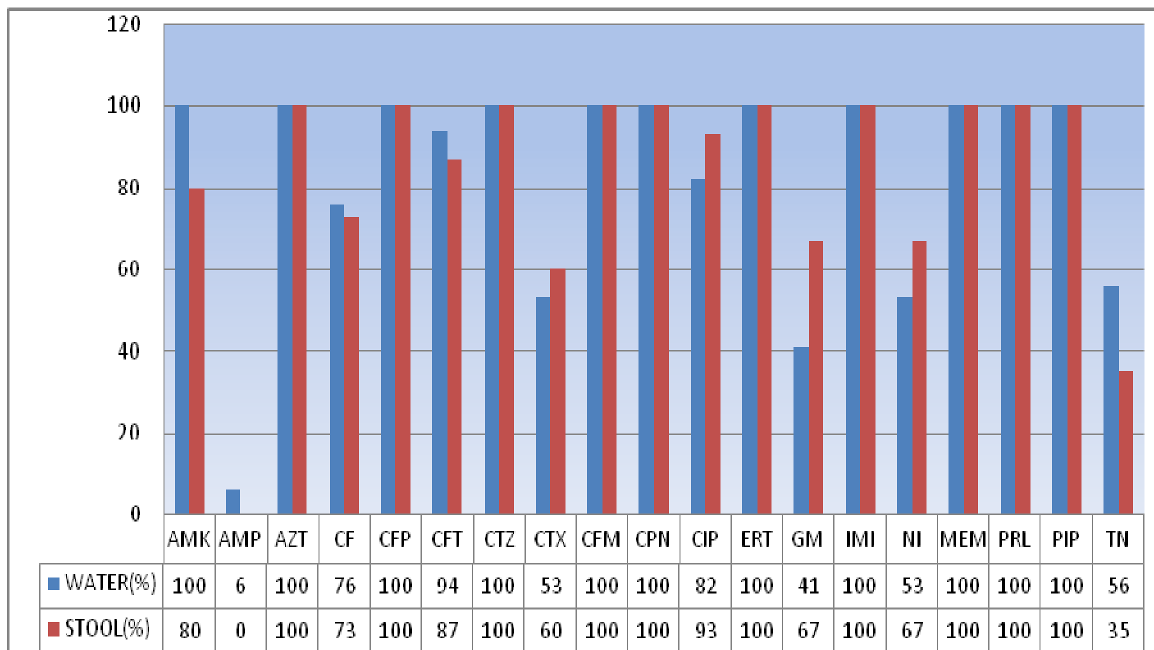


Figure 5.1: Gel electrophoresis depicting the RAPD banding pattern obtained with primer H1. Lane 1: Marker (100bp to 10 000bp). Lane 2:E001. Lane 3:E002. Lane 4:E003. Lane 5:E004. Lane 6:E005 .Lane 7:E006. Lane 8: Marker (100bp to 10 000bp). Lane 9:S001. Lane 10:S002. Lane 11:S003. Lane 12:S004. Lane 13:S005. Lane 14: S006 Lane 15: Marker (100bp to 10 000bp)



	AMK	AMP	AZT	CF	CFP	CFT	CTZ	CTX	CFM	CPN	CIP	ERT	GM	IMI	NI	MEM	PRL	PIP	TN
WATER(%)	100	6	100	76	100	94	100	53	100	100	82	100	41	100	53	100	100	100	56
STOOL(%)	80	0	100	73	100	87	100	60	100	100	93	100	67	100	67	100	100	100	35

Figure 5.2. antimicrobial activtites of all tested isolates from water and stool samples

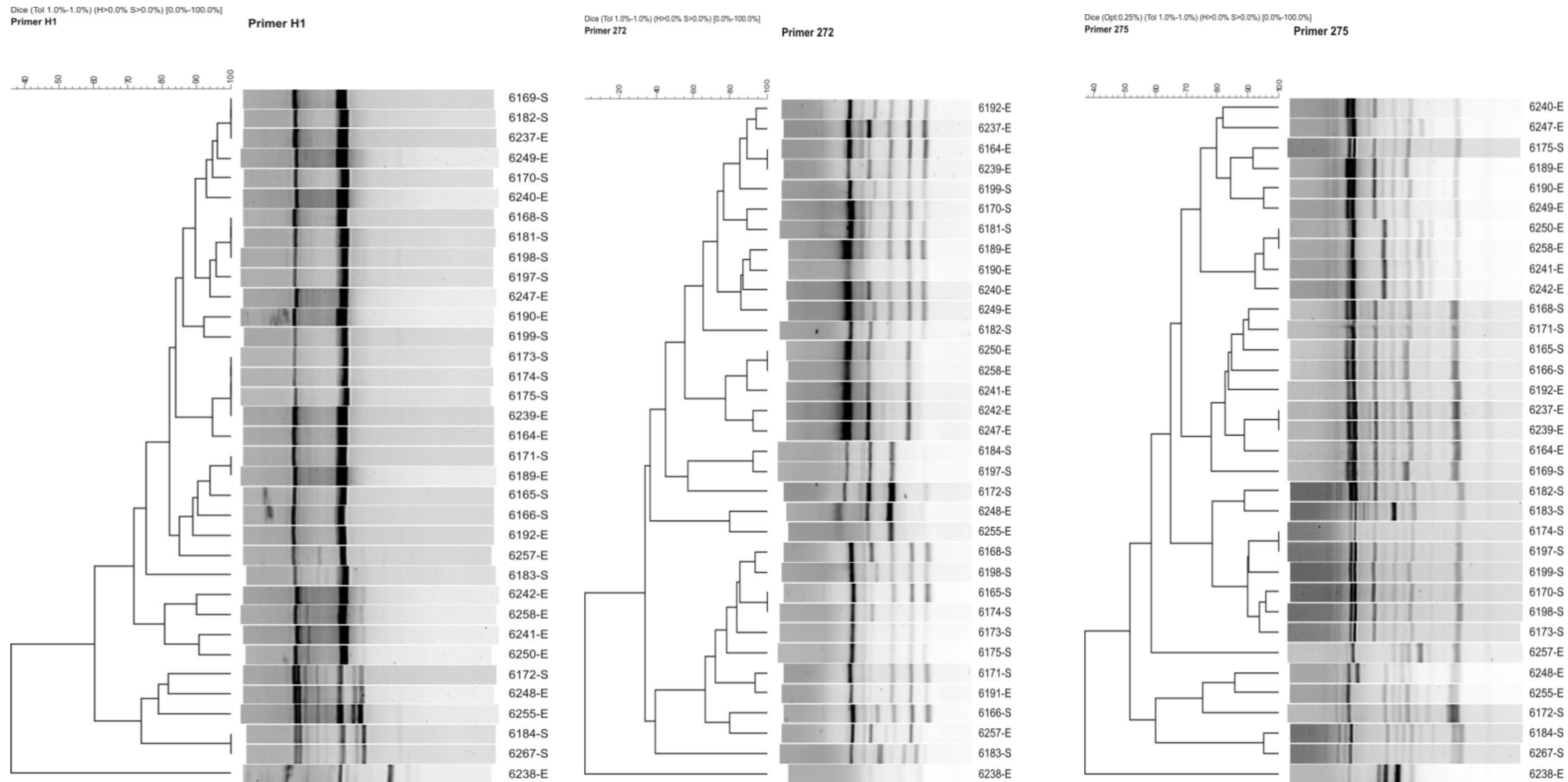


Figure 5.3: Dendogram obtained through RAPD typing with primers H1, 272 and 275 depicting the relationship between *A.hydrophila* stool isolates and water isolates obtained from Limpopo Province, South Africa

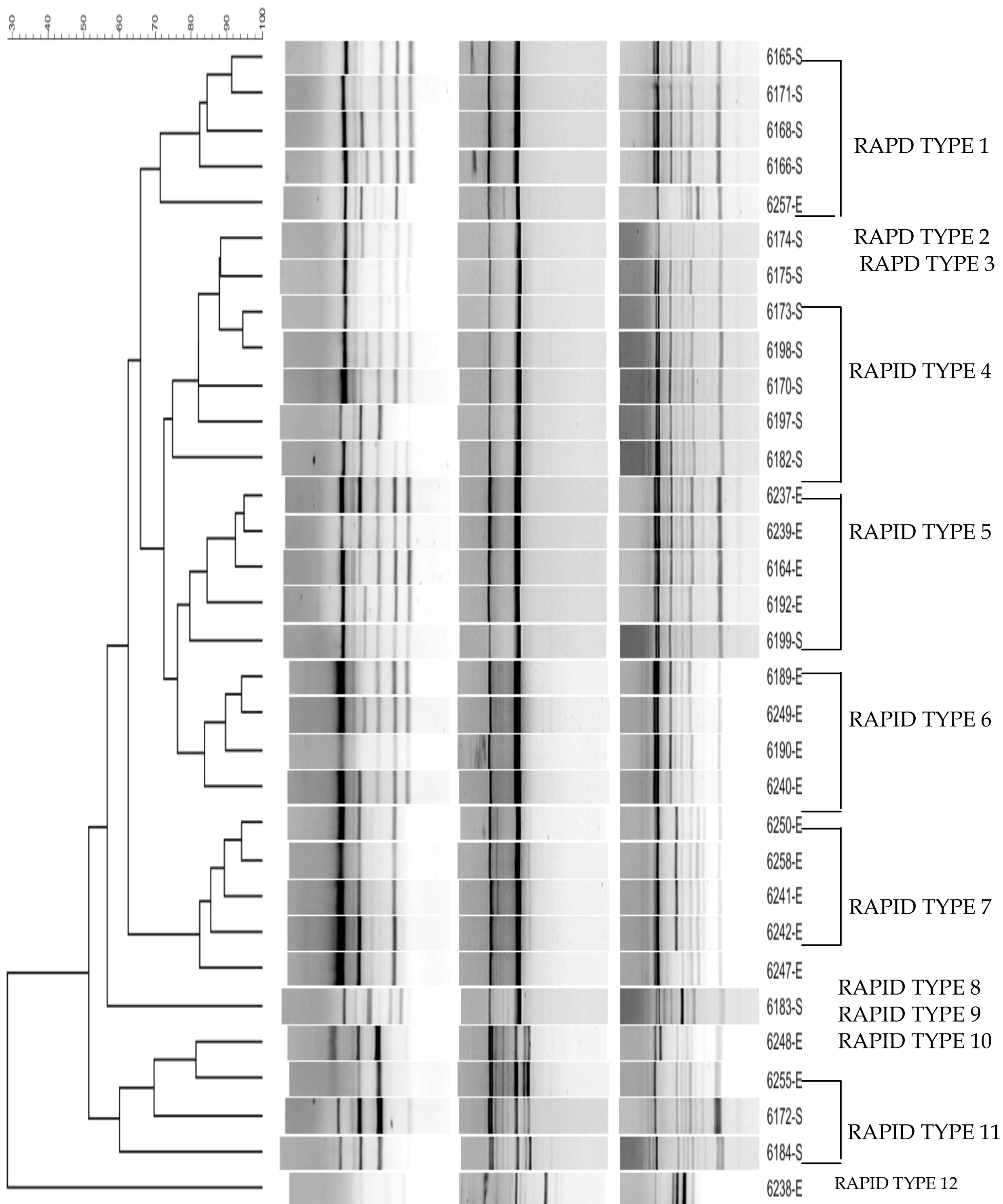


Figure 5.4: Dendogram obtained through RAPD typing with primers 277, H1 and 275 depicting the relationship between *A.hydrophila* stool isolates and water isolates obtained from Limpopo Province, South Africa

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Chapter six

Antibacterial activity profiles of *Pyrenacantha grandiflora Baill* and *Ficus sycomorus* used in Rural Venda communities in South Africa against *Aeromonas Hydrophila*

6.1 Abstract

The present study was conducted to investigate the antibacterial activities of *Pyrenacantha grandiflora* Baill and *Ficus sycomorus* against *Aeromonas hydrophila* in the era of HIV/AIDS. The ground plant materials were extracted with different extractants and screened for anti-microbial activity using the disk diffusion and micro-dilution techniques. Inoculated strains were serially diluted and plated at time intervals of 0, 2, 4, 6, 8 and 24h. Results obtained showed that *F. sycomorus* extracts killed all the bacterial cells of *A. hydrophila* at a concentration of 3 mg/ml in 8h. A higher concentration of 6mg/ml had the same effect after 6h. Acetone extracts were most active for both plants with the MIC of 1.5mg/ml and MBC of 1.5mg/ml. Results have revealed the strong in vitro activity of *Pyrenacantha grandiflora* Baill and *Ficus sycomorus* extracts against *Aeromonas hydrophila.* This study may mark a reference document on the activities of *Pyrenacantha grandiflora* Baill and *Ficus sycomorus* in the Venda region of South Africa against *Aeromonas hydrophila* and justifies their use by local traditional healers in the treatment of gastrointestinal related infections.

6.2 Introduction

Diarrheal diseases occur worldwide and cause about 3.2% of all deaths including 1.8 million people globally each year, mostly children in developing countries (WHO, 2004). It is estimated that one in five children in developing countries die before their fifth birth as a result of diarrhoeal diseases (Haque *et al*., 2003). They are of immense clinical importance and are widely spread in the environment, especially in surface water and sewage; they also occur in untreated and treated drinking water (Obi *et al*., 2007, Samie *et al*., 2007). In humans, *Aeromonas* spp. are responsible for gastroenteritis, chronic diarrhea, wound infections, respiratory tract infections, peritonitis, urinary tract infections, and septicemia (Obi *et al*., 2007). Among *Aeromonas*-associated infections of humans, *A. hydrophila*, *A. caviae*, and *A. veronii* are the predominating species, whereas *A. eucrenophila*, *A. popoffi* and *A. culicicola* are rarely found in clinical samples.

Despite the efficacy and success of modern medicine, plant remedies have gained immense popularity in recent years for the treatment of several ailments. With emphasis being on scientific validation, a number of plants have been chemically and biologically evaluated for their properties.

A lot of medicinal plant preparations have been recognized for treating diarrhea, although their modes of action in the elimination of organisms causing diarrheal diseases have not been fully elucidated.

About 70% of the population in Africa reportedly rely on plants for the prevention or cure of many diseases (Kamanzi *et al.,* 2002). Previous studies had highlighted the activities of several South African medicinal plants against bacteria, parasites and viruses pathogenic to man (Obi *et al.,* 2002, 2003, Ranasamy *et al*., 2007, Samie *et al*., 2007, Gundiza *et al*., 2008). For example the extract of *Euclea divinorum, Rhus lancea, Piper auritum, Peltoforum africanum* and *Withanea somnifera* demonstrated inhibitory activities against members of enterobactericiae (Obi *et al*., 2003, Samie *et al*., 2007 and Gundiza *et al*. 2008). *Euphorbia hirta, Hollarena floribunda* and *Harungana madagascariensis* extracts

showed substantial activities against *E. histolytica* (Kraft *et al*., 2003; McGaw *et al*., 2000, Samie *et al*, 2007). Apart from the antimicrobial activities, plant extracts have also demonstrated immunomodulatory effects on different cell cultures and in experimental animals (Tshibangu *et al*., 2002, Nakamura *et al*., 1999).

In the Venda region of South Africa, medicinal plants are used widely for the treatment and management of diarrhea. Such plants include *P. grandiflora* and *F. sycomorus*. The purpose of this study was to determine the antimicrobial activities of *Pyrenacantha grandiflora Baill* and *Ficus sycomorus* used by traditional healers in Limpopo Province for the treatment and management of *Aeromonas* diarrhoeal related infections.

6.3 Materials and methods

6.3.1 Study site and Patients

The study was carried out in the Venda region of South Africa, between December 2004 and January 2008. Stool samples were collected from HIV/AIDS patients attending healthcare facilities whereas water samples were obtained from stored household drinking water.

6.3.2 Isolation, identification and susceptibility of Aeromonas species

A total of 1,369 samples (660 stool samples and 709 water samples) were collected and screened for the presence of *Aeromonas* species. The specimens were cultured using the method as previously described by Obi *et al*., 2007. Briefly, freshly collected stool specimens were plated onto MacConkey agar (Difco/BD Diagnostics Systems, Sparks, MI, USA) and xylose deoxycholate citrate agar (XDCA) while water samples were plated on Cysteine Lactose Electrolytes Deficient (CLED) agar and MacConkey agar (Difco/BD Diagnostics Systems, Sparks, MI, USA) . Cultures were incubated at 37°C for 18-24 hours, after which non-lactose–fermenting colonies on MacConkey agar; Cysteine Lactose Electrolytes Deficient (CLED) agar and non-xylose-fermenting on xylose

deoxycholate citrate agar (XDCA) were screened for production of oxidase. All oxidase –positive colonies were further identified and confirmed as Aeromonas using API 20E and API 20NE system (bioMerieux, Marcy-l'Etoile, France).

6.3.3 Microorganisms

The plant extracts were tested against 300 isolates of *Aeromonas hydrophila;* bacterial strains were stored at -70°C in Brain heart infusion (BHI) with glycerol.

6.3.4 Plant collection

Pyrenacantha grandiflora Baill and *Ficus sycomorus* used by local population to cure different ailments such as diarrhea or as prophylaxis against diarrhea were collected with the help of traditional healers and local village chief. Appropriate plant parts were collected between January, 2007 and July 2008 at Makwarani and Tswinga villages, in the Venda region of South Africa. Plants and parts used as well as other ethno medicinal information are shown in Table 6.1

6.3.5 Plant sample preparations

Plant materials collected from were dried at room temperature for about 2 weeks or using an incubator at 40°C for 2 to 3 days. The dried plant materials were ground into powder form using a grinder followed by a warring blender.

6.3.6 Extraction

50 g of the ground materials of each plant was extracted in 500ml of methanol under continuous shaking for 24 h. The extract was filtered through a 22 μm paper filter. The filtrate was evaporated to dryness using a rotatory evaporator at 40°C. The residues in the form of powder were preserved in sterile glass bottles in a cool dark place until use.

6.3.7 Screening of antibacterial activity

Screening of antibacterial activity was performed by standard disc diffusion method (Samie *et al*., 2005) with some modifications. Briefly, sterilized discs of filter paper (6 mm diameter) were soaked in 1 ml of infusion and decoction separately for 1-2 minutes and then used for screening. The potency of each disc was 10 µl. Mueller- Hinton agar (MHA) (Merck) was used as base medium and Mueller-Hinton broth (MHB) was used for the preparation of inoculum. Four to five pure isolated colonies of tested organisms were picked by sterile inoculating loop and inoculated in tubes of MHB (5 ml each). The inoculated tubes were incubated at 35-37°C for 24 hours and adjusted to match with 0.5 McFarland turbidity standards. A sterile cotton swab was dipped into the standardized bacterial test suspension to inoculate entire surface of a MHA plate. Discs of infusion were placed on the surface of inoculated plates with the help of sterile forceps. The inoculated plates were incubated at 35-37°C for 24 hours. After incubation inhibition zone diameters were measured to the nearest millimeter (mm). The experiments were done in triplicate.

6.3.8 Microdilution assay

The microdilution method was used to determine the minimum inhibitory concentrations (MICs) of the plant extracts using 96 well microtitration plates as previously described (Samie *et al*., 2005). Briefly, 185µl of the broth was added into each well in the first row of the microtitration plate and 100µl to the rest of the wells from the second row downwards. 15µl of the plant extracts was then added into each well on the first row (row A), starting with the positive control (ampicillin, Roche), followed by the negative control (the 20% DMSO used to dissolve the plant extracts) and the plant extracts in the rest of the wells in that row. A twofold serial dilution was done by mixing the contents in each well of the first row and transferring 100 µl to the second well of the same column and the same was done up to the last well of the same column and the last 100 µl from the last well was discarded. Then 100µl of Vibrio Cholera culture

suspensions was added. The results were observed after 24 h incubation at 37°C followed by the addition of 40 µl of a 0.2% Iodo Nitro Tetrazolium (INT) solution after a further incubation of 4 h at 37°C. The minimum bacterial concentration (MBC) was determined by inoculating the contents from the MIC plates onto Mueller- Hinton agar plates and the results were observed after 18 to 24 h incubation at 37°C. The presence of the bacterial colonies on agar plates were an indication that the plant extract only inhibited the growth of the organisms without killing them and the absence indicated that the plant extract was able to kill the organisms. The least concentration of the plant extracts that was able to kill the microorganisms was considered as the minimum bacterial concentration.

6.3.9 Cellular toxicity

Haemolysis was used to determine cellular toxicity of the extract. Plant extracts at concentrations ranging from 6 to 32 mg/ml, were incubated with an equal volume of 1% human red blood cells in phosphate buffered saline (10mM PBS, pH 7.4) at 37°C for 1 h. Non-hemolytic and 100% hemolytic controls were the buffer alone and the buffer containing 1% Triton X-100, respectively. Cell lysis was determined by measuring the release of hemoglobin spectrophotometrically at 540 nm

6.3.10 Time kill curve

Time-kill curve was performed as described by Samie et al 2010, using Mueller-Hinton broth (MHB). Inocula were prepared as described above by Samie et al., 2010. The resultant suspension was diluted 1:100 with fresh sterile broth and used to inoculate 50 ml volumes of the broth incorporated with extracts a final cell density of approximately 2 × 106 cfu/ml. The flasks were incubated at 37°C on an orbital shaker at 100 rpm. A 500 µl sample was removed from cultures at 0, 2,4,6,8, 18 and 24 h, diluted serially and 100 µl of the diluted samples were plated on agar plates and incubated at 37°C for 24 h.

Statistical analysis: Mean diameter of zone of inhibition and standard deviations were calculated.

Table 6.1: Ethnobotanical information of selected Venda medicinal plants used in the study

Family	Botanical Names	Vernacular Names	Form	Traditional uses
Icacinaceae	*Pyrenacantha grandiflora* Baill.	Gwere,Velavhahleka, Mbengelele	Climber	Dysentery, inflammation, tooth pain
Moraceae	*Ficus sycomorus*	Muhuyu	Bark	Wounds infection , management of diarrhoea

6.3.11 Antimicrobial evaluation of medicinal plants by the disc diffusion method

The disc diffusion method was used as described previously (Obi *et al.*, 2007). Briefly; brain heart infusion broth was supplemented with 0.01% tween 80 to enhance the solubilisation of extract. 100 µl of an 18hour culture of test organism was spread on the agar surface using a sterile swab and the plate was left for 30min in order to air dry. Whatman No.1 paper was used to prepare discs of 6mm diameter and sterilized by autoclaving. The blank sterile discs were deposited on top of the seeded Mueller-Hinton agar (Difco, USA) and 15 µl (3mg) of each extract concentration was added on top of the disc. All the plates were incubated aerobically at 35˚C for 24 hours. All experiments were repeated three times using 10µl of a 50mg/ml Gentamycin as a positive control and 15µl (6%) of DMSO as negative control using *Escherichia coli* ATCC25922 as a control organism. The antibacterial activity was expressed as the mean diameter of the inhibition zones (mm) produced by the plant extracts. Diameters of zones of inhibition were measured in mm and recorded.

6.4 Statistical analysis

The proportion difference was determined by the chi-square test. A p value of <0.05 was considered statically significant.

6.5 Results

The results showed that acetone and methanol extracts (Table 6.2) effectively inhibited the growth of *Aeromonas hydrophila*. Further, the killing curve was determined (figures 6.1 to 6.6). The means (X) of readings obtained at each concentration plated for each organism are presented graphically in Figures 1-6. As presented, micro-organisms were consistently killed at concentrations of 6 mg/ml using *P.grandiflora* and 3mg/ml with *F. sycomorus* respectively. The initial inoculum contained 2.05×10^{4}cfu/ml of the organism. After 24h, the population had been reduced to 1.34×10^{2}cfu/ml. Concentrations of 3 mg/ml completely wiped out all viable bacteria after 8h. Stronger concentrations of *P.grandiflora* the extracts (3 mg/ ml and 6 mg/ml) completely killed all viable *A.hydrophila* cells in 6h. In the highest concentration tested, (>6mg/ml) the initial inoculum was substantially reduced to 1.01×10^{1}cfu/ml in 24h. A concentration of >6mg/ml however had some little effects in the first 6h.

TABLE 6.2: Antimicrobial Activity of Medicinal Plant Extracts against isolates of A*eromonas hydrophila*

Plants species	**Percentage Susceptible and Range of MIC_{90} of the isolates to antibiotics**			
	Water(n=80)	Water(**MIC_{90}**)	Stool (n=80)	stool(**MIC_{90}**)
Ficus sycomorus	96	1.5,3	75	1.5,3,6
Pyrenacantha grandiflora Baill.	46	3,6	26	1.5,3,6

MIC_{90} = MIC of 90% of isolates tested

Table 6.3 Percentage susceptibilities of tested species of *Aeromonas hydrophila* against medicinal plants

Plants extract	**Extractant**	**>6mg/ml**	**6mg/ml**	**3mg/ml**	**1.5mg/ml**	**0.75mg/ml**
P.grandiflora	Methanol	43	33	25	0	0
	Hexane	26	74	0	0	0
	Acetone	5	79	16	0	0
F.sycomorus	Methanol	33	35	32	0	0
	Hexane	46	47	7	0	0
	Acetone	2.3	42	40	2	0

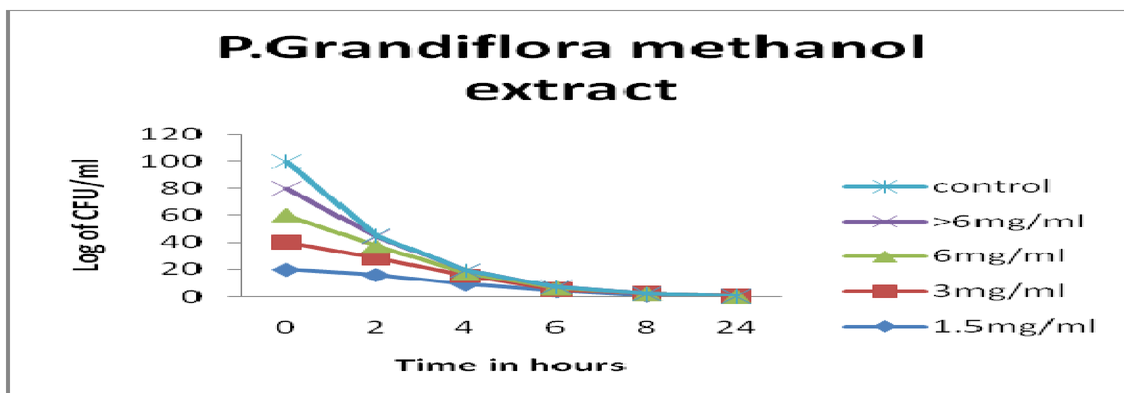

Figure 6.1: Activity of varying concentrations of *P.grandiflora* extracts against selected isolates of *A. hydrophila* using methanol extract

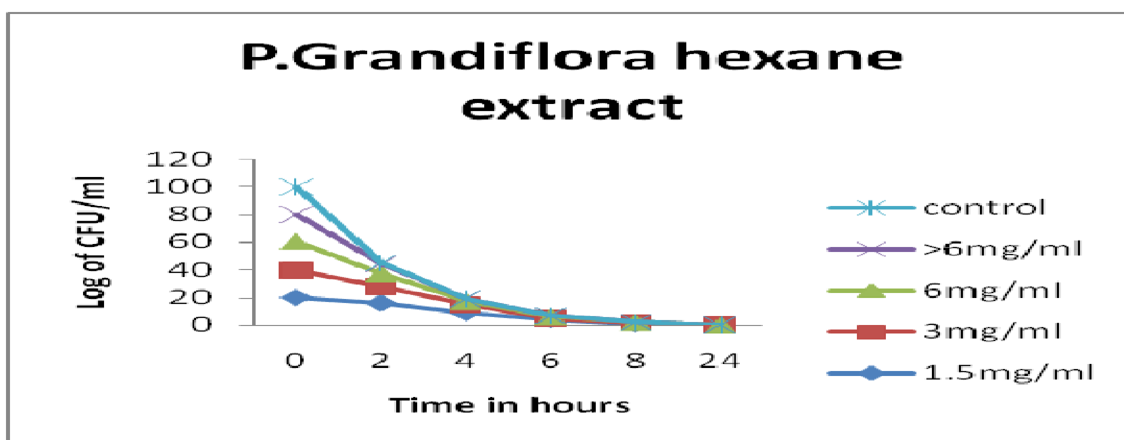

Figure 6.2: Activity of varying concentrations of *P.grandiflora* extracts against selected isolates of *A. hydrophila* using hexane extract

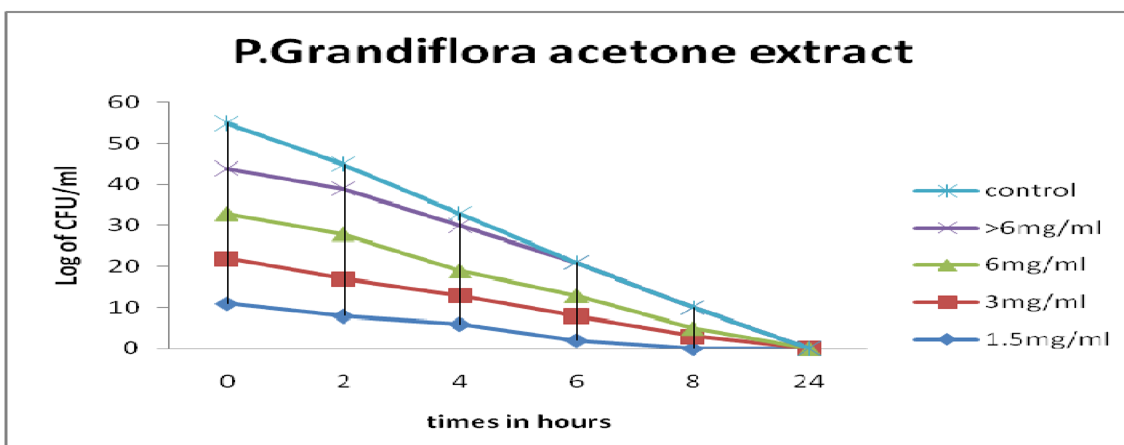

Figure 6.3: Activity of varying concentrations of *P.grandiflora* extracts against selected isolates of *A. hydrophila* using acetone extract

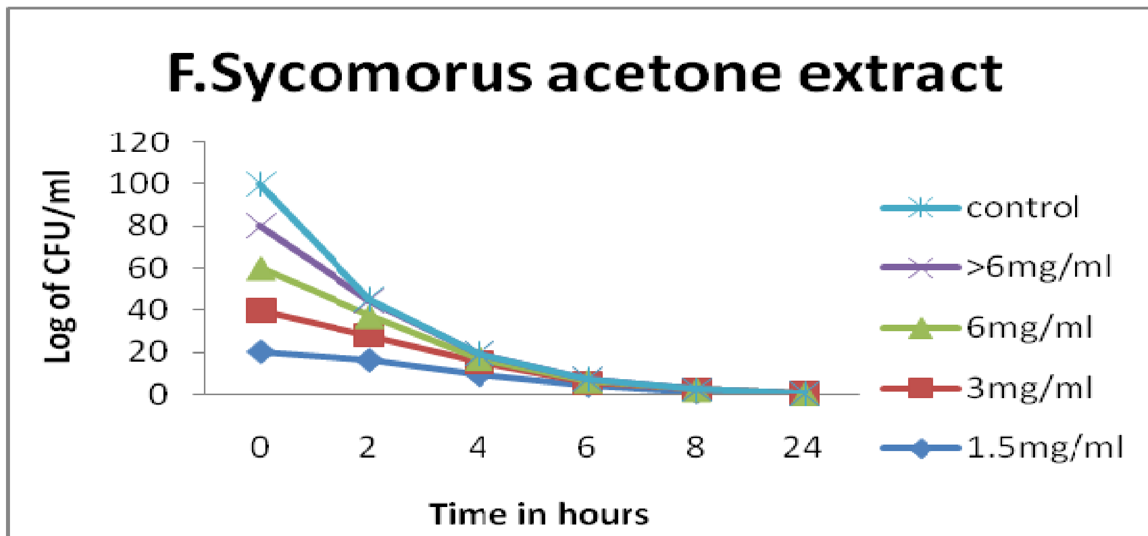

Figure 6.4: Activity of varying concentrations of *F.Sycomorus* extracts against selected isolates of *A. hydrophila* using acetone extract

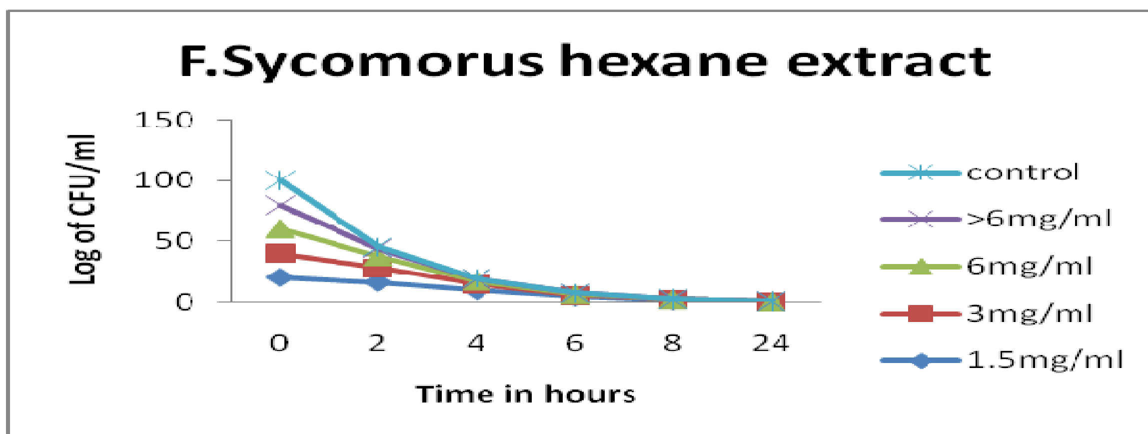

Figure 6.5: Activity of varying concentrations of *F.Sycomorus* extracts against selected isolates of *A. hydrophila* using hexane extract

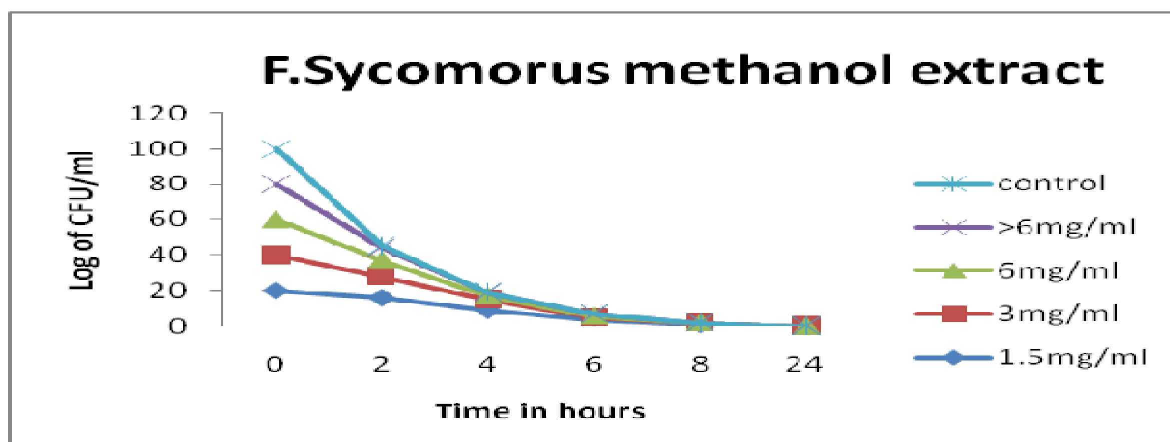

Figure 6.6: Activity of varying concentrations of *F.Sycomorus* extracts against selected isolates of *A. hydrophila* using methanol extract

6.6 Discussion

Ficus sycomorus and *P. grandiflora* are commonly employed in the treatment of diarrhoeal related infections in some rural communities in South Africa. However scientific studies to guage their efficacies are scanty. Diarrhoeal diseases are known to be responsible for a wide array of morbidities and mortalities in different age groups and these have been compounded by the epidemy of HIV/AIDS in South Africa. Indeed diarrhoea has been identified as one of the major symptoms associated with HIV/AIDS in developing countries because over 70% of HIV/AIDS patients suffer from chronic diarrhea. Diarrhoeagenic pathogens are diverse and include parasites, fungi, viruses and bacteria. Among the bacterial pathogens, *Aeromonas* species had been reported to be emerging pathogens in the Limpopo province (Obi *et al.*, 2007). *Aeromonas* species were also isolated from HIV/AIDS patients as well as from water samples in previous studies (Obi *et al.*, 2003, Samie *et al.*, 2005, Samie *et al.*, 2007). The

management of persistent diarrhea requires the use of antibiotics but the resistance of bacteria to a wide range of antibiotics imposes serious limitations and hence the quest for alternative sources of treatment. Due to reports on the usefulness of medicinal plants in the treatment of microbial infections (Mathebe *et al.* 2006, Tshibangu *et al*., 2002, Nakamura *et al*., 1999), we decided to investigate the *in vitro* antimicrobial activities of the extracts of *F.sycomorus* and *P.grandiflora* against *A. hydrophila* isolated from both clinical and environmental samples using different methods including the time-kill-curve assay. The medicinal plants demonstrated bactericidal activities against most of the clinical and environmental isolates, with *F.Sycomorus extracts* demonstrating stronger activities using all extractants employed for extractions than *P.grandiflora* extracts with very less activities against both isolates as demonstrated in Figs. 6.1 to 6.6. Among the two plants, bactericidal activities of *P.grandiflora* extracts were less profound than those of *F.Sycomorus*.

6.7 Conclusion

Consequently the strong inhibitory activities observed for *F.Sycomorus* in this study points to the potential of unraveling effective sources of management of *Aeromonas* related infections by use of medicinal plants.

Studies on the in vitro activities of these plants on other organisms such as protozoans, viruses, helminthes and other bacteria are recommended in order to determine the scope of activities.

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Chapter Seven: General conclusions and recommendations

7.1 Conclusions

The study indicates that Vitek 2 and Micro Scan Walkway could be used for the identification and determination of antimicrobial susceptibility profiles of *A. hydrophila* isolates from both environmental and clinical sources.

The reported prevalence of ESBLs in *A. hydrophila* emphasizes the importance of constant surveillance of clinical isolates to determine the prevalence of antibiotic resistant genes. The dissemination of beta-lactamases may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of diarrheal infections due to ESBL-producing *A. hydrophila*.

The results of the present study further revealed the possibility of an intricate web of relationship between *Aeromonas hydrophila* isolates from stool and water samples in the Limpopo province of South Africa, as indicted by the phylogenetic tree, indicating the possibilities of cross contaminations and also pointing to the need for effective control measures.

It was also concluded that specific genotypes might be related to antibiotic resistance.

Our study further demonstrated that many medicinal plants used in the Venda region of the Limpopo province of South Africa had a wide range of antibacterial activity and confirms the traditional use of these plants in the management of infections caused by the pathogens.

High levels of multi resistance to most commonly used antibiotics may be indicative of the horizontal and vertical spread of resistance genes among isolates.

The novel finding of class 1 integrons amongst ESBL-producing *Aeromonas hydrophila* in South Africa , strongly suggest a possible role in the dissemination of ESBL-mediated resistance among the isolates.

7.2 RECOMMENDATIONS

- Constant surveillance of clinical isolates to determine the prevalence of antibiotic resistant genes is recommended because of the noted occurrence of ESBLs in *Aeromonas hydrophilia*.
- Monitoring and evaluation of emerging antibiotic resistance genes in bacteria such as *A. hydrophila* is recommended in order to gauge the efficacy of control measures.
- A better understanding of the processes of gene transfer in natural environments is crucial in order to assess the risk of antibiotic resistance among ubiquitous agents of diarrhoea such as the motile aeromonads.
- Further studies on the virulence and cytotoxicity of *A. hydrophilia* isolates to further clarify the pathogenic characteristics of strains in the region are warranted.
- Extracts of medicinal plants studied exhibited varying degrees of antimicrobial activities against *A. hydrophila* isolates. It is therefore recommended that the medicinal plants extracts should be further studied phytochemically to elucidate the active compounds for potential application as candidate drugs.
- Studies on the activities of medicinal plants against other organisms such as protozoans, viruses, and helminthes and the determination of their phytochemistry are recommended.